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

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New & Noteworthy

We examined whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow resistive breathing, if 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. Only when sufficiently strenuous, inspiratory flow resistive breathing elevates plasma F_2-isoprostanes, and our novel data show this is not related to a reduction in transdiaphragmatic twitch pressure.

ABSTRACT

We questioned whether the respiratory muscles of humans contribute to systemic oxidative stress following inspiratory flow resistive breathing, if 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 4 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 1 resistive load per visit, and the order that 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 to 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 and total antioxidant capacity. Further, 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 only when sufficiently strenuous, 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}.

INTRODUCTION

Increased respiratory muscle work is encountered during strenuous whole body exercise, asthma attacks, 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 inspiratory mouth pressure (P_{Imax}) (57). 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, 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, if 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 prior to their first trial, which was then replicated prior to all subsequent trials. Participants reported that they were recreationally active, which included playing sports, and participating in aerobic and resistance exercise 3-4 days per week. 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]

Experimental design

Participants attended the laboratory for 4 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 1 resistive load per visit, and the order that 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 non-rebreathing 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 non-rebreathing valve. Inspiratory maneuvers for

204 P_{Imax} and P_{dimax} were performed while seated, initiated from residual volume, and sustained
205 for at least 1 s. Repeat efforts separated by 30 s were performed until three serial measures
206 differed by no more than 10% or 10 cmH_2O, whichever was smallest (33). The highest value
207 recorded was used for subsequent analysis.

208

209 ***Respiratory muscle pressures***

210 Respiratory muscle pressures were quantified by measuring esophageal (P_e) and gastric (P_g)
211 pressures using two 10 cm balloon-tipped latex catheters (Model 47-9005; Ackrad
212 Laboratories, Cranford, NJ) which were attached to calibrated differential pressure
213 transducers (MLT844; AD Instruments, Dunedin, New Zealand) (33, 34). The esophageal
214 and gastric balloons were filled with 1 ml and 2 ml of air, respectively. During the first
215 experimental trial, the distance from the tip of the nares to the most distal point of the
216 catheters was recorded and replicated in subsequent trials. P_{di} was calculated automatically
217 using LabChart Pro software (AD Instruments, Bella Vista, Australia) by subtracting P_e from
218 P_g. To estimate respiratory muscle energy expenditure (16), P_{di} and P_e were integrated over
219 the period of inspiratory flow and multiplied by breathing frequency and labeled the
220 diaphragm pressure-time product (PTP_{di}) and the inspiratory muscle pressure-time product
221 (PTP_e), respectively. Nonphysiological flows and pressures that resulted from swallowing,
222 coughing, and breath holding were visually identified and removed. Raw pressure data were
223 recorded continuously at 200 Hz using a 16-channel analog-to-digital data acquisition system
224 (PowerLab 16/35; AD Instruments, Dunedin, New Zealand).

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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 prior to stimulation were instructed to hold 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 non-potentiated P_{diTW} responses and the final three potentiated P_{diTW} responses were 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.

252 ***Inspiratory flow resistive breathing***

253 Following cervical magnetic phrenic nerve stimulation, participants remained seated and
254 continued to wear a nose clip. Resting measurements were collected for 5 min whilst
255 participants breathed through a mouthpiece to a two-way non-rebreathing valve. For
256 P_{dimax}50% and P_{dimax}70% trials, the custom-built variable sized aperture was adjusted to
257 narrow its diameter. This was continued until participants could match the target P_{di} which
258 was displayed on a screen in front of them and monitored continuously to ensure adequate
259 pressure development. Participants were asked to maintain tidal volumes close to those
260 achieved at rest, and the proportion of P_{di} contributed by P_g and P_e was not controlled. In the
261 event that the partial pressure of end-tidal carbon dioxide fell from resting concentrations,
262 carbon dioxide was added to the inspirate to maintain isocapnia and avoid the deleterious
263 effects of hypocapnia (e.g., light-headedness, confusion, paresthesia, tetany). This occurred in
264 two participants after ~3 min during the P_{dimax}70% trial when end-tidal carbon dioxide partial
265 pressure fell below 30 mmHg. Once isocapnia was restored, these participants were coached
266 to maintain expired volumes close to that achieved at rest to prevent further episodes of
267 hypocapnia. Participants maintained a breathing frequency of 15 breaths·min^{-1} and a duty
268 cycle of 0.5 by listening to a computer-generated audio signal with distinct inspiratory and
269 expiratory tones.

270

271 ***Cardiorespiratory responses***

272 Standard ventilatory responses were measured on a breath-by-breath basis using a metabolic
273 cart (JAEGER® Vyntus; CareFusion, San Diego, CA) with the flow sensor inserted into the
274 mouth port of the two-way non-rebreathing valve. Cardiac frequency and estimated arterial
275 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 mL 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 2500 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 F2-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, bis(trimethylsilyl)trifluoroacetamide 99% and trimethylchlorosilane 1% were added and incubated at 45°C for 20 min. Finally, hexane was added and samples were mixed,

299 then 1 ml was injected for analysis using gas chromatography mass spectrometry (Varian;
300 Belrose, Australia) in negative chemical ionization mode. The laboratory coefficient of
301 variation for this assay is 4.5%.

302

303 ***Plasma protein carbonyls***

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

318

319 ***Plasma total antioxidant capacity***

320 Total antioxidant capacity was measured using a modified version (36) of an assay previously
321 described (47, 62), and adapted for a Cobas Mira autoanalyser (Cobas Mira, Roche
322 Diagnostica, Switzerland). Briefly, plasma was incubated with metmyoglobin and 2,20-

azino-bis(3-ethylbenzothiazoline-6-sulphonic 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 8 participants would be required to demonstrate a 10% increase in plasma F_2-isoprostanes with an alpha of 0.05 and power 0.8. All data was 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 repeated 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 x 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 (I) flow resistive load; and (II) 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 Figure 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 to 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 to Control (Figure 1). RPD was elevated during the 5th and final min of P_{dimax}70% compared to 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 to Control. There was a time x 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 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 Figure 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 to Control and P_{dimax}50% (Figure 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.

[TABLE 2] [FIGURE 1] [FIGURE 2]

Transdiaphragmatic twitch pressures

A plateau (i.e., no significant increase in amplitude with increasing stimulation intensity) in P_{diTW} amplitude (Figure 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 to Baseline, $P_{dimax}50\%$ and $P_{dimax}70\%$ were reduced at the End and at +30 min after inspiratpry flow resistive breathing (Figure 4). There were no main effects of resistive load or time x resistive load interactions (Figure 4).

[FIGURE 3] [FIGURE 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} (Figure 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 $P_{dimax}70\%$ (Figure 6).

[FIGURE 5] [FIGURE 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, if 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 and total antioxidant capacity. Further, 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 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). Further, 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

420 flow resistive breathing at P_{dimax}50%. This may be due to the intensity of the loading that was
421 insufficient to observe increased appearance rates of ROS to exceed the ability of
422 antioxidants to counteract their effects. Indeed, it has been previously reported that F_2-
423 isoprostane concentrations are higher following high-intensity intermittent rather than
424 constant load cycling exercise (14).

425

426 We did not observe an increase in plasma protein carbonyl concentration and total
427 antioxidant capacity. Plasma protein carbonyl concentrations are a marker of protein
428 oxidation. They are elevated in the diaphragms of rats when they are exposed to inspiratory
429 flow resistive breathing, and concentrations are higher after 8 and 12 days, compared to 4
430 days (51). However, certain exercise conditions can result in a net decrease in plasma protein
431 carbonyl concentrations, which occurs in parallel with increases in other biomarkers of
432 oxidative stress. Greater inspiratory flow resistive intensities and/or durations may be
433 required to elicit increases in markers of oxidative stress. Exercise intensity (>70% maximal
434 oxygen uptake) and prolonged duration (>60 min) appear to be the main contributing factors
435 in the observed post-exercise increases in plasma protein carbonyl concentration (59).
436 However, it must be noted that whole body exercise engages a significantly greater muscle
437 mass than inspiratory flow resistive breathing. The factors influencing decreases in protein
438 carbonyls are more difficult to interpret, but likely involve the clearance of oxidized proteins
439 from plasma, potentially by plasma proteasomes, excretion, or uptake into active tissues (59).

440

441 Total antioxidant capacity is a marker of exogenous antioxidant utilization (30). Other studies
442 using maximal treadmill exercise have also found no changes to plasma total antioxidant
443 capacity immediately post exercise (2, 15). However, others have observed significant

increases at 30 min (58) and 1 h (60). The timing of measurements may therefore be important for total antioxidant capacity, and plasma protein carbonyl measurements. For example, around 50 min of exercise resulted in a 32% increase in protein carbonyls 30 min post-exercise and 94% 4 h later (31). Our experimental design unfortunately 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

Similar to others (19), we observed a reduction in potentiated and non-potentiated P_{diTW} following inspiratory resistive breathing which 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 non-potentiated 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. Firstly, 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 Figure 5 do not indicate that there are any sex differences, but this warrants further investigation. Secondly, 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. Thirdly, 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}.

492 ***Conclusion***

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

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697 experiments; D.R.B., K.V., D.E.M., performed the experiments; D.R.B., M.A.J., G.R.S.,
698 J.S.C., D.E.M., analyzed the data; D.R.B., K.V., M.A.J., G.R.S., J.S.C., D.E.M., wrote the
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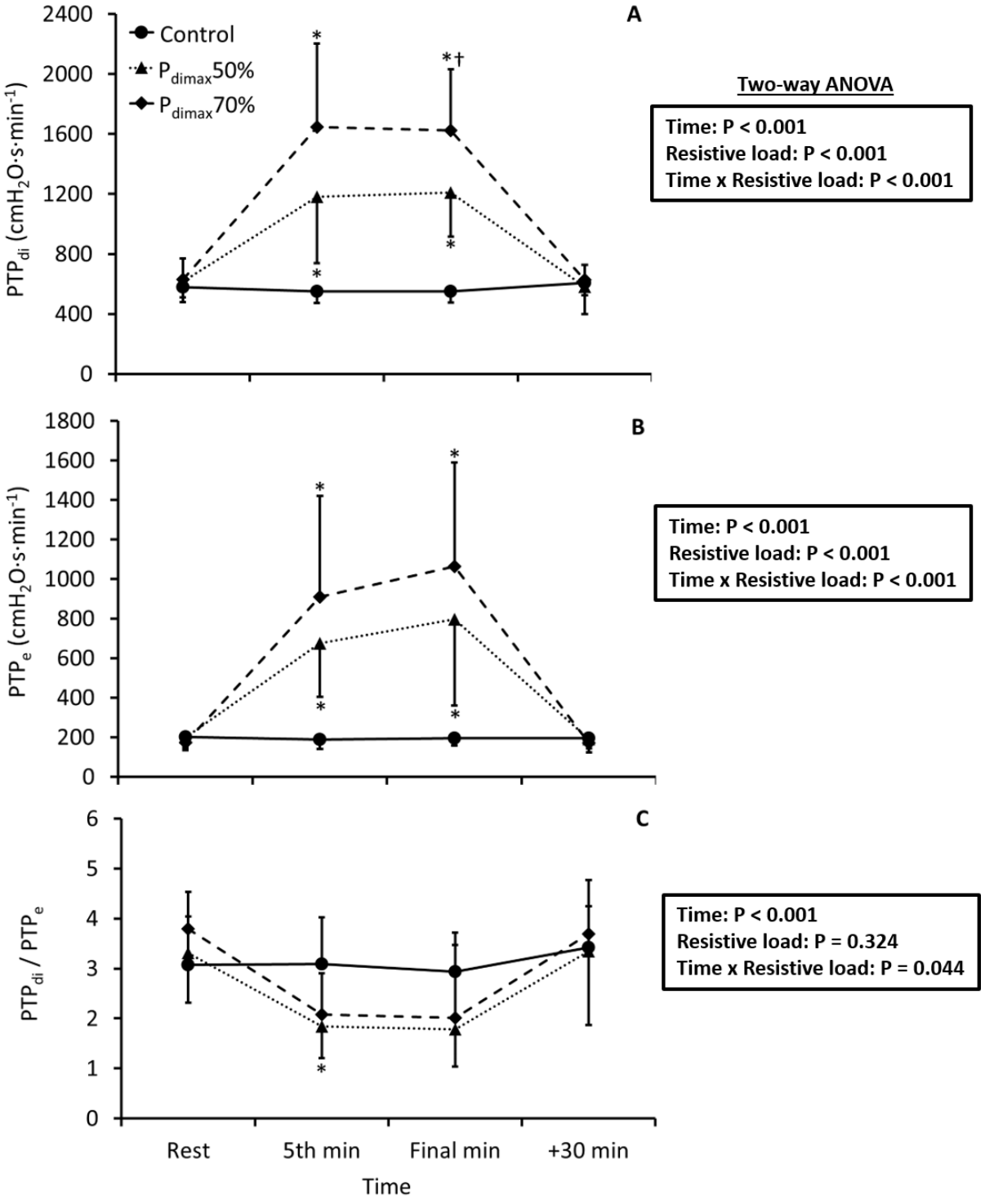
716 FIGURES



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718 Figure 1. Diaphragm pressure-time product (PTP_{di}; A), inspiratory muscle pressure-time
719 product (PTP_e; B) and the relative contribution of diaphragm to the inspiratory muscle
720 pressure-time product (PTP_{di}/PTP_e; C) responses to inspiratory flow resistive breathing for
721 Control and at 50 and 70% of peak transdiaphragmatic pressure (P_{dimax}50% and P_{dimax}70%).
722 Values are mean ± SD. * Significantly different from Control ($P < 0.05$). † Significantly
723 different from P_{dimax}50% ($P < 0.05$).

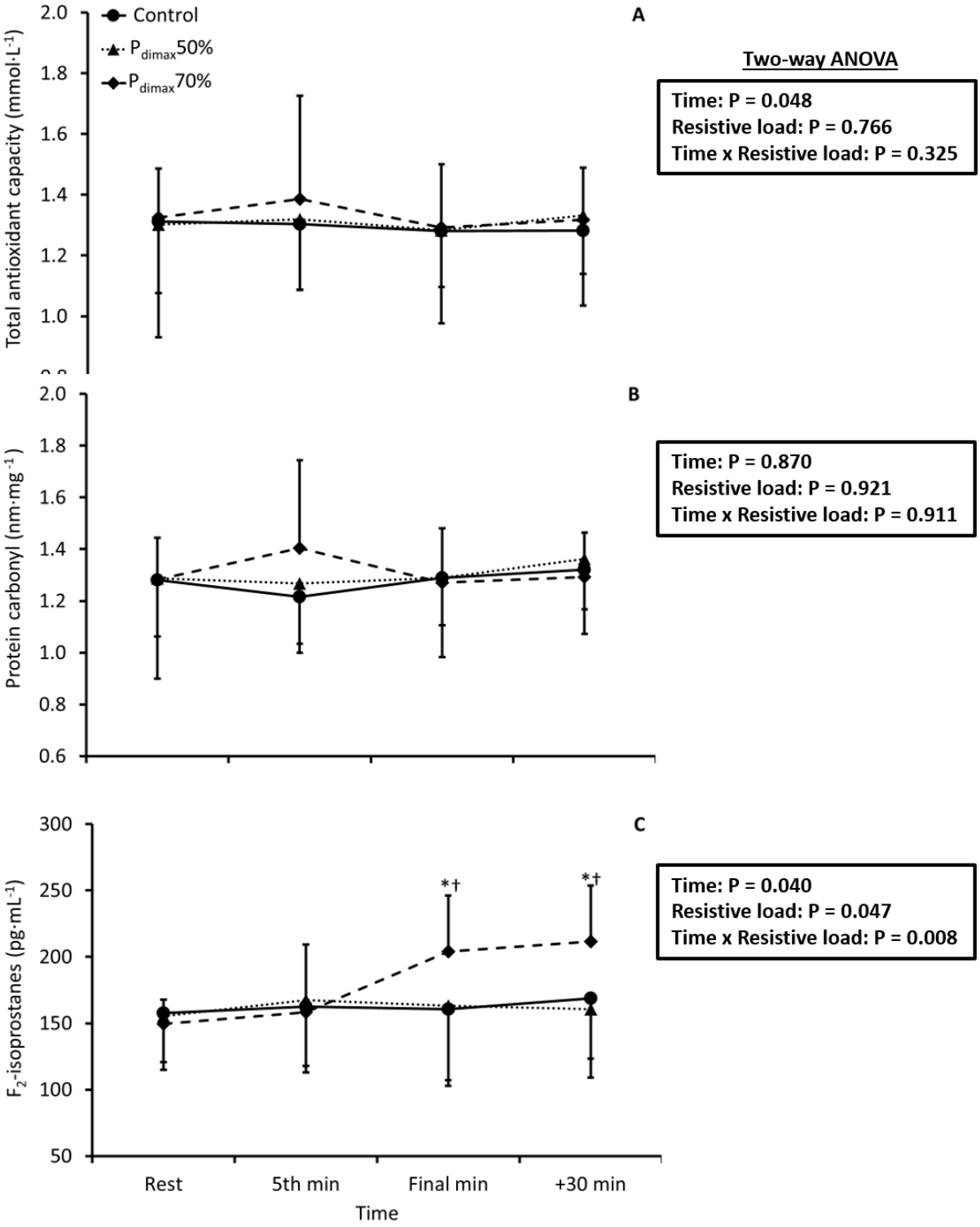

Figure 2. Plasma total antioxidant capacity (A), protein carbonly (B) and F_2-isoprostane (C) responses to inspiratory flow resistive breathing for Control and at 50 and 70% of peak transdiaphragmatic pressure (P_{dimax}50% and P_{dimax}70%). Values are mean ± SD. * Significantly different from Control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

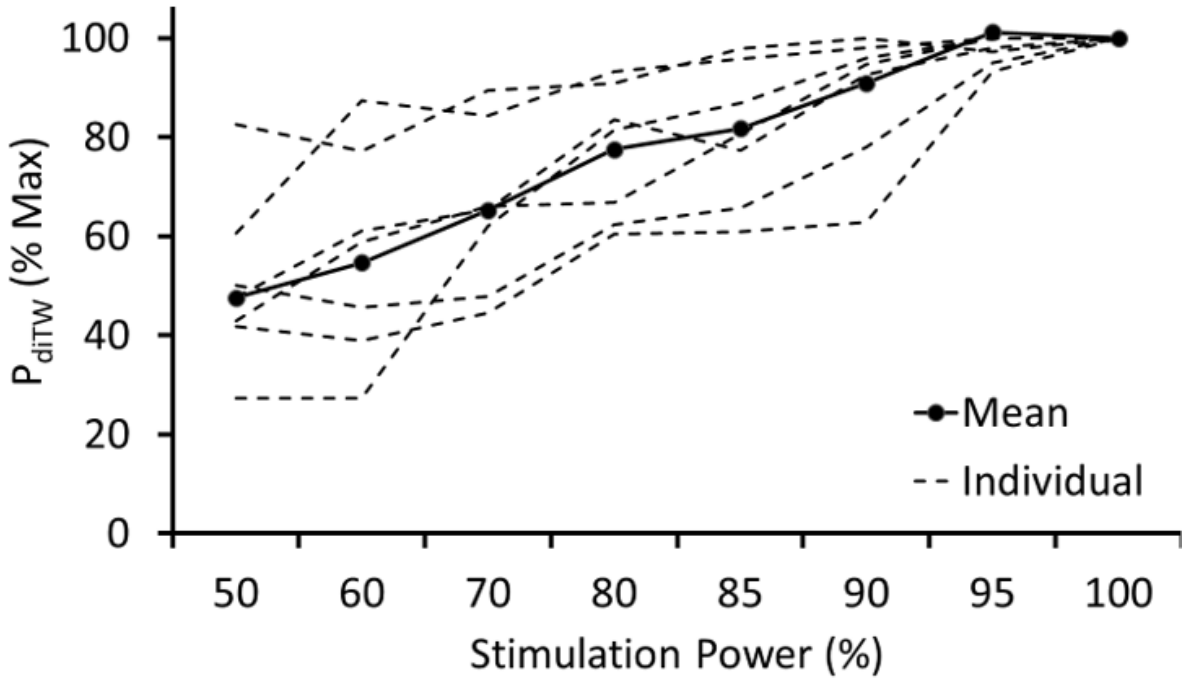

Figure 3. Individual and group mean transdiaphragmatic twitch pressure (P_{diTW}) in response to cervical magnetic stimulation of increasing stimulation intensity.

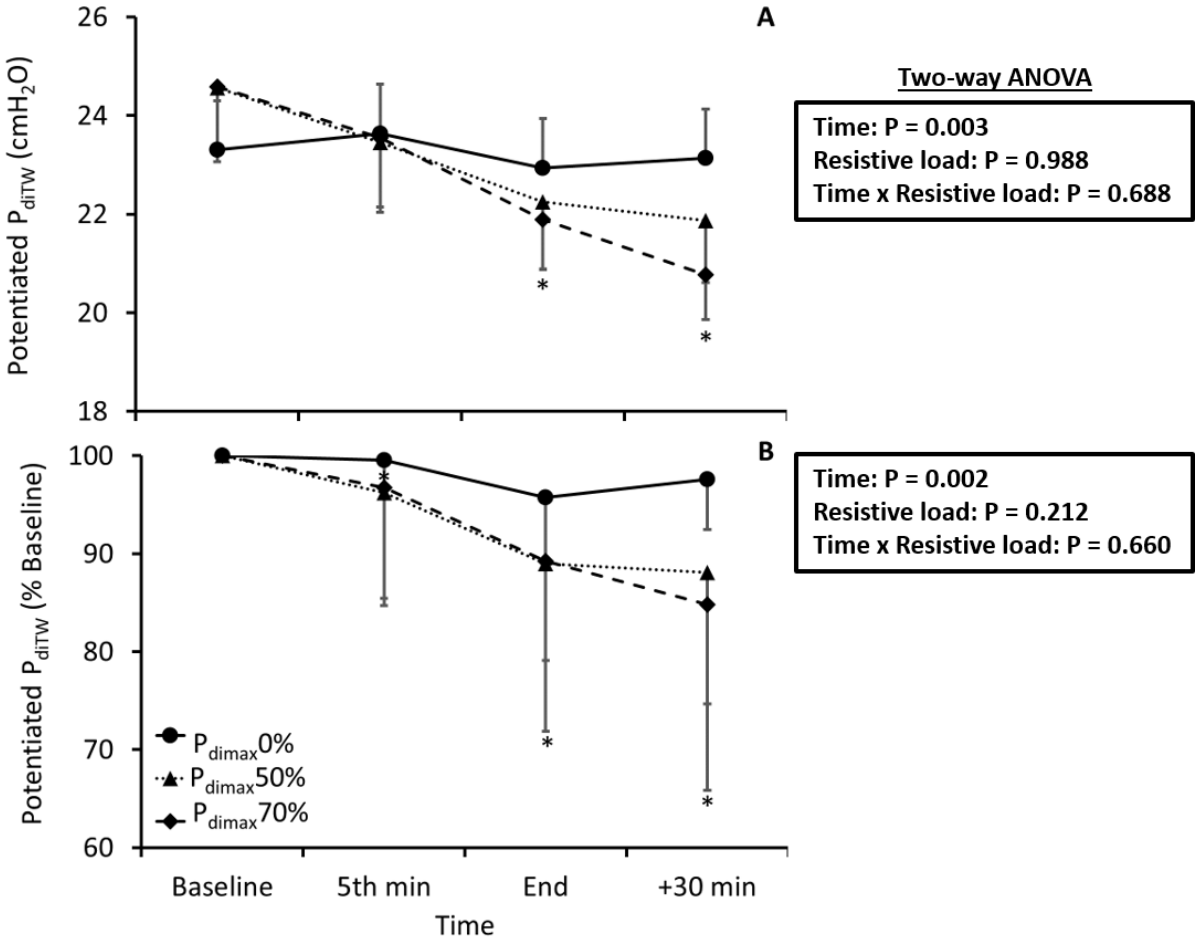

Figure 4. Absolute (A) and relative (A) and potentiated transdiaphragmatic twitch pressure (P_{diTW}) responses to inspiratory flow resistive breathing for Control and at 50 and 70% of peak transdiaphragmatic pressure (P_{dimax}50% and P_{dimax}70%). Values are mean ± SD. * Significantly different from Baseline for P_{dimax}50% and P_{dimax}70% ($P < 0.05$).

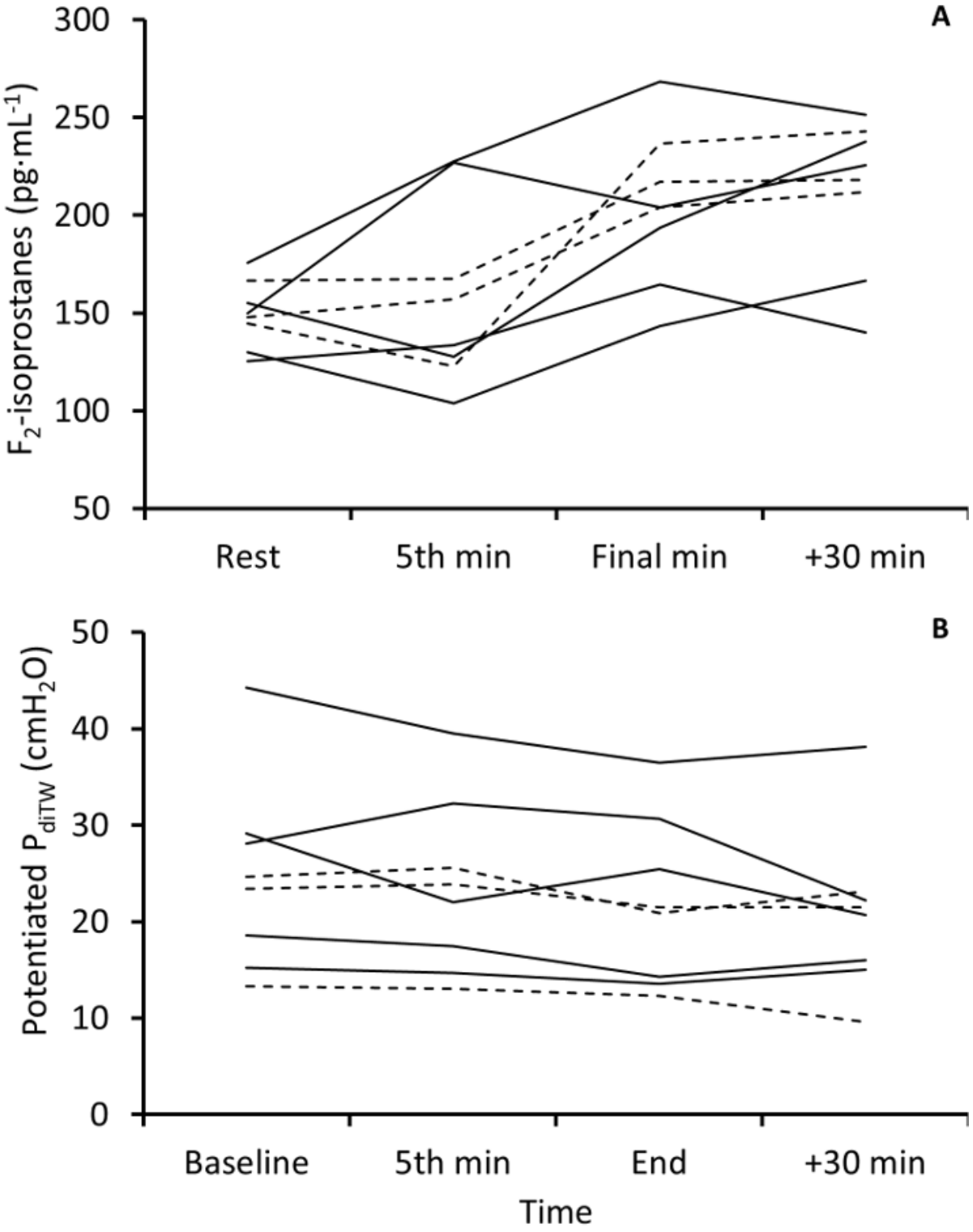

Figure 5. Individual male (solid line) and female (dashed line) plasma F_2-isoprostanes (A) and absolute potentiated transdiaphragmatic twitch pressure (P_{diTW}) (B) responses to inspiratory flow resistive breathing at 70% of peak transdiaphragmatic pressure.

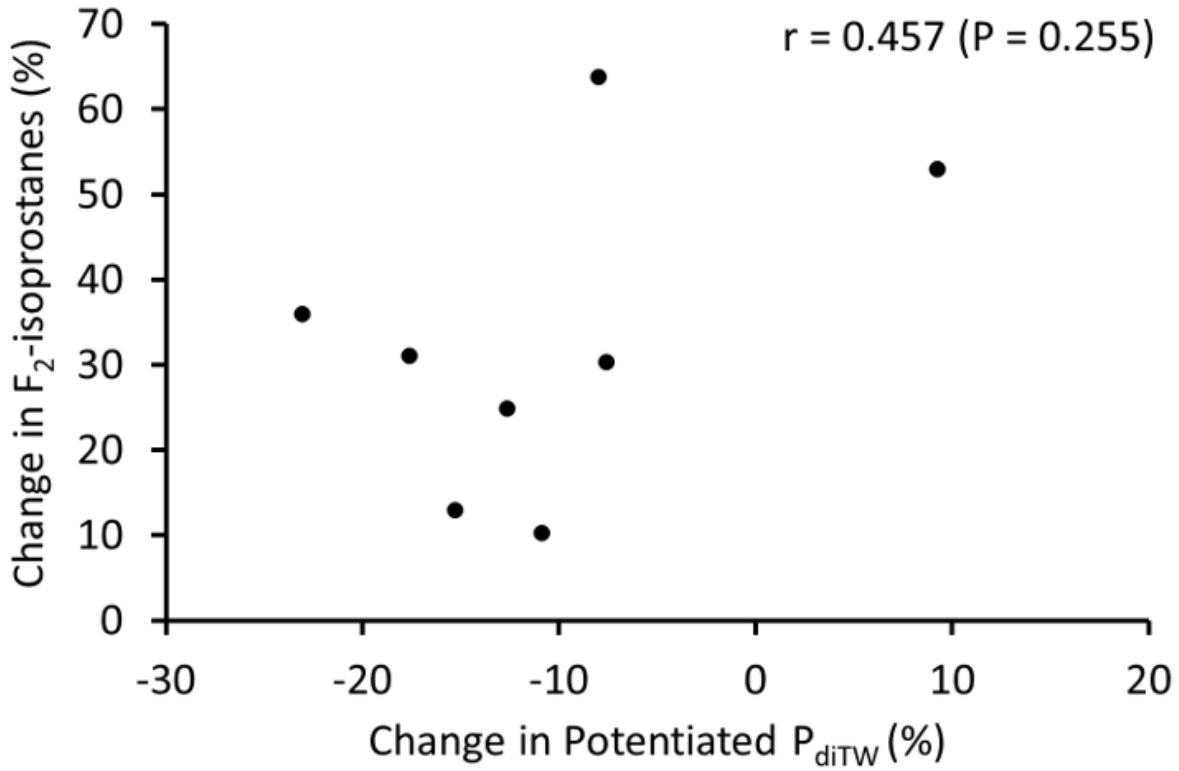

Figure 6. Percentage change from Rest to Final min for plasma F_2-isoprostanes vs. percentage change from Baseline to End during inspiratory flow resistive breathing at 70% of peak transdiaphragmatic pressure.

780 TABLES

781

782 Table 1. Participant anthropometrics and respiratory function. Values are mean ± SD.

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	Male (n = 5)	Female (n = 3)
Age, years	26 ± 5	26 ± 4
785 Height, cm	176 ± 7	164 ± 9
Body mass, kg	91 ± 8	70 ± 9
786 FVC, L	5.01 ± 0.79	4.30 ± 0.94
FVC, % predicted	101 ± 4	109 ± 17
787 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
788 FEV_1/FVC, % predicted	96 ± 8	97 ± 3
P_{Imax}, cmH_2O	101 ± 35	117 ± 48
P_{Imax}, % predicted	92 ± 22	131 ± 14
789 P_{dimax}, cmH_2O	90 ± 27	98 ± 24

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

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Table 2. Cardiorespiratory and perceptual responses to inspiratory flow resistive breathing for Control and at 50 and 70% of peak transdiaphragmatic pressure (P_{dimax}50% and P_{dimax}70%). Values are mean ± SD.

Variable	Resistive load	Rest	5th min	Final min	+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*
$\dot{V}_E$, $L \cdot min^{-1}$	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, $breaths \cdot min^{-1}$	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, $beats \cdot min^{-1}$	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
SpO_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

P_{Ipeak}, peak inspiratory mouth pressure; P_{epeak}, peak esophageal pressure; P_{dipeak}, peak transdiaphragmatic pressure; RPD, rating of perceived dyspnea; $\dot{V}_E$, minute ventilation; f_B, breathing frequency; V_T, tidal volume; T_I/T_{TOT}, duty cycle; f_C, cardiac frequency; SpO_2, estimated arterial oxygen saturation; $P_{ET}CO_2$, end tidal carbon dioxide pressure. *

819 Significantly different from Control at the same time point ($P < 0.05$). † Significantly
820 different from $P_{dimax}50\%$ at the same time point ($P < 0.05$).

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