

Respiratory muscle induced systemic oxidative stress

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

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20 **Running Head:** Respiratory muscle induced systemic oxidative stress.

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32 **New & Noteworthy**

33 We examined whether the respiratory muscles of humans contribute to systemic oxidative
34 stress following inspiratory flow resistive breathing, if the amount of oxidative stress is
35 influenced by the level of resistive load, and whether the amount of oxidative stress is related
36 to the degree of diaphragm fatigue incurred. Only when sufficiently strenuous, inspiratory
37 flow resistive breathing elevates plasma F₂-isoprostanes, and our novel data show this is not
38 related to a reduction in transdiaphragmatic twitch pressure.

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62 **ABSTRACT**

63 We questioned whether the respiratory muscles of humans contribute to systemic oxidative
64 stress following inspiratory flow resistive breathing, if the amount of oxidative stress is
65 influenced by the level of resistive load, and whether the amount of oxidative stress is related
66 to the degree of diaphragm fatigue incurred. Eight young and healthy participants attended
67 the laboratory for 4 visits on separate days. During the first visit, height, body mass, lung
68 function and maximal inspiratory mouth and transdiaphragmatic pressure (P_{dimax}) were
69 assessed. During visits 2-4, participants undertook inspiratory flow resistive breathing with
70 either no resistance (Control) or resistive loads equivalent to 50 and 70% of their P_{dimax}
71 ($P_{\text{dimax}50\%}$ and $P_{\text{dimax}70\%}$) for 30 min. Participants undertook 1 resistive load per visit, and
72 the order that they undertook the loads was randomized. Inspiratory muscle pressures were
73 higher ($P < 0.05$) during the 5th and final min of $P_{\text{dimax}50\%}$ and $P_{\text{dimax}70\%}$ compared to
74 Control. Plasma F_2 -isoprostanes increased ($P < 0.05$) following inspiratory flow resistive
75 breathing at $P_{\text{dimax}70\%}$. There were no increases in plasma protein carbonyls and total
76 antioxidant capacity. Further, although we evidenced small reductions in transdiaphragmatic
77 twitch pressures (P_{diTW}) after inspiratory flow resistive breathing at $P_{\text{dimax}50\%}$ and $P_{\text{dimax}70\%}$,
78 this was not related to the increase in plasma F_2 -isoprostanes. Our novel data suggest that
79 only when sufficiently strenuous, inspiratory flow resistive breathing in humans elicits
80 systemic oxidative stress evidenced by elevated plasma F_2 -isoprostanes, and based on our
81 data this is not related to a reduction in P_{diTW} .

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86 **INTRODUCTION**

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

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93 Reactive oxygen species (ROS) form as products under normal physiological conditions due
94 to the partial reduction of molecular oxygen (42, 43). Oxidative stress is defined as
95 macromolecular oxidative damage along with a disturbance of redox signaling and control
96 and usually results from either excessive ROS production, mitochondrial dysfunction,
97 impaired antioxidant system, or a combination of these factors (42, 43). ROS produced under
98 oxidative stress can damage all cellular biomolecules including lipids, proteins, carbohydrates
99 and DNA (42, 43). The measurement of oxidative stress *in vivo* is difficult as ROS are highly
100 reactive and/or have a very short half-life (<1 s for some), so they can be estimated from
101 changes in free radicals, radical mediated damages to lipids, proteins and nucleic acids, and
102 antioxidant enzyme activity or concentration (39). Therefore, a battery of different markers
103 that are reliable are essential to summarize the effects of oxidative stress (39). Systemic
104 measurements can include protein carbonyls as a marker of protein oxidation, total
105 antioxidant capacity for exogenous antioxidant utilization, and F₂-isoprostanes for lipid
106 peroxidation, which is widely regarded as a gold standard because of their chemical stability
107 and prevalence in all human tissues and biological fluids (35, 38, 64).

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109 Oxidative stress is elevated in the diaphragms of animals exposed to inspiratory flow resistive
110 breathing, and the amount of oxidative stress is positively associated with the level of
111 resistive load (1, 7, 12, 13, 51). Supplementation with a combination of antioxidants also
112 reduces the response of plasma cytokines in humans following 45 min of inspiratory flow
113 resistive breathing undertaken at 75% of maximal inspiratory mouth pressure ($P_{I_{max}}$) (57).
114 Mild and acute exposure to exogenous ROS generally increases the muscles ability to
115 generate force (11, 24, 61), whereas stronger or prolonged exposure as occurs during flow
116 resistive breathing (1, 7, 12, 13, 51), significantly reduces respiratory muscle force generation
117 (19, 45). Indeed, *in vitro* studies have shown that ROS released from diaphragm fibers
118 promotes low-frequency diaphragm fatigue (5, 25, 46, 52), which in humans can be measured
119 objectively using phrenic nerve stimulation (29). Supplementation with the antioxidant N-
120 acetylcysteine before inspiratory resistive breathing or heavy exercise may also attenuate
121 respiratory muscle fatigue (21, 56). In patients with severe chronic obstructive pulmonary
122 disease, diaphragm fatigue can contribute to muscle dysfunction (6, 17), and the development
123 of respiratory failure (41). Taken together, these animal, *in vitro* and supplementation studies
124 indicate that resistive breathing leads to increased oxidative stress, that the amount of
125 oxidative stress is associated with the level of resistive load, and that this is related to
126 diaphragm fatigue. The findings for the animal and *in vitro* studies, however, have not been
127 repeated in humans.

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129 Accordingly, we questioned whether the respiratory muscles of humans contribute to
130 systemic oxidative stress following inspiratory flow resistive breathing, if the amount of
131 oxidative stress is influenced by the level of resistive load, and whether the amount of
132 oxidative stress is related to the degree of diaphragm fatigue incurred. We utilized a battery
133 of oxidative stress markers including plasma F₂-isoprostanes, protein carbonyls and total

134 antioxidant capacity and objectively measured low-frequency diaphragm fatigue using
135 phrenic nerve stimulation. We hypothesized that oxidative stress would be increased
136 following exposure to inspiratory flow resistive breathing, and greater with increased
137 resistive loads, and the increase in oxidative stress measures would be related to the degree of
138 diaphragm fatigue incurred.

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155 **METHODS**

156 *Participants*

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

170 [TABLE 1]

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172

173 *Experimental design*

174 Participants attended the laboratory for 4 visits on separate days. Each laboratory visit was
175 separated by a minimum of 48 h and took place at the same time of day. During the first visit,
176 height, body mass, lung function, $P_{I_{max}}$ and maximum transdiaphragmatic pressure (P_{dimax})
177 were assessed according to published guidelines and statements (4, 32). Subsequently,
178 participants were familiarized with all other measurements and inspiratory flow resistive
179 breathing. During visits 2-4, participants undertook inspiratory flow resistive breathing with

180 either no resistive load (Control), or loads equivalent to 50 and 70% of their P_{dimax} ($P_{\text{dimax}50\%}$
181 and $P_{\text{dimax}70\%}$) for 30 min. These reflected “low”, “moderate” and “heavy” flow resistive
182 loads, respectively. Participants undertook 1 resistive load per visit, and the order that they
183 undertook the loads was randomized. Participants were naïve to the prescribed resistive load
184 and the resistive loading device was hidden from view. The resistive loads were chosen
185 because, through our pilot studies and other work (3, 8, 23), they were sustainable for 30 min
186 and would elicit varying degrees of diaphragm fatigue. Transdiaphragmatic twitch pressures
187 (P_{diTW}) were measured at Baseline, at 5 min, at the End, and +30 min after the completion of
188 inspiratory flow resistive breathing trials. Blood samples for oxidative stress measures,
189 respiratory pressures, cardiorespiratory data, and rating of perceived dyspnea (RPD; Borg
190 modified CR10 scale (9) as a measure of the effort required to overcome the resistance) were
191 measured at rest, during the 5th min, in the Final min, and +30 min after the completion of
192 inspiratory flow resistive breathing trials.

193

194 ***Pulmonary function and maximal inspiratory mouth and transdiaphragmatic pressure***

195 Pulmonary function was assessed using a calibrated testing system (JAEGER® Vyntus;
196 CareFusion, San Diego, CA). P_{Imax} and P_{dimax} were assessed using the same experimental
197 equipment used for the inspiratory flow resistive breathing. Participants inspired through a
198 two-way non-rebreathing valve (Model 2730; Hans Rudolph, Shawnee Mission, KS) with
199 resistance provided by a custom-built variable sized aperture with a length of 2 mm placed
200 into the inspiratory port. To assess P_{Imax} and P_{dimax} , the aperture was closed and incorporated
201 a 1 mm orifice to prevent glottic closure during inspiratory efforts. Mouth pressure was
202 measured using a calibrated transducer (MLT844; AD Instruments, Dunedin, New Zealand)
203 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 cmH₂O, 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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228 ***Cervical magnetic phrenic nerve stimulation***

229 Cervical magnetic phrenic nerve stimulation was applied via a double 70 mm coil connected
230 to a Magstim 200² stimulator (Magstim, Dyfed, UK). Participants initially rested for 20 min
231 to minimize postactivation potentiation. Subsequently, while participants were sat upright and
232 the neck flexed, the coil was placed over the midline between the 5th (C5) and 7th (C7)
233 cervical vertebrae (50). The optimal coil position was defined as the vertebral level that when
234 stimulated at 50% of maximum stimulator output evoked the highest P_{diTW} . This location was
235 marked with indelible ink and used for subsequent stimulations. During stimulations,
236 participants wore a noseclip, and prior to stimulation were instructed to hold breathing effort
237 at functional residual capacity, which was inferred from visual feedback of P_e . To determine
238 supramaximal phrenic nerve stimulation, three single twitches were obtained every 30 s at
239 intensities of 50, 60, 70, 80, 85, 90, 95, and 100% of maximal stimulator output. A plateau in
240 P_{diTW} responses with increasing stimulation intensities indicated maximum depolarization of
241 the phrenic nerves.

242

243 Maximum P_{diTW} was assessed at each measurement point every 30 s using three stimuli at
244 100% of maximal stimulator output. Additionally, P_{diTW} at each measurement point was
245 followed by the assessment of the potentiated P_{diTW} response. Participants performed a 3 s
246 maximal Müller maneuver and ~5 s later a single stimuli was delivered. This procedure was
247 repeated six times with each measure separated by 30 s. The average of the three individual
248 non-potentiated P_{diTW} responses and the final three potentiated P_{diTW} responses were used for
249 analysis. This procedure was undertaken at Baseline, after 5 min of inspiratory flow resistive
250 breathing, at the End, and +30 min after the completion of inspiratory flow resistive breathing
251 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 $P_{\text{dimax}50\%}$ and $P_{\text{dimax}70\%}$ 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 $P_{\text{dimax}70\%}$ 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,

276 Finland) and fingertip pulse oximeter (Radical-7 Pulse CO-Oximeter, Masimo Corporation,
277 Irvine, CA), respectively.

278

279 ***Blood sampling***

280 Ten mL of venous blood was sampled at each time point from an antecubital vein via an
281 indwelling 21-G cannula. Blood was transferred into precooled tubes containing K₃E EDTA
282 (BD vacutainers; Franklin Lakes, NJ). Samples were stored on ice before being centrifuged at
283 2500 rpm for 10 min at 4°C. Plasma was then aliquoted and stored at -80°C until biochemical
284 assays were performed.

285

286 ***Plasma F₂-isoprostanes***

287 Samples were analyzed in duplicate using an optimized method for quantification of total F₂-
288 isoprostanes using gas chromatography–tandem mass spectrometry (10). Isoprostanes were
289 extracted from plasma after saponification with methanolic NaOH. Samples were spiked with
290 8-iso-PGF₂α-d₄ (Cayman Chemicals, Ann Arbor, MI) as an internal standard and incubated
291 at 42°C for 60 min. Samples were then acidified to pH 3 with hydrochloric acid, and hexane
292 was added and samples were mixed for 10 min before centrifugation. The supernatant was
293 removed, and the remaining solution extracted with ethyl acetate and dried under nitrogen.
294 Samples were reconstituted with acetonitrile, transferred into vials with silanized glass inserts
295 and dried. Derivatization with pentafluorobenzylbromide and diisopropylethylamine and
296 incubation at room temperature for 30 min followed. Samples were then dried under nitrogen
297 before pyridine, bis(trimethylsilyl)trifluoroacetamide 99% and trimethylchlorosilane 1% were
298 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-

323 azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). After incubation, hydrogen peroxide was
324 added, and the sample was incubated again. Absorbance was measured
325 spectrophotometrically to determine total antioxidant capacity. The laboratory coefficient of
326 variation for this assay is 1.9%.

327

328 ***Statistical analysis***

329 Statistical analyses were performed using SPSS for Windows (IBM, Chicago, IL). An initial
330 power calculation was performed on the basis of previous work (36) showing that 8
331 participants would be required to demonstrate a 10% increase in plasma F₂-isoprostanes with
332 an alpha of 0.05 and power 0.8. All data was confirmed as parametric via a Shapiro-Wilk test
333 for normality. The data from supramaximal phrenic nerve stimulation was analyzed using a
334 one-way ANOVA. The data from the three inspiratory flow resistive breathing trials were
335 analyzed using a two way repeated measures ANOVA procedure to determine the effects of
336 ‘time’ (Rest/Baseline, 5th min, Final min/End and +30 min) and ‘resistive load’ (Control,
337 P_{dimax}50% and P_{dimax}70%). Following significant time x resistive load interaction effects,
338 planned pairwise comparisons were made using the Bonferroni method. Pearson’s product
339 moment correlation coefficient was used to examine the relationship between the degree of
340 oxidative stress incurred and (I) flow resistive load; and (II) degree of diaphragm fatigue
341 incurred. Reliability was assessed using a coefficient of variation calculated from a pooled
342 mean of all trials. Statistical significance was set at P < 0.05. Results are presented as means
343 ± SD.

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345

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347 **RESULTS**

348 *Cardiorespiratory and perceptual responses*

349 Inspiratory muscle pressures and estimates of respiratory muscle energy expenditure during
350 inspiratory flow resistive breathing are shown in Table 2 and Figure 1, respectively. PTP_{di} ,
351 PTP_e , P_{Ipeak} , P_{epeak} and P_{dipeak} were higher during the 5th and final min of $P_{dimax50\%}$ and
352 $P_{dimax70\%}$ compared to Control. The relative contribution of the diaphragm to the inspiratory
353 muscle pressure-time product (PTP_{di}/PTP_e) was lower during the 5th min of $P_{dimax50\%}$
354 compared to Control (Figure 1). RPD was elevated during the 5th and final min of $P_{dimax70\%}$
355 compared to both $P_{dimax50\%}$ and Control (Table 2). Duty cycle was increased during the 5th
356 and final min of $P_{dimax70\%}$ and 5th min of $P_{dimax50\%}$ compared to Control. There was a time
357 x resistive load interaction effect ($P = 0.003$) for cardiac frequency (Table 2), but no pairwise
358 differences. There were no differences between Control, $P_{dimax50\%}$ and $P_{dimax70\%}$ for minute
359 ventilation, breathing frequency, tidal volume, estimated arterial oxygen saturation and end
360 tidal carbon dioxide pressure (Table 2).

361

362 *Markers of oxidative stress*

363 Markers of oxidative stress during inspiratory flow resistive breathing are shown in Figure 3.
364 Plasma F_2 -isoprostanes were higher during the final min and at +30 min of inspiratory flow
365 resistive breathing at $P_{dimax70\%}$ compared to Control and $P_{dimax50\%}$ (Figure 2). There was a
366 main effect of time ($P = 0.048$) for total antioxidant capacity, but no main effect of resistive
367 load. There were no differences between Control, $P_{dimax50\%}$ and $P_{dimax70\%}$ for plasma
368 protein carbonyls and total antioxidant capacity.

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

370

371 ***Transdiaphragmatic twitch pressures***

372 A plateau (i.e., no significant increase in amplitude with increasing stimulation intensity) in
373 P_{diTW} amplitude (Figure 3) was observed in response to supramaximal cervical magnetic
374 phrenic nerve stimulation, indicating maximal depolarization of the phrenic nerves. The
375 within and between coefficient of variation for P_{diTW} and potentiated P_{diTW} at rest was <5%.
376 Absolute ($P = 0.03$) and relative potentiated P_{diTW} decreased ($P = 0.02$) following inspiratory
377 flow resistive breathing at $P_{dimax}50\%$ and $P_{dimax}70\%$. Compared to Baseline, $P_{dimax}50\%$ and
378 $P_{dimax}70\%$ were reduced at the End and at +30 min after inspiratory flow resistive breathing
379 (Figure 4). There were no main effects of resistive load or time x resistive load interactions
380 (Figure 4).

381 [FIGURE 3] [FIGURE 4]

382 ***Time Course and relationship between markers of oxidative stress and diaphragm fatigue***

383 Although the time course of the increase in plasma F_2 -isoprostanes during inspiratory flow
384 resistive breathing at $P_{dimax}70\%$ corresponded with the decrease P_{diTW} (Figure 5), there were
385 no significant relationships between the individual percentage change from Rest for plasma
386 F_2 -isoprostanes and percentage change from Baseline for potentiated P_{diTW} after $P_{dimax}70\%$
387 (Figure 6).

388 [FIGURE 5] [FIGURE 6]

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396 DISCUSSION

397 ***Main findings***

398 The aim of this study was to examine whether the respiratory muscles of humans contribute
399 to systemic oxidative stress following inspiratory flow resistive breathing, if the amount of
400 oxidative stress is influenced by the level of resistive load, and whether the amount of
401 oxidative stress is related to the degree of diaphragm fatigue incurred. The main finding was
402 that the only measured marker of oxidative stress to increase was plasma F₂-isoprostanes
403 following inspiratory flow resistive breathing at P_{dimax}70%. There were no increases in
404 plasma protein carbonyls and total antioxidant capacity. Further, although we evidenced
405 small reductions in P_{diTW} after inspiratory flow resistive breathing at P_{dimax}50% and
406 P_{dimax}70%, this was not related to the increase in plasma F₂-isoprostanes.

407

408 ***Markers of oxidative stress***

409 We observed an increase in plasma F₂-isoprostanes following inspiratory flow resistive
410 breathing at P_{dimax}70%, but not at P_{dimax}50%. We chose to measure F₂-isoprostanes in blood,
411 and because of their chemical stability and prevalence in all human tissues and biological
412 fluids, this measurement is widely regarded as gold standard for the assessment of oxidative
413 stress (35, 38, 64). F₂-isoprostanes represent a marker of lipid peroxidation and acute exercise
414 and muscle contractions generally increase concentrations in skeletal muscle and plasma (37).
415 Further, F₂-isoprostanes are elevated in the diaphragms of rats exposed to prolonged periods
416 of inspiratory flow resistive breathing (51). Thus, we infer that the increase in plasma F₂-
417 isoprostanes that we observed following inspiratory flow resistive breathing at P_{dimax}70% are
418 released from the contracting respiratory muscles into the systemic circulation. In contrary to
419 our hypothesis, we did not see an elevation of plasma F₂-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

444 increases at 30 min (58) and 1 h (60). The timing of measurements may therefore be
445 important for total antioxidant capacity, and plasma protein carbonyl measurements. For
446 example, around 50 min of exercise resulted in a 32% increase in protein carbonyls 30 min
447 post-exercise and 94% 4 h later (31). Our experimental design unfortunately did not allow us
448 to take measurements beyond 30 min after inspiratory flow resistive breathing as we wanted
449 to mirror the time course of the reduction in P_{diTW} . We acknowledge that this is a limitation of
450 our study design, and future research would aim to undertake blood sampling at later time
451 points. We must also note that whole body exercise engages a significantly greater muscle
452 mass than inspiratory flow resistive breathing.

453

454 ***Diaphragm fatigue and relationship between markers of oxidative stress***

455 Similar to others (19), we observed a reduction in potentiated and non-potentiated P_{diTW}
456 following inspiratory resistive breathing which is indicative of low-frequency peripheral
457 fatigue. The underlying mechanisms are thought to be reduced Ca_2^+ release from the
458 sarcoplasmic reticulum, reduced Ca_2^+ sensitivity of the myofibrils, and/or damaged
459 sarcomeres caused by overextension of the muscle fiber (20). Mild and acute exposure to
460 exogenous ROS generally increases the muscles ability to generate force (11, 24, 61),
461 whereas stronger or prolonged exposure as occurs during flow resistive breathing (1, 7, 12,
462 13, 51), significantly reduces respiratory muscle force generation (19, 45). Indeed, *in vitro*
463 studies have shown that ROS released from diaphragm fibers promotes low-frequency
464 diaphragm fatigue (5, 25, 26, 46, 52). Supplementation with the antioxidant N-acetylcysteine
465 before inspiratory resistive breathing or heavy exercise may also attenuate respiratory muscle
466 fatigue (21, 56). Therefore, we hypothesized that the amount of oxidative stress that we
467 observed would be related to the degree of diaphragm fatigue incurred. However, although

468 the time course of the increase in plasma F₂-isoprostanes during inspiratory flow resistive
469 breathing at P_{di}max70% corresponded with the decrease P_{di}TW, there were no significant
470 relationships between the absolute and relative changes in potentiated and non-potentiated
471 P_{di}TW. These indirect measures of lipid peroxidation and respiratory muscle force generation
472 in our systemic *in vivo* experiment may not be strong enough to demonstrate significant
473 relationships and warrant further experimentation. The source of the increase in plasma F₂-
474 isoprostanes could also be the lung, as previous research had demonstrated that inspiratory
475 resistive breathing in animal models can lead to lung injury and oxidative stress (18, 53-55).
476 This may also explain the lack of relationship between the increases in plasma F₂-
477 isoprostanes and the reduction in P_{di}TW.

478

479 ***Methodological limitations***

480 There are several methodological limitations to our study that need to be acknowledged.
481 Firstly, sex differences occur in respiratory physiology (28, 48), and we acknowledge that our
482 data may be confounded by including both male and female participants. Although in a small
483 sample size, the individual responses presented in Figure 5 do not indicate that there are any
484 sex differences, but this warrants further investigation. Secondly, we did not control the
485 contributions of P_e to P_{di}, which allowed participants to possibly preferentially use their rib
486 cage muscles rather than the diaphragm and to alternate between these muscle groups.
487 Thirdly, the outcome assessor was not blinded to the level of inspiratory resistance or other
488 participant information as they undertook both the experimental testing and analyses. Finally,
489 as our oxidative stress markers are indirect measurements they may have contributed to the
490 lack of association with P_{di}TW.

491

492 **Conclusion**

493 In conclusion, inspiratory flow resistive breathing undertaken at $P_{\text{dimax}}70\%$ 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 $P_{\text{dimax}}50\%$ and $P_{\text{dimax}}70\%$, 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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695 **Competing Interests:** The authors declare no conflict of interest

696 **Author Contributions:** D.R.B., M.A.J., G.R.S., J.S.C., D.E.M., conceived and designed the
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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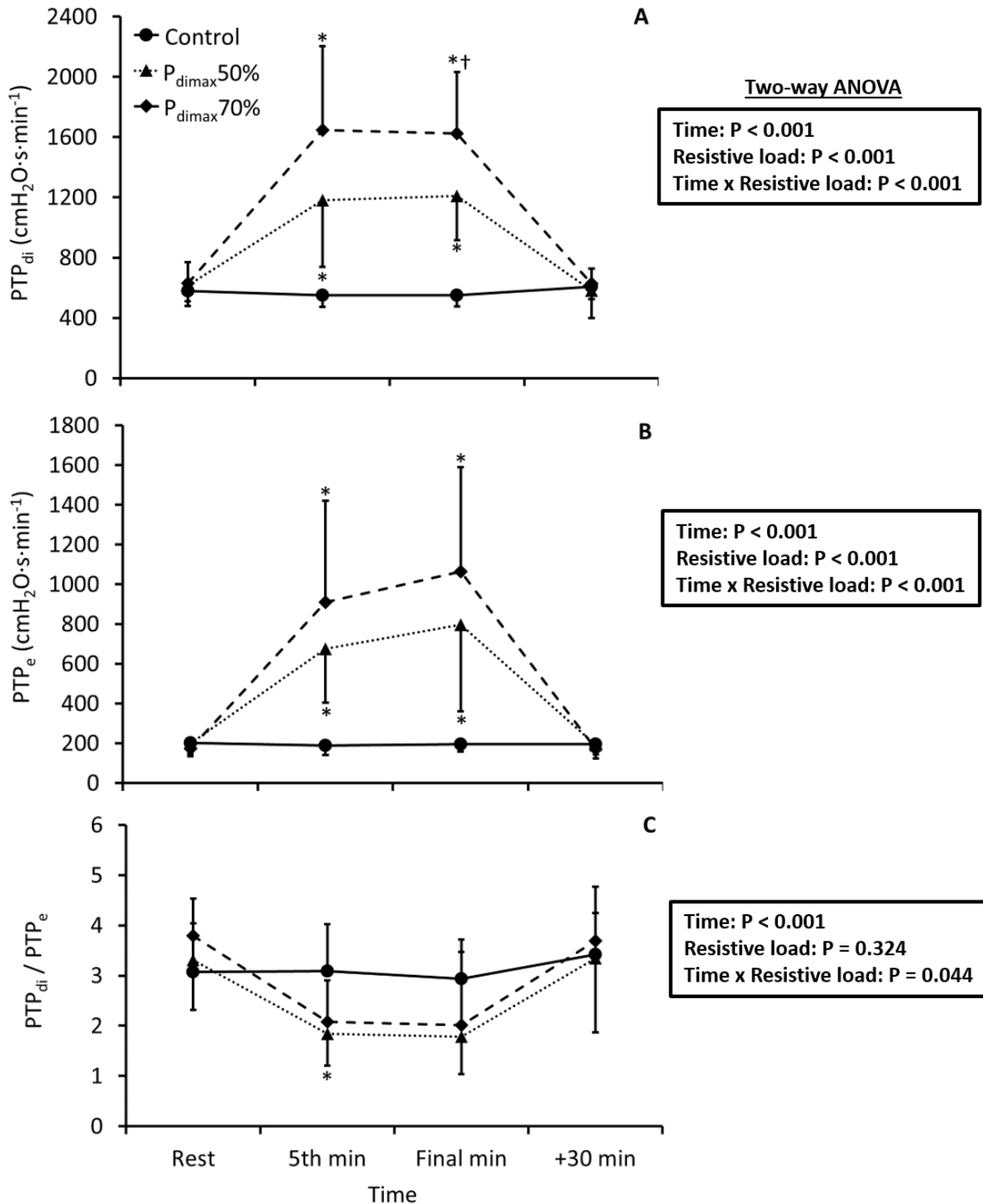
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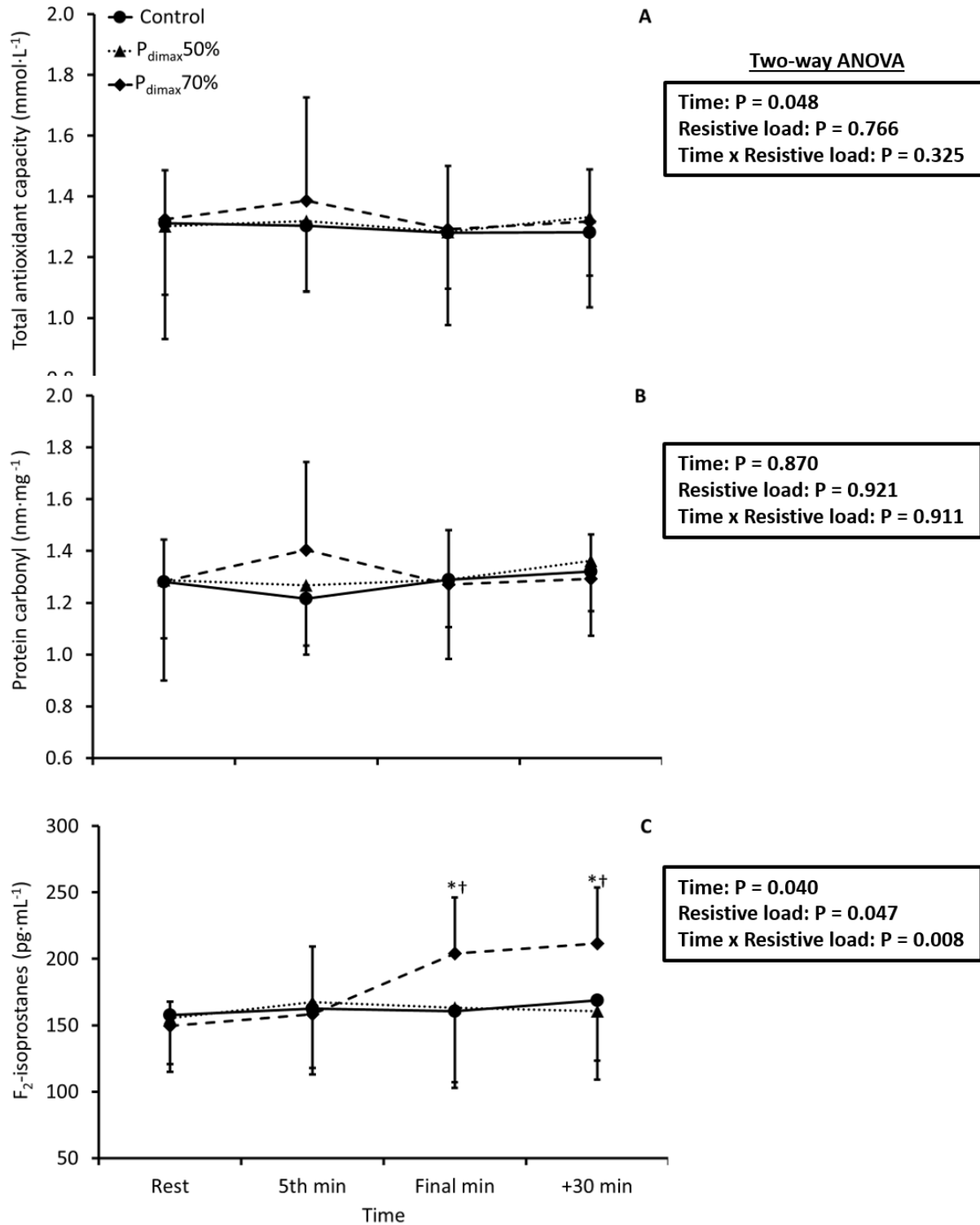
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716 FIGURES



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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).

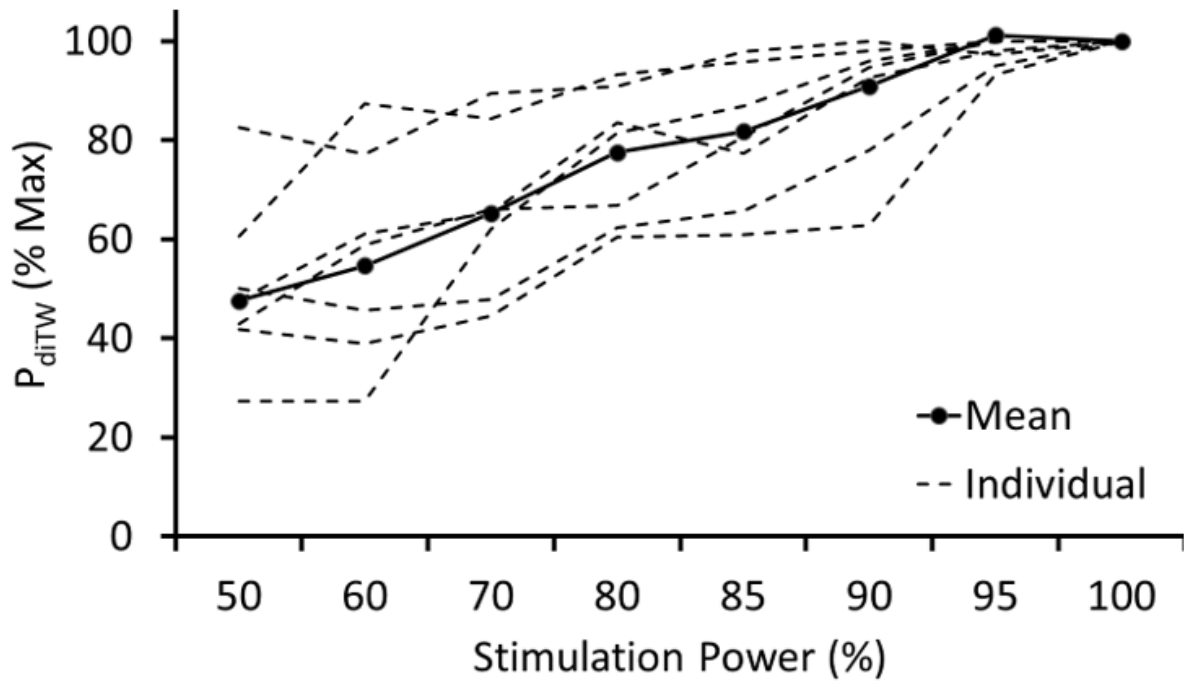


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725 Figure 2. Plasma total antioxidant capacity (A), protein carbonyl (B) and F₂-isoprostane (C)
 726 responses to inspiratory flow resistive breathing for Control and at 50 and 70% of peak
 727 transdiaphragmatic pressure (P_{dimax}50% and P_{dimax}70%). Values are mean ± SD. *
 728 Significantly different from Control (P < 0.05). † Significantly different from P_{dimax}50% (P <
 729 0.05).

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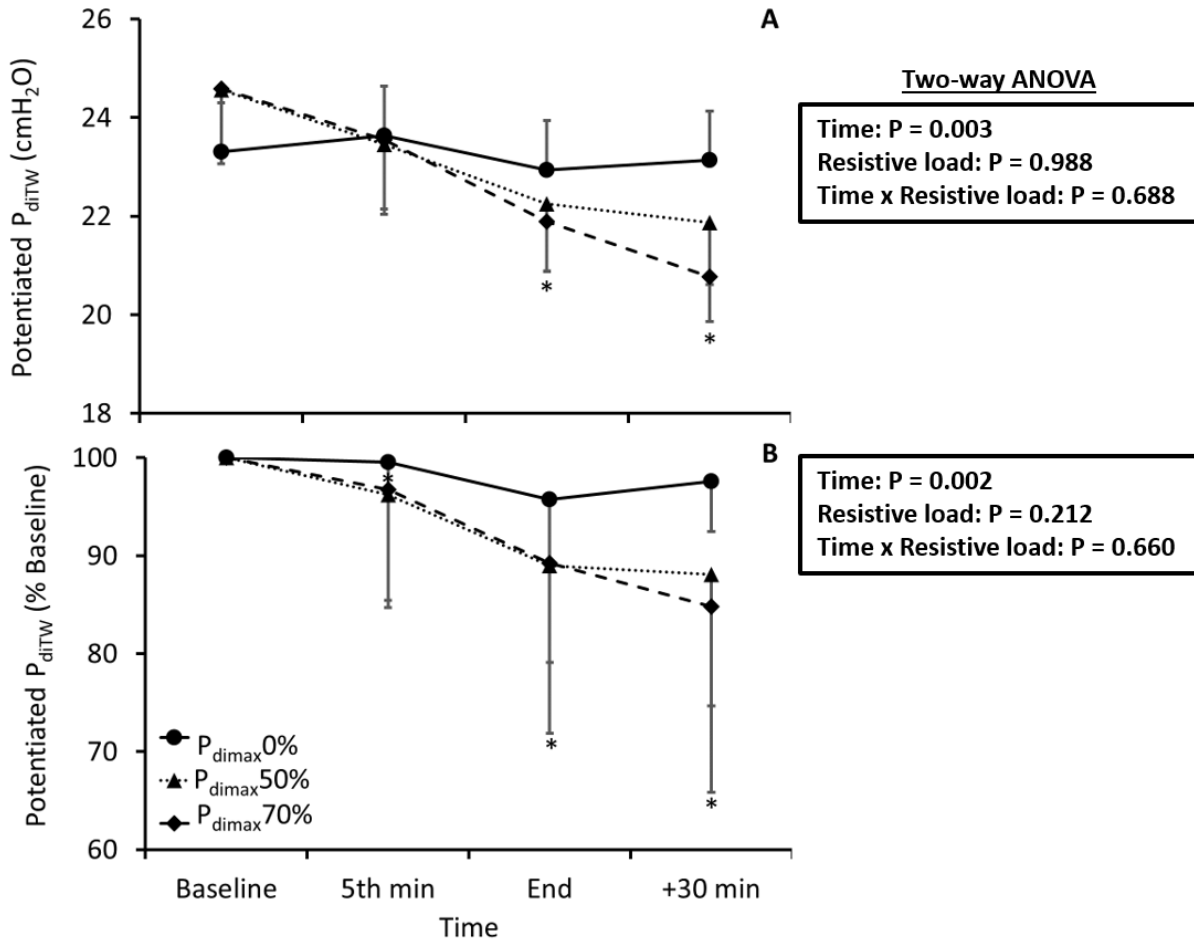
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733 Figure 3. Individual and group mean transdiaphragmatic twitch pressure (P_{diTW}) in response
734 to cervical magnetic stimulation of increasing stimulation intensity.

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

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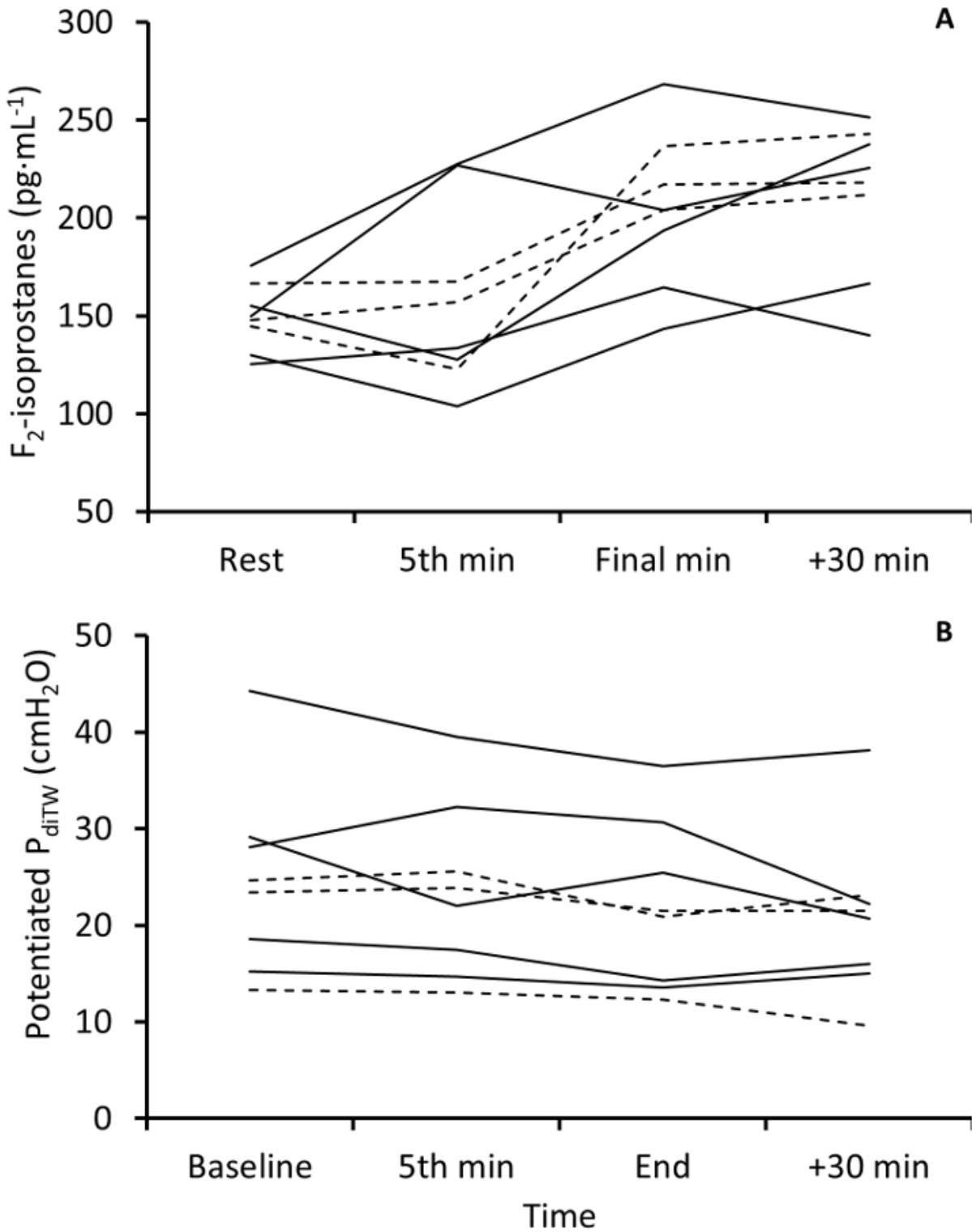
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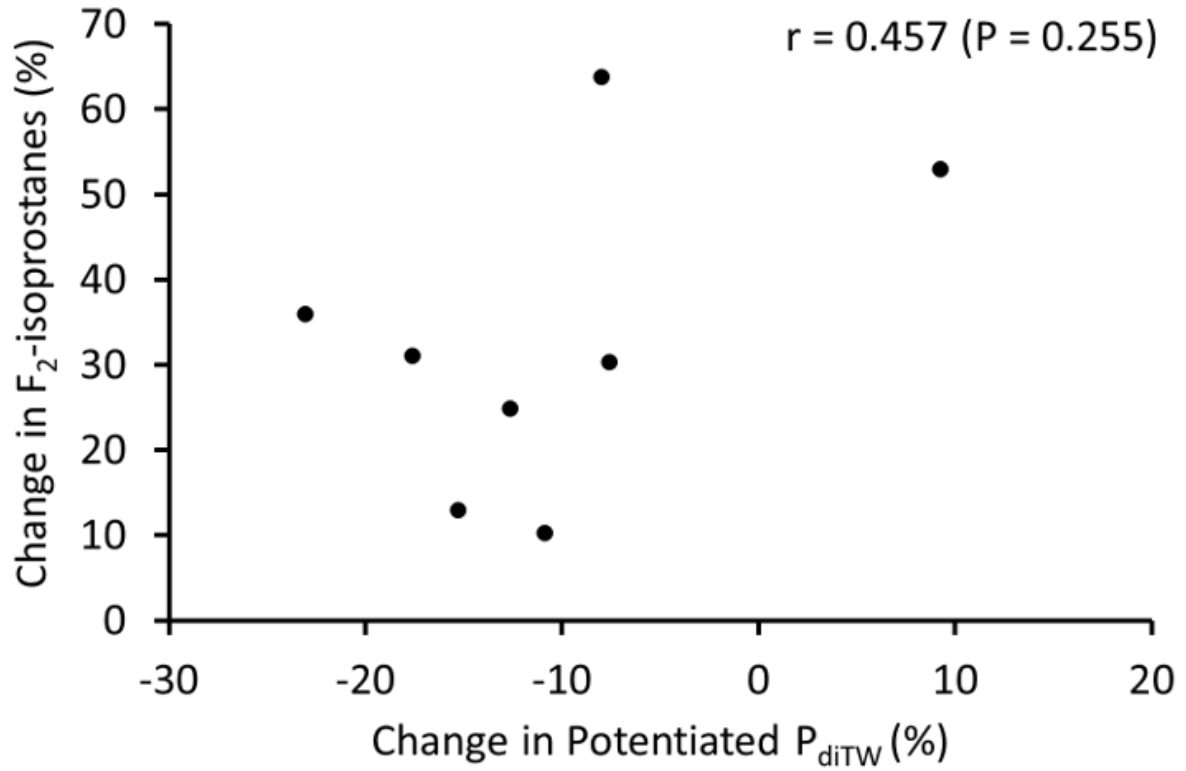
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754 Figure 5. Individual male (solid line) and female (dashed line) plasma F_2 -isoprostanes (A)
 755 and absolute potentiated transdiaphragmatic twitch pressure (P_{diTW}) (B) responses to
 756 inspiratory flow resistive breathing at 70% of peak transdiaphragmatic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

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

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780 TABLES

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

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	Male (n = 5)	Female (n = 3)
Age, years	26 \pm 5	26 \pm 4
785 Height, cm	176 \pm 7	164 \pm 9
Body mass, kg	91 \pm 8	70 \pm 9
786 FVC, L	5.01 \pm 0.79	4.30 \pm 0.94
FVC, % predicted	101 \pm 4	109 \pm 17
787 FEV ₁ , L	4.11 \pm 0.76	3.59 \pm 0.73
FEV ₁ , % predicted	99 \pm 12	106 \pm 15
FEV ₁ /FVC, %	79.8 \pm 7.0	80.7 \pm 1.9
788 FEV ₁ /FVC, % predicted	96 \pm 8	97 \pm 3
P _I max, cmH ₂ O	101 \pm 35	117 \pm 48
789 P _I max, % predicted	92 \pm 22	131 \pm 14
P _{dimax} , cmH ₂ O	90 \pm 27	98 \pm 24

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791 FVC, forced vital capacity; FEV₁, 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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811 Table 2. Cardiorespiratory and perceptual responses to inspiratory flow resistive breathing for
 812 Control and at 50 and 70% of peak transdiaphragmatic pressure ($P_{\text{dimax}50\%}$ and $P_{\text{dimax}70\%}$).
 813 Values are mean \pm SD.

Variable	Resistive load	Rest	5th min	Final min	+30 min
P_{Ipeak} , cmH ₂ O	Control	-1.2 \pm 0.2	-1.2 \pm 0.2	-1.2 \pm 0.2	-1.2 \pm 0.2
	$P_{\text{dimax}50\%}$	-1.1 \pm 0.4	-24.7 \pm 14.1*	-34.5 \pm 22.0*	-1.1 \pm 0.4
	$P_{\text{dimax}70\%}$	-1.3 \pm 0.3	-35.2 \pm 22.1*	-42.1 \pm 25.7*	-1.3 \pm 0.4
P_{epeak} , cmH ₂ O	Control	-10.0 \pm 2.0	-9.5 \pm 1.5	-9.3 \pm 1.3	-10.0 \pm 2.0
	$P_{\text{dimax}50\%}$	-9.6 \pm 2.5	-28.9 \pm 12.2*	-37.3 \pm 19.9*	-9.8 \pm 3.5
	$P_{\text{dimax}70\%}$	-9.4 \pm 2.4	-37.4 \pm 19.8*	-42.6 \pm 20.2*	-9.2 \pm 1.4
P_{dipeak} , cmH ₂ O	Control	28.5 \pm 5.3	27.6 \pm 5.4	26.6 \pm 4.6	30.3 \pm 5.9
	$P_{\text{dimax}50\%}$	27.1 \pm 7.8	47.4 \pm 11.5*	48.5 \pm 11.4*	26.6 \pm 6.6
	$P_{\text{dimax}70\%}$	32.4 \pm 6.2	63.2 \pm 17.1*	60.2 \pm 11.6*	30.4 \pm 5.0
RPD	Control	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0
	$P_{\text{dimax}50\%}$	0.0 \pm 0.0	1.8 \pm 1.3	2.8 \pm 1.4*	0.1 \pm 0.2
	$P_{\text{dimax}70\%}$	0.0 \pm 0.0	4.7 \pm 2.4*†	6.5 \pm 2.6*†	0.3 \pm 0.4*
\dot{V}_E , L·min ⁻¹	Control	9.0 \pm 1.6	11.0 \pm 7.3	9.9 \pm 4.0	9.3 \pm 2.5
	$P_{\text{dimax}50\%}$	10.2 \pm 2.8	10.8 \pm 2.9	11.8 \pm 4.2	9.8 \pm 2.4
	$P_{\text{dimax}70\%}$	8.6 \pm 2.2	11.1 \pm 3.9	9.6 \pm 1.6	9.8 \pm 2.7
f_B , breaths·min ⁻¹	Control	16 \pm 5	15 \pm 0	15 \pm 1	14 \pm 4
	$P_{\text{dimax}50\%}$	14 \pm 3	15 \pm 0	15 \pm 0	15 \pm 5
	$P_{\text{dimax}70\%}$	16 \pm 7	14 \pm 1	15 \pm 0	15 \pm 5
V_T , L	Control	0.68 \pm 0.11	0.88 \pm 0.58	0.80 \pm 0.32	0.88 \pm 0.23
	$P_{\text{dimax}50\%}$	0.95 \pm 0.31	0.88 \pm 0.24	0.94 \pm 0.34	0.89 \pm 0.41
	$P_{\text{dimax}70\%}$	0.71 \pm 0.25	0.98 \pm 0.29	0.78 \pm 0.13	0.86 \pm 0.32
T_I/T_{TOT}	Control	0.44 \pm 0.04	0.45 \pm 0.04	0.44 \pm 0.04	0.44 \pm 0.04
	$P_{\text{dimax}50\%}$	0.43 \pm 0.04	0.52 \pm 0.06*	0.50 \pm 0.08	0.43 \pm 0.05
	$P_{\text{dimax}70\%}$	0.42 \pm 0.06	0.54 \pm 0.06*	0.55 \pm 0.07*	0.43 \pm 0.03
f_C , beats·min ⁻¹	Control	65 \pm 9	66 \pm 9	64 \pm 11	65 \pm 11
	$P_{\text{dimax}50\%}$	70 \pm 16	75 \pm 14	75 \pm 14	67 \pm 17
	$P_{\text{dimax}70\%}$	68 \pm 13	77 \pm 12	80 \pm 13	66 \pm 11
SpO ₂ , %	Control	97.1 \pm 1.2	97.6 \pm 1.1	97.4 \pm 1.2	98.0 \pm 0.9
	$P_{\text{dimax}50\%}$	97.6 \pm 0.9	97.6 \pm 1.1	98.1 \pm 0.7	97.9 \pm 0.7
	$P_{\text{dimax}70\%}$	97.5 \pm 1.1	98.0 \pm 0.6	97.1 \pm 1.4	98.2 \pm 0.7
P_{ETCO_2} , mmHg	Control	36.3 \pm 5.4	34.1 \pm 8.0	34.5 \pm 7.4	35.2 \pm 5.4
	$P_{\text{dimax}50\%}$	34.5 \pm 4.5	34.0 \pm 5.4	35.3 \pm 5.1	34.8 \pm 4.7
	$P_{\text{dimax}70\%}$	34.9 \pm 4.9	35.7 \pm 9.4	35.0 \pm 6.1	34.1 \pm 3.6

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815 P_{Ipeak} , peak inspiratory mouth pressure; P_{epeak} , peak esophageal pressure; P_{dipeak} , peak
 816 transdiaphragmatic pressure; RPD, rating of perceived dyspnea; \dot{V}_E , minute ventilation; f_B ,
 817 breathing frequency; V_T , tidal volume; T_I/T_{TOT} , duty cycle; f_C , cardiac frequency; SpO₂,
 818 estimated arterial oxygen saturation; P_{ETCO_2} , end tidal carbon dioxide pressure. *

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

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