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INTEGRATIVE PHYSIOLOGY

Effects of non-fatiguing respiratory muscle loading induced by expiratory flow limitation during strenuous incremental cycle exercise on metabolic stress and circulating natural killer cells

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Abstract Exercise induces release of cytokines and increase of circulating natural killers (NK) lymphocyte during strong activation of respiratory muscles. We hypothesised that non-fatiguing respiratory muscle loading during exercise causes an increase in NK cells and in metabolic stress indices. Heart rate (HR), ventilation (VE), oesophageal pressure (Pes), oxygen consumption (VO_2), dyspnoea and leg effort were measured in eight healthy humans (five men and three women, average age of 31 ± 4 years and body weight of 68 ± 10 kg), performing an incremental exercise testing on a cycle ergometer under control condition and expiratory flow limitation (FL) achieved by putting a Starling resistor. Blood samples were obtained at baseline, at peak of exercise and at iso-workload corresponding to that reached at the peak of FL exercise during control exercise. Diaphragmatic fatigue was evaluated by measuring the tension time index of the diaphragm. Respiratory muscle overloading caused an earlier interruption of exercise. Diaphragmatic fatigue did not occur in the two conditions. At peak of flow-limited exercise compared to iso-workload, HR, peak inspiratory and expiratory Pes, NK cells and norepinephrine were significantly higher. The number of NK cells was significantly related to ΔPes (i.e. difference between the most and the less negative Pes) and plasmatic catecholamines. Loading of respiratory muscles is able to cause an increase of NK cells provided that activation of respiratory muscles is intense enough to induce a significant metabolic stress.

Keywords Respiratory muscle · Strenuous exercise · Physiology · Immunology · Dyspnoea

Introduction

Whole-body exercise induces increase of circulating lymphocyte subpopulations, particularly natural killer (NK) lymphocytes, occurring soon after the start of exercise [33]. The lymphocyte concentration increases during exercise is due to recruitment of all lymphocyte subpopulations from other tissue pools (spleen, lymph nodes and gastrointestinal tract) to the vascular compartment. The number of cells that enter the circulation is determined by the intensity of the stimulus. CD4 and CD8 T cells, CD19 B cells, CD16 natural killer (NK) cells and CD56 NK cells increase in number during exercise and decline after intense exercise lasting at least 1 h. Generally, NK cell activity is increased when measured immediately after or during both moderate and intense exercise of a few minutes, being these definitions of moderate and intense exercise in accordance with the American College of Sports Medicine [10]. If the exercise has lasted for a long period

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and has been very intense (e.g. a triathlon race), only a modest increase in NK cells is found post-exercise, and the functions of NK cells are suppressed. The mechanisms underlying exercise-associated lymphocyte changes are multifactorial and include neuroendocrinological factors such as adrenaline and to a lesser degree noradrenaline responsible for acute exercise effects on lymphocyte dynamics, including exercise effects on NK cell activity [9, 22, 23, 30, 34, 37].

Whole-body exercise also induces increase of cytokines: effects on cytokines are based on the intensity and duration of the physical exercise [29]. After a marathon race, TNF-alpha and IL-1beta levels increase twofold and IL-6 levels increase up to 100-fold [32]. Measurements from cycle ergometer and treadmill running show no response of short-term exercise and an increase in IL-6 after 1 h of intense cycling exercise, and 3-h alternating cycling and treadmill running showed high values of IL-6, IL-1β and TNF-α [27, 41]. The mechanical disruption of myofibers initiates local and systemic production of cytokines. Exercise is not characterised by a fully developed systemic proinflammatory response. This lack for systemic response may be due to only a transient cytokine release in response to exercise. Alternatively, this may reflect an adaption to the cytokine response (e.g. increased ability to induce effective natural occurring inhibitory cytokines and cytokine receptors) [34]. Exercise-induced release of cytokines has been also observed during strong activation of respiratory muscles, as shown by the observation that 45-min strenuous inspiratory resistive breathing causing diaphragmatic fatigue induces a cytokine response analogue, even if of a lesser intensity, occurring during strenuous whole-body exercise [43, 44].

During exercise in healthy subjects, breathing requires an increase in both expiratory and inspiratory muscle activity [1, 38]. Fatigue in respiratory muscles can affect exercise tolerance, leading to dyspnoea and increased forced respiration [14, 15, 39]. Expiratory muscles may be even more prone to fatigue than inspiratory muscles as several studies have shown that expiratory muscles are in general less oxidative than inspiratory muscles [19, 42] and because expiratory muscle fatigue enhances neuromuscular activity of respiratory muscles [21].

Beside blood flow distribution and oxygen delivery [3, 4], little is known on the effects of non-fatiguing respiratory muscle loading during strenuous incremental exercise on lymphocyte and cytokine modifications [3, 4] in healthy subjects. We did investigate the impact of combined incremental strenuous cycling exercise and overloading respiratory muscles caused by a Starling resistor-induced expiratory flow limitation on lymphocyte subpopulations, cytokine production, neuroendrocrinological factors and muscle damages. The primary aim of our study was to investigate changes induced by exercise performed with expiratory flow limitation on lymphocyte subpopulation, cytokine quantification, plasmatic catecholamines and muscle enzymes. The second aim was to determine the impact of neuroendocrinological factors in the mechanisms underlying exercise-associated lymphocyte changes.

Methods

To test these hypotheses, in eight normal subjects, we compared the changes in transdiaphragmatic pressure, dyspnoea intensity, lymphocyte subpopulations, proinflammatory cytokines and plasmatic catecholamine release observed during a standard maximal incremental cycling exercise with those observed during a maximal incremental cycling exercise performed while overloading respiratory muscles by placing a Starling resistor on the expiratory line, causing an expiratory flow limitation.

Subjects

Eight healthy humans (five men and three women, average age of 31 ± 4 years and body weight of 68 ± 10 kg) were included. All were non-smokers and have experience in physiological studies and in performing respiratory manoeuvres. All the subjects had a normal forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) (114 ± 12 and 111 ± 10% of predicted values, respectively) [35]. The research was carried out in accordance with the principles outlined in the Declaration of Helsinki. The study was approved by the local ethics committee, and all the subjects signed written, informed consent after a detailed description of the protocol at the time of their first assessment.

Protocol

The study was carried out over 3 days. All the experiments were carried out in the morning while the participants were on fasting.

On day 1, the eight subjects performed a routine spirometry, with the measurement of static and dynamic lung volumes, according the recommendations of the American Thorax Society (ATS)/European Respiratory Society (ERS) [26]. The forced expiratory volume at the first second (FEV_1) and the forced vital capacity (FVC) were measured during maximal expiratory manoeuvres by Vmax (SensorMedics, Yorba Linda, CA) according to the ATS/ERS recommendations [26]. The functional residual capacity (FRC) was measured by the wash out N_2 technique (Vmax SensorMedics, Yorba Linda, CA). Predicted normal values were derived from standard equations. Values were expressed as percentages of the

predicted normal values calculated according to gender, weight and age. Reference equations were taken from the ERS [35].

Subsequently, to familiarise the subjects with the procedure, all the subjects performed a maximal incremental cycle exercise test according with the ATS/ACCP 2003 standards [36].

On day 2, the eight subjects performed flow-limited exercise: subjects seated on a cycloergometer and breathed through a mouthpiece and a Fleisch type 3 pneumotachograph attached to a Hans Rudolph valve, which separated inspiratory from expiratory flow. Dead space of the system was 75 mL. The subjects performed a symptom-limited incremental exercise test seated on a cycle ergometer (Ergometrics 800S, SensorMedics, Yorba Linda, CA). The equipment was calibrated before each test. All exercise tests consisted of a steady-state resting period of 6 min and a 3-min warm-up of unloaded pedalling, followed by an incremental test in which the work rate (WR) was increased in 1-min intervals by increments of 10 W until the point of symptom limitation (peak exercise). The subjects were instructed to maintain the pedalling rate at 60 rpm. Flow limitation was achieved by putting a Starling resistor that limited expiratory flow to ~ 0.8–1 L/s on the expiratory port of the valve. Expired carbon dioxide ($PETCO_2$) was sampled continuously at the mouth by an infrared carbon dioxide meter (Datex Normocap; Helsinki, Finland). Oxygen uptake (VO_2), carbon dioxide output (VCO_2) and minute ventilation (VE) were analysed using breath-by-breath analysis from the Vmax system. Heart rate (HR) and arterial oxygen saturation (SaO_2) were assessed with a pulse oxymeter (Masimo Rad 9, USA) by means of an ear probe. Exercise variables were measured continuously and averaged over the last 30 s of each minute and at peak exercise. Peak work rate (WRpeak), peak oxygen uptake ($V'O_2$peak) and peak ventilation (V'Epeak) were defined, respectively, as the highest level of exercise and the highest $V'O_2$ and V'E that could be sustained for at least 30 s during the last stage of exercise.

On day 3, the eight subjects performed control exercise: the experimental procedure was the same as that in the second day, without expiratory flow limitation; *rest* was the steady-state period after at least 3 min of breathing on the mouthpiece before exercise began; *peak* was the last 30 s of loaded pedalling; and *iso-workload* was defined as the highest equivalent exercise WR achieved by all the participants during their respective flow-limited exercises. To determine iso-workload and time to perform analysis, design needed expiratory flow limitation on the first place (day 2). The sequence of exercise test was the same for all the subjects and was not randomised. Although the first experimental session was performed to familiarise the subjects with the procedure, it is unlikely that in these healthy subjects, this might have influenced the data collected during the two exercise bouts performed later, independently of order of test.

Of note, flow-limited exercise corresponds to whole-body exercise because of both components of the applied stress on the legs (cycle exercise) and on the respiratory muscles (strenuous resistive breathing).

Pleural and transdiaphragmatic pressures

Oesophageal pressure (Pes) was measured by a standard balloon-tipped catheter, introduced via the nose and connected to a pressure transducer (± 100 cm H_2O, Validyne, Northridge, CA) and was used as index of pleural pressure (Ppl). The balloon was positioned in the midoesophagus and contained 0.4 mL of air. Gastric pressure (Pg) was simultaneously measured with a similar balloon-catheter system connected to a second differential pressure transducer. This balloon was positioned in the stomach 65–70 cm balloon tip to nares, and contained 2 mL of air. Transdiaphragmatic pressure (Pdi) was obtained by subtracting Pes to Pg. From the oesophageal pressure signal, we determined the less negative oesophageal (expiratory) pressure (Pesmax), the most negative oesophageal (inspiratory) pressure (Pesmin), and the difference between the most negative (peak value during inspiration) and the less negative pleural pressure (at end expiration) during tidal breathing (ΔPes).

Inspiratory muscle strength was assessed by measuring minimal (i.e. the greatest negative) inspiratory pleural pressure sniff (Pessn) and maximal transdiaphragmatic pressure sniff (Pdisn) at FRC during sniff manoeuvres [25]. The subjects were repeatedly encouraged to try as hard as possible and they had a visual feedback of generated pressure. The manoeuvres were repeated until three measurements with less than 5% variability were recorded, and the lowest Pessn and the highest Pdisn values obtained were used for analysis. The presence of diaphragmatic fatigue was evaluated by measuring the tension time index of the diaphragm according to Bellemare and Grassino [5]. In brief, we calculated the tension time index of the diaphragm by multiplying the inspiratory duty cycle (inspiratory time over total time to breathe, TI/TTOT) by the Pdi measured at end-inspiration during the last step of exercise test and expressed as percent of the maximal Pdi. The obtained values were compared with the threshold value for fatigue (0.15) calculated by Bellemare and Grassino [5].

Pressure and flow signals were recorded onto an IBM-compatible personal computer by a 16-channel analog/digital board at 50-Hz sampling rate (National Instrument DAQCard 6024E).

Dyspnoea and leg sensation

Dyspnoea, or breathlessness, was defined as "the unpleasant sensation of laboured or difficult breathing," and leg discomfort as "the level of leg discomfort experienced during

exercise" as previously described [16–18]. By pointing to a 10-point Borg scale [7] from 0 (none) to 10 (maximal), the subjects rated the magnitude (intensity) of their perceived breathlessness and leg discomfort at rest, every minute and at peak exercise.

Biochemical variables

Lymphocyte subpopulations

Lymphocyte subpopulations were measured at baseline of each exercise, at iso-workload and at peak of each exercise. Lymphocyte subpopulations were not measured at 3 and 6 h after each exercise. To evaluate the percentages of lymphocyte, NK, T and B lymphocyte, 2×10^5 cells were collected and stained with anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD16, anti-CD20 or anti-CD56 fluorochrome-conjugated MAbs (Becton Dickinson). Cells were analysed on a FACSCalibur cytofluorimeter, using the CellQuest software (Becton Dickinson). The area of positivity was determined by use of an isotype-matched MAb.

Cytokine quantification

Cytokine quantification was performed at baseline of each exercise, at iso-workload, at peak of each exercise, and at 3 and 6 h after each exercise. Blood was drawn into sterile syringes and transferred to precooled sterile EDTA tubes. Samples were immediately spun in a refrigerated centrifuge to separate plasma from cells and thus avoid ex vivo cytokine secretion, and were next placed in polystyrene tubes and stored at −70 °C until assayed. Plasma levels of IL-6, IL-1β and TNF-α were measured with commercially available ELISA kits (Quantikine, R&D Systems, Minneapolis, MN). In consideration of the variable response kinetics of exercise-related cytokines (sometimes late increase toward the end of exercise or immediately after or hours late after exercise), their plasmatic level was also measured 3 and 6 h after the end of exercise [34].

Plasmatic catecholamines

Catecholamines (noradrenaline, adrenaline) were measured at baseline of each exercise, at iso-workload and at peak of each exercise by a radioenzymatic method as previously described [28]. Catecholamines were not measured at 3 and 6 h after each exercise.

Muscle enzymes

Creatine kinase (CK) and myoglobin (Mb) serum concentrations were measured as indexes of muscle damage at baseline of each exercise, at iso-workload, at peak of each exercise, and at 3 and 6 h after each exercise. Serum samples were separated from whole blood by using Vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) by centrifugation at 1000*g* for 10 min after the blood was allowed to clot at room temperature for 30 min. Serum CK activity was measured by using biochemical assay kits (Baxter Diagnostics, Deerfield, IL, USA); the normal limits were 20–160 U/ml. Mb concentration was measured by using chemiluminescent (CL) enzyme immunoassay kits; the normal limits were 0–75 ng/ml.

Statistics

We studied eight non-smoking consecutive healthy subjects. This is a complex and heavy study involving two strenuous cycle exercise testing (control vs flow-limited) with pressure-related respiratory mechanics measurements (oesophageal and gastric balloons and pressures) and several blood samples obtained during exercises. Nevertheless, this small sample size yet provides an 80% power to detect a significant difference (two-sided $\alpha = 0.05$) in dyspnoea intensity (Borg scale) measured at a standardised work rate during incremental cycle cardiopulmonary exercise testing, based on a relevant difference in Borg ratings of ~ 1.5, a standard deviation of ~ 1.0 for Borg rating changes having been found in our laboratory. Values are expressed as mean ± standard error (SE), unless otherwise specified (standard deviation, SD). To determine the significance of differences in various parameters between control and flow-limited exercises, we used the parametric Student's matched pair *t* test, and two-way analysis of the variance (ANOVA) for repeated measures. The continuous variables were compared by means of a two-way ANOVA, followed if positive, by a posterior comparison by Duncan multiple range test. Correlations were estimated with Bravais-Pearson correlation coefficients. The level of significance was set up at $p < 0.05$. All statistical procedures were carried out using the SPSS 14.0 for Windows (SPSS Inc., Chicago IL, USA).

Results

Incremental exercise test

Concerning the results of incremental exercise testing, all relative values here are expressed as mean ± SD.

Although HR at peak control exercise (days 1 and 3) was relatively low for such young subjects (151 ± 5 beats/min, which corresponds to 80 ± 9% of predicted maximal HR), we were satisfied that a maximal symptom-limited exercise was achieved in all the healthy subjects during control exercise (days 1 and 3) based on motivational effort: the subjects

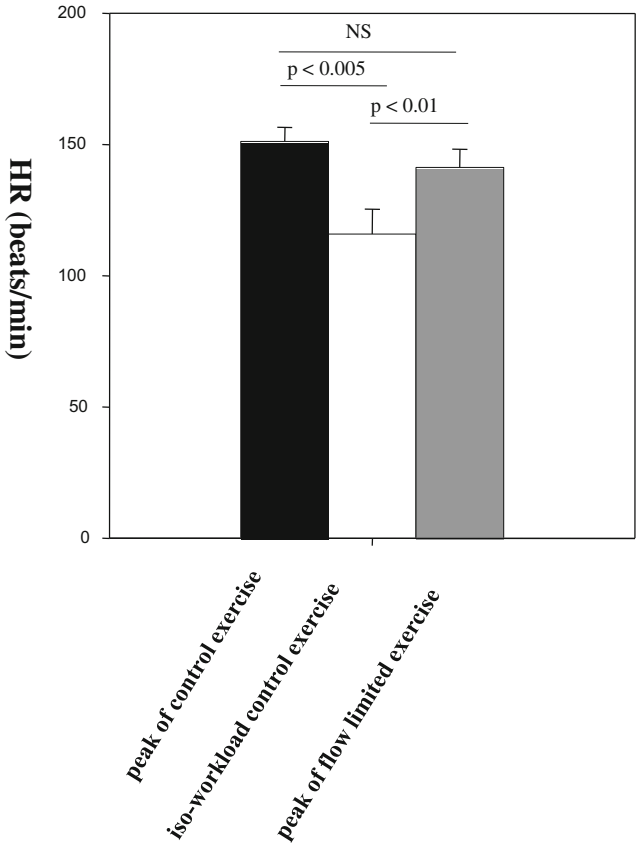

Fig. 1 Changes in heart rate induced by exercise. Black column: peak of control exercise; white column: iso-workload control exercise; grey column; peak of flow-limited exercise. *HR* heart rate

reported intolerable exertional leg discomfort at peak exercise (9 ± 1 arbitrary units), had a respiratory exchange ratio at peak > 1.10 (RER = 1.15 ± 0.05) and a plateau in peak VO_2 for the majority [36]. SaO_2 was pretty stable during control exercise and did not fall (97 ± 0.5%).

Exercising with expiratory flow limitation caused an earlier interruption of exercise compared to control test: the maximal achieved workload was 158.75 ± 33.1 W in control test and 97.5 ± 23.1 W during flow-limited exercise ($p < 0.002$). The maximal oxygen consumption (VO_2) was 2.49 ± 0.42 L/min (92.38 ± 11.61% of predicted value) [12] during control exercise and 1.85 ± 0.33 L/min (69.1 ± 13.3% of predicted value) during flow-limited exercise; at iso-workload conditions, VO_2 was 1.54 ± 0.27 L/min. VO_2 at peak of flow-limited exercise was lower compared to the value observed at the peak of control exercise ($p < 0.0001$), but significantly greater compared at the value observed at iso-workload ($p < 0.01$).

Heart rate was similar at the peak of the two exercise tests, while at iso-workload, it was lower than both peak values (Fig. 1).

The maximal achieved ventilation was 81.9 L/min in control conditions and 38.8 with expiratory flow limitation; at iso-workload in control conditions, it was 48.9 L/min. Ventilation at peak flow-limited exercise was significantly lower than that both at peak control exercise ($p < 0.0001$) and at iso-workload ($p < 0.002$).

The maximal $PETCO_2$ was 34 ± 1 mmHg during control exercise and 48 ± 2 mmHg during flow-limited exercise; at iso-workload conditions, $PETCO_2$ was 39 ± 1 mmHg. $PETCO_2$ at peak of flow-limited exercise was significantly higher compared to the value observed at the peak of control exercise ($p = 0.0002$), and compared with the value observed at iso-workload ($p = 0.002$).

Pleural and transdiaphragmatic pressures

Values of end-expiratory and end-inspiratory oesophageal/pleural pressure during flow-limited and control exercises are showed in Fig. 2, left panel.

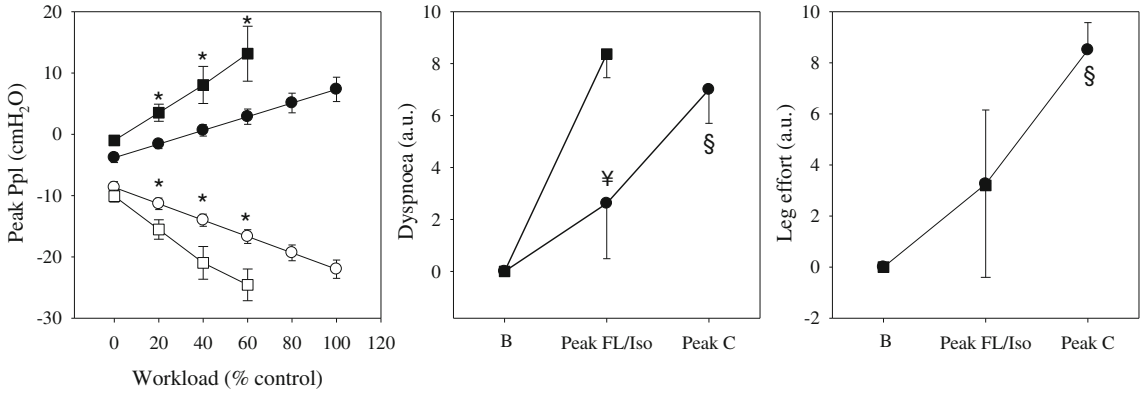

Fig. 2 Left panel: end-expiratory (filled symbols) and end-inspiratory (empty symbols) pleural pressure (Ppl—Pes in the manuscript) values during control (circles) and flow-limited (squares) exercise. The distance between end-inspiratory and end-expiratory values is ΔPes. Middle and right panels: dyspnoea sensation and leg fatigue sensation scores, respectively, at baseline, at peak of both control and flow-limited exercises and at iso-workload. Circles: control exercise, squares: flow-limited exercise. B: baseline, peak C: peak of control exercise, peak FL: peak of flow-limited exercise, iso: iso-workload. * indicates a significant difference (< 0.05) between control and flow-limited exercises at standardised work load expressed as percentage of control exercise (20, 40 and 60%). § indicates a significant difference (< 0.05) between values at peak FL and at peak C. ¥ indicates a significant difference (< 0.05) between values at iso-workload and at peak FL

At peak of flow-limited exercise, values of Pesmax and Pesmin did not show significant difference when compared to peak of control exercise (Table 1).

At peak of flow-limited exercise, ΔPes, which is the expression of the global activation of both inspiratory and expiratory muscles, was significantly higher than at peak of control exercise (Table 1).

At iso-workload, values of Pesmax, Pesmin and ΔPes were significantly lower than at peak of control exercise (Table 1).

At iso-workload, values of Pesmax, Pesmin and ΔPes were significantly lower than at peak of flow-limited exercise (Table 1).

Pdi values measured at end-inspiration during the last step of exercise test and expressed as percent of the maximal Pdi (Pdi sniff) showed an average value of 16 ± 2.4% during control exercise and 16 ± 2.6% during flow-limited exercise. The tension time index of the diaphragms was 0.079 ± 0.012 and 0.052 ± 0.007, during control and flow-limited exercises, respectively.

Dyspnoea and leg sensation

All values here are expressed as mean ± SD.

Dyspnoea progressively increased during both exercise trials. Borg score reached a maximum mean value of 7.0 ± 1.1 during control exercise and 8.0 ± 1.0 during expiratory flow-limited exercise ($p < 0.035$) (Fig. 2, middle panel). At iso-workload, Borg score was 3.0 ± 2.0, which is significantly lower compared to that recorded at the peak of flow-limited exercise ($p < 0.0001$).

Leg discomfort sensation progressively increased during exercise, reaching a maximum mean value of 9.0 ± 1.0 during control exercise and 3.0 ± 4.0 during flow-limited exercise ($p < 0.003$) (Fig. 2, right panel). At iso-workload, leg fatigue sensation was not different from that observed at the peak of flow-limited exercise (3.0 ± 3.0 vs 3. 0 ± 4.0, p = ns).

Table 1 Pesmax, Pesmin and ΔPes at peak of control exercise, at iso-workload and at peak of flow-limited exercise

	Pesmax (cm H_2O)	Pesmin (cm H_2O)	ΔPes (cm H_2O)
(A) Peak control	9.3 ± 2.1	− 22.9 ± 1.7	32.6 ± 2.5
(B) Iso-workload	2.3 ± 1.8	− 16.5 ± 1.1	18.6 ± 2.4
(C) Peak flow-limited	17.1 ± 5.1	−26.1 ± 2.5	40.6 ± 4.5
A vs B, p =	0.002	0.024	0.001
A vs C, p =	0.086	0.156	0.034
B vs C, p =	0.005	0.011	0.0001

Values are expressed as mean ± standard error

Pesmax less negative pressure developed during tidal breathing, *Pesmin* more negative pressure developed during tidal breathing, *ΔPes* difference between Pesmax and Pesmin

Biochemical variables

Lymphocyte subpopulations

During both control and flow-limited exercises, total lymphocytes significantly increased compared to those at baseline (respectively, $p < 0.0001$ and $p < 0.001$). Peak values during flow-limited exercise were not different from control peak values, but significantly greater compared to those at iso-workload ($p < 0.025$).

During both control and flow-limited exercises, CD16+/CD56+ natural killer lymphocytes (as percent of total lymphocytes) significantly increased compared to those at baseline (respectively, $p < 0.001$ and $p < 0.0001$). Peak values during flow-limited exercise were not different from control peak values, but significantly greater than those at iso-workload ($p < 0.003$) (Fig. 3).

No significant modulation of other cell types was observed (data not showed).

Cytokine quantification

Plasmatic values of IL-6, IL-1 IL-1β and TNF-α did not show any significant change from rest to peak exercise and 3 and 6 h after the end of exercise, either during control or flow-limited exercise (NS for both tests, data showed in Fig. 4).

Plasmatic catecholamines

During both control and flow-limited exercises, plasmatic norepinephrine and epinephrine ($p < 0.0001$ for both) increased significantly (Fig. 5). Norepinephrine peak values of both control and flow-limited tests were significantly higher than those at baseline ($p < 0.0001$ for both) and iso-workload ($p < 0.001$ for both), with no difference between them. For epinephrine, peak values of control exercise were significantly higher than those at baseline and iso-workload ($p < 0.0001$ and $p < 0.001$, respectively), while peak values of flow-limited exercise were higher than those at baseline ($p < 0.018$), but not different from those of both iso-workload and peak control exercises (Bonferroni's test).

Values of both norepinephrine and epinephrine measured at baseline, peak flow-limited exercise, iso-workload and peak control exercise significantly related to ΔPes values recorded in the same conditions (pooled data, $r = 0.74$, $p < 0.0001$ and $r = 0.54$, $p < 0.001$, respectively) (Fig. 6, right upper panels). Moreover, the number of $CD16^+$ and $CD56^+$ NK cells (as percentage of total lymphocytes) observed at baseline, peak flow-limited exercise, iso-workload and peak control exercise significantly related to both norepinephrine (pooled data, $r = 0.66$, $p < 0.0001$ and $r = 0.62$, $p < 0.0001$, respectively) values (Fig. 6, left lower panels) and epinephrine (pooled data, $r = 0.42$, $p < 0.018$ and

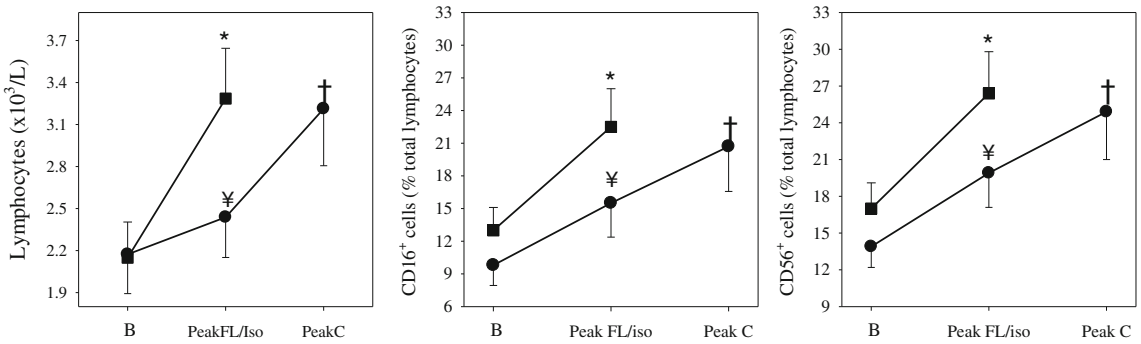

Fig. 3 Total blood lymphocytes (left panel), expressed as absolute number, CD16+ lymphocytes (middle panel) and CD56+ lymphocytes (right panel), expressed as percentage of total lymphocytes, during both control (circles) and flow-limited (squares) exercises at baseline, iso-workload and peak exercise. B: baseline, peak C: peak of control exercise, peak FL: peak of flow-limited exercise, iso: iso-workload. * indicates a significant difference (< 0.05) between values at peak FL and at baseline. † indicates a significant difference (< 0.05) between values at peak C and at baseline. ¥ indicates a significant difference (< 0.05) between values at iso-workload and at peak FL

$r = 0.42$, $p < 0.016$, respectively) values (Fig. 6, right lower panels) and to ΔPes values (pooled data, $r = 0.6$, $p < 0.0001$ and $r = 0.62$, $p < 0.0001$, respectively) (Fig. 6, left upper panels) recorded in the same conditions.

Muscle enzymes

Serum CK showed a small but significant increase from rest to peak during control exercise ($p = 0.003$), even if values were still within the limits of the normal range in most subjects, while no change was observed at 3 and 6 h after control exercise and during flow-limited exercise (NS) (data showed in Fig. 4). Serum myoglobin did not change in either test (NS for both) (data showed in Fig. 4).

Discussion

The novel findings of this study are as follows: (i) loading of respiratory muscles during an incremental exercise test causes an increase of the indices of metabolic stress (norepinephrine and heart rate) and of NK cells similar to that observed during a control exercise at a higher work rate, and (ii) indices of metabolic stress, number of circulating NK cells and intensity of respiratory muscle exercise are closely related.

Exercising with a respiratory flow limitation affected significantly exercise performance: maximal achieved workload was reduced by 39%, minute ventilation decreased and both inspiratory and expiratory peak pressure increased significantly (see Fig. 1), these changes being in line with the results of previous studies [2, 4, 11].

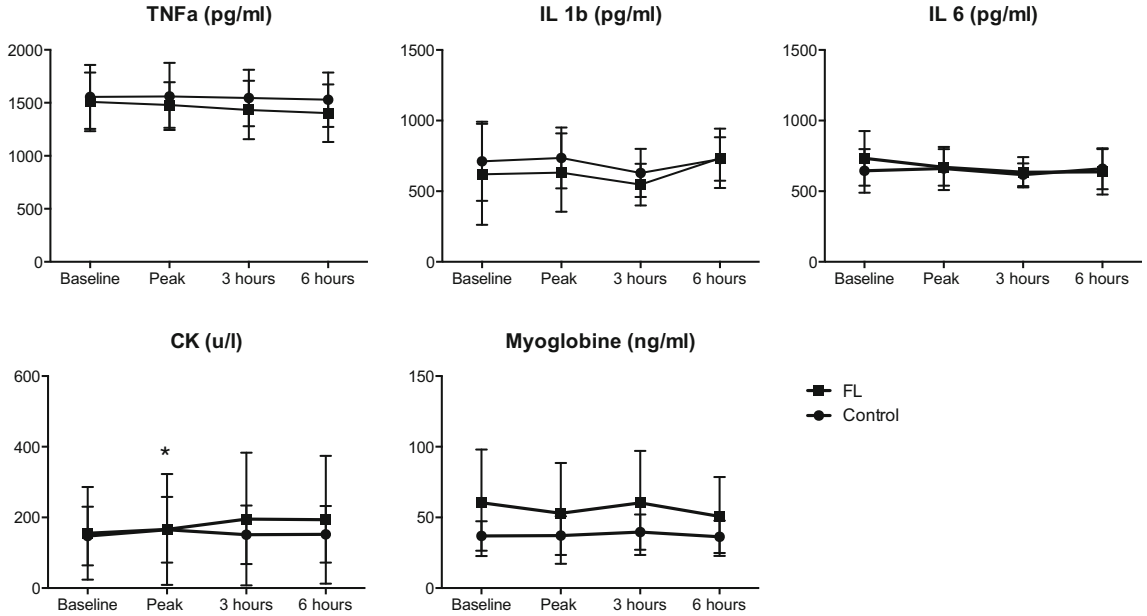

Fig. 4 Figure showing the data regarding cytokines (e.g. IL-6, IL-1b, TNF-α) and muscle damage markers (e.g. CK (creatine kinase), myoglobin) during baseline (B), peak control exercise (C), peak flow-limited exercise (FL), 3 h and 6 h. * indicates a significant difference (< 0.05) between values at peak C and at baseline

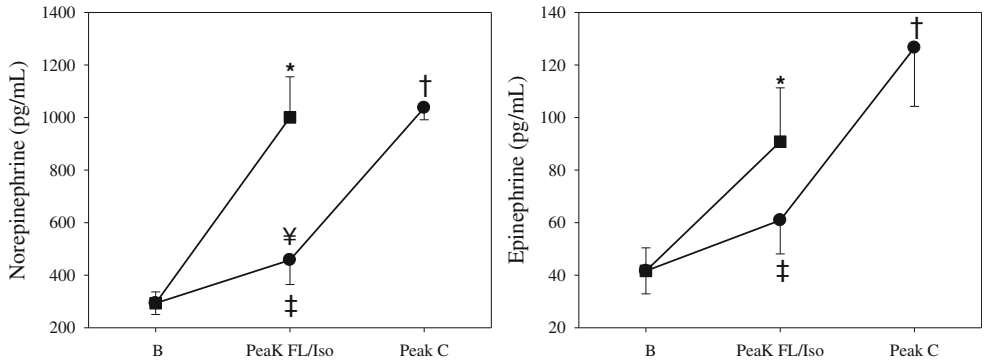

Fig. 5 Norepinephrine (left panel) and epinephrine (right panel) plasmatic levels during both control (circles) and flow-limited (squares) exercises at warm-up, iso-workload and peaks of exercise. B: baseline, peak C: peak of control exercise, peak FL: peak of flow-limited exercise, iso: iso-workload. * indicates a significant difference (< 0.05) between values at peak FL and at baseline. † indicates a significant difference (< 0.05) between values at peak C and at baseline. ‡ indicates a significant difference (< 0.05) between values at iso-workload and at peak C. ¥ indicates a significant difference (< 0.05) between values at iso-workload and at peak FL

The high inspiratory and expiratory pleural pressures are expression of a greater activation of both inspiratory and expiratory muscles due to a greater neural drive to breathe, and demonstrate that during the flow-limited test, respiratory muscles performed a strenuous exercise. Our results are in line with those of previous reports showing that imposition of flow limitation, by causing an increase in mean expiratory pleural pressure, alveolar pressure and abdominal pressure, leading to expiratory muscle recruitment [3], and that increase of the load on the respiratory muscles during exercise causes an increase in blood flow to these muscles at the expense of blood flow to the locomotor muscles, as their metabolic demand increased, reflecting their activity [4].

Dyspnoea sensation increased during respiratory muscle loading reaching a value that was more than twice the control exercise value at the same workload. Those results confirm that non-fatiguing respiratory muscle loading during strenuous incremental exercise contributes to the perception of exertion-related respiratory sensations, notably dyspnoea [15]. Even if the pressures developed by the expiratory muscles significantly contribute to dyspnoea [15], the mechanisms responsible for this sensation are not yet clearly elucidated. Aliverti et al. [3] show that expiratory flow limitation during exercise impaired exercise performance because of severe dyspnoea, but dynamic hyperinflation was not a prominent feature of their results. Indeed, in two of their six subjects, it

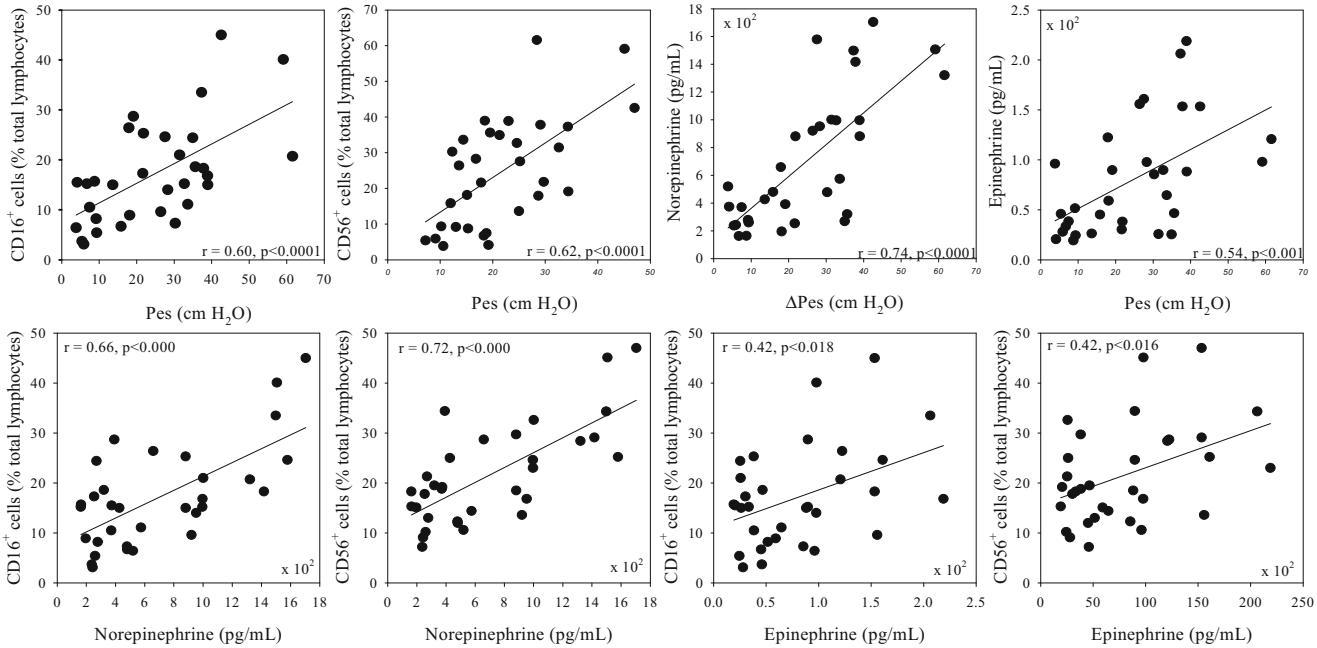

Fig. 6 Relationship of $CD16^+$ (left panel) and $CD56^+$ (right panel) NK cells (as percentage of total lymphocytes) vs ΔPes, norepinephrine and epinephrine values. Pooled data obtained at baseline, peak flow-limited exercise, iso-workload and peak control exercise. ΔPes: difference between the most negative and the less negative oesophageal pressure

did not occur at all, and in the remaining four, it only occurred at the end of the flow-limited run. Before that, during the Starling run, end-expiratory chest wall volumes were similar to or less than during control exercise, whereas dyspnoea assessed by the Borg scale was considerably greater at the same exercise level under control conditions. In healthy rats, Loverdos et al. have recently shown that expiratory resistive breathing results in dynamic hyperinflation and intrinsic positive end-expiratory pressure induction; a potential proinflammatory role for dynamic hyperinflation per se has been proposed, mainly attributable to cellular stretch [20]. In COPD patients, things are more complex: although expiratory muscles are usually recruited during exercise in most patients with COPD, they do not influence the dynamic hyperinflation and exertional dyspnoea, the relatively early respiratory mechanical constraints or the attendant perceived inspiratory difficulty [18].

It is well known that strenuous exercise is accompanied by blood lymphocyte modifications consisting in a rapid increase of circulating lymphocyte subpopulations, particularly NK lymphocytes, occurring soon after the start of exercise, and in an optional later release of several "proinflammatory" cytokines [33]. Increases in lymphocyte subpopulation and cytokine release are different phenomena not only for the time necessary for their induction but also for the pathophysiologic mechanism [33], fatigue and muscular damage being involved in the release of plasmatic cytokines [8, 31, 43] and the metabolic stress induced by the muscle work in the effects on lymphocytes [9, 24, 32, 39].

As to lymphocyte changes, scarce information is available on the effects of respiratory muscle overloading during exercise on lymphocytes. There is only one report in abstract form, describing increases in the percentage of T-cytotoxic lymphocytes, T-cytotoxic naïve lymphocytes and NK cells after strenuous resistive breathing following the same fatiguing breathing causing a cytokine release [14], but it is not known if a non-fatiguing exercise of respiratory muscles, as in our work, is able to induce a rapid increase of lymphocytes similarly to what is observed during whole-body exercise.

Furthermore, during whole-body exercise, Nieman et al. suggest that exercise is principally associated with an increase in NK cells (two third of the increase), with little or no change in other subpopulation (one third of CD8 cells and CD4 stayed relatively level), and that nearly all of the increase in CD8 cells was of the dimly fluorescent type (which function primarily as natural killer cells) [30]. In other investigations engaging cycling exercise, increases in T and B lymphocytes are heterogeneous and vary widely across studies from 10 to 100% [30, 34]. Finally, Pederson et al. suggest that the percentage of B cells among blood mononuclear cells does not change in relation to exercise [33]. During whole-body exercise, NK cells are rapidly mobilized into the circulation most likely by increased shear stress caused by a substantial increase in the peripheral blood flow and catecholamine-induced down-regulation of adhesion molecule expression [6, 13, 40]. Changes in NK lymphocytes are closely associated with the metabolic stress induced by the strenuous physical exercise, as indicated by the relationship among plasmatic catecholamines, exercise intensity [22, 23] and circulating $CD16^+$ and $CD56^+$ cells [9, 30, 37]. Those data are in agreement with the data reported. In our report, NK lymphocyte number increased during the control exercise test, and in particular, a great increase was observed for the NK lymphocytes ($CD16^+$, $CD56^+$ phenotypes). At the peak of flow-limited exercise, changes in NK lymphocytes were similar to those observed at peak of control exercise at a significantly higher workload and significantly greater than those observed at the same workload in control conditions (iso-workload), indicating that strenuous activation of respiratory muscles exerts on lymphocytes, the same effects exerted by strenuous activation of limb muscles. As to the mechanism of increased NK cell number, the significant relationship between changes in overall respiratory muscle output (ΔPes) and changes in NK cells suggests an important role of the increased work of respiratory muscles. Moreover, during both control and flow-limited exercises, there was a significant increase of the indices of stress: at the peak of flow-limited exercise test, heart rate, epinephrine and norepinephrine were significantly greater than those at iso-workload and not different from the values observed at the peak of control exercise, indicating that breathing with an expiratory flow limitation was a powerful stressful stimulus, similar to a whole-body maximal exercise test. Moreover, the number of circulating $CD16^+$ and $CD56^+$ cells was significantly related to plasmatic catecholamine levels, which in turn was significantly related to the pressure developed by respiratory muscles, demonstrating that the close association among respiratory muscle activity, plasma catecholamine concentration and change in circulating NK cells, already observed during strenuous limb muscle exercise [9, 22, 23, 30, 37], which occurs also with strenuous respiratory muscle exercise. These data confirm that the NK increase depends on the intensity of the metabolic stress induced by strenuous muscle exercise [33, 34].

As to cytokine release, Vassilakopoulos et al. [43] conclude that strenuous resistive breathing induces an immune response consisting of the elevation of plasma cytokines. This is in contrast with our report.

This might be explained by the fact that elevation of cytokines occurs only if the exercise is intense and prolonged enough to induce muscle damage, as shown by the increase of CK and by the relationship between CK and IL-6 increase [8]. Exercise of respiratory muscles is able to cause cytokine release, but only when the level of muscle exercise is sufficiently high to cause diaphragm fatigue, whereas moderate resistive breathing not-inducing diaphragm fatigue did not exert any effect [43]. In our subjects, increased work of

respiratory muscles was not accompanied by the development of fatigue, as shown by the low value of tension time index of the diaphragm, which did not reach the threshold value of 0.15 [5], and by the low percentage of Pdimax developed during the test (maximal value 27%, with fatigue occurring when Pdi is > 60% of Pdimax). Fatigue in the respiratory muscles may require a reliable measurement of respiratory muscle fatigue, such as magnetic stimulation to objectively assess respiratory muscle fatigue to confirm that the flow-limited incremental exercise indeed induced greater respiratory muscle fatigue compared to the control condition. Nonetheless, in our study, the absence of signs of muscle damage combined with a normal tension time index of the diaphragm is likely to be suggestive of the absence of occurrence of respiratory muscle fatigue and may explain the lack for systemic pro-inflammatory response.

In addition, exercise is not characterised by a fully developed systemic proinflammatory response. There are several possible explanations for the variable results on proinflammatory and inflammation-responsive cytokines in relation to exercise. The lack for systemic response may be due to only a transient cytokine release in response to exercise. Alternatively, this may reflect an adaption to the cytokine response (e.g. increased ability to induce effective naturally occurring inhibitory cytokines and cytokine receptors) [34].

Thirdly, previous studies provide indirect evidence that cytokines are upregulated in the diaphragm, secondary to exercise, but the intradiaphragmatic cytokine production does not prove that these cytokines are released in the circulation and can account for the plasma cytokine elevation, secondary to resistive breathing [44]. Other potential tissues/cells of cytokine origin, secondary to resistive loading, are still not elucidating, and it is possible that the plasma cytokine induction during resistive breathing is differentially regulated by various stimuli [43]. Finally, even if cytokines may not increase immediately after exercise [34], as at 3 and 6 h after the end of exercise, no significant changes appear in plasmatic cytokine concentration; these data indicate that no significant systemic proinflammatory response occurred during the exercise tests.

Based on these data, the answer to the hypothesis of this study appears to be positive, i.e. strenuous exercise of respiratory muscles, inducing neither fatigue nor muscle damage, is able to cause lymphocyte subpopulation changes, provided that it is intense enough to induce a significant metabolic stress. To this regard, it is interesting to compare the behaviour of dyspnoea and leg effort sensation: at peak of flow-limited exercise, dyspnoea was greater and leg effort sensation the same compared to that at iso-workload, indicating a greater respiratory muscle involvement than leg muscle involvement; on the other side, at peak of control exercise, dyspnoea was only slightly lower and leg effort sensation much greater compared to peak of flow-limited exercise, indicating a greater leg muscle involvement than respiratory muscle activation. This may suggest that during control exercise, increased leg muscle activation was the main responsible of lymphocyte changes and respiratory muscles contributed to a lesser extent, while during flow-limited exercise, the role of the limb and respiratory muscles was inverted. Finally, maximal achieved workload during flow-limited exercise was 39% less than that of control exercise, even if the changes in lymphocytes were the same, confirming the notion that the amount of these changes depends more on the intensity than on the duration of the physical work [33].

In conclusion, our results suggest that non-fatiguing respiratory muscle loading is able to cause a significant metabolic stress responsible of lymphocyte changes in the absence of respiratory muscle fatigue (as indicated by the normal value of tension time index of the diaphragm and by the low percentage of Pdimax developed during the test) or damage (for cytokine release). These responses appear to be correlated to increase of circulating NK cells similar to that observed during leg exercise. Additional interesting experimental conditions in the context of the present study would have been to use progressively increasing voluntary ventilation with and without flow limitation while maintaining the subject at rest, thereby mimicking the ventilation levels observed during the exercise conditions in order to determine the relative contribution of respiratory vs locomotor muscles in the responses of plasma cytokines and circulating natural killer lymphocytes. Confirming in a further large and prospective study on the effect of NK cells on exercise tolerance, dyspnoea and forced respiration, it would be interesting to see whether dyspnoea could be modulated by counteracting the immune response.

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