The combination of cold exposure training and a breathing exercise attenuates the inflammatory response in humans

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Abstract

Background - We previously showed that a training intervention encompassing two breathing exercises and exposure to cold enables for voluntary activation of the sympathetic nervous system, reflected by profoundly increased plasma adrenaline levels, and subsequent attenuation of the endotoxin-induced inflammatory response. Herein, we determined the contribution of the different elements of the training, assessed if the training duration is of importance, and whether it can be provided by an independent trainer instead of the well-known individual who devised it.

Methods – Forty healthy male volunteers were randomized to either a short or extensive training in both breathing exercises (i.e. cyclic hyperventilation with or without prolonged breath retention) by either the creator of the intervention or an independent trainer. In a subsequent study, 48 healthy male volunteers were randomized to cold exposure training, training in the established optimal breathing exercise, a combination of both, or no training. All 48 subjects were subsequently intravenously challenged with 2 ng/kg lipopolysaccharide to induce endotoxaemia.

Results - Both breathing exercises were equally effective in enhancing plasma adrenaline concentrations and this response was also independent from the length of the training or the individual who provided it. Cold exposure training alone did not result in relevant modulation of the endotoxin-induced cytokine response, although flu-like symptoms were markedly reduced compared with the untrained group. Whereas subjects who received training in the breathing exercise alone displayed attenuated plasma levels of pro-inflammatory cytokines IL-6, IL-8, IP-10, MCP-1, MIP-1 α , and MIP-1 β (-34%, -14%, -48%, -37%, and -28%, respectively) during endotoxemia, combined training resulted in enhanced concentrations of anti-inflammatory IL-10 (+44%) and reduced concentrations of TNF- α , IL-6, IL-8, IP-10, MCP-1, MIP-1 α , and MIP-1 β (-32%, -35%, -30%, -48%, -29%, -35%, -30%, respectively) compared with untrained individuals.

Conclusions - The combination of cold exposure training and a hyperventilation breathing exercise attenuates the in vivo inflammatory response most potently. Our study demonstrates that the immunomodulatory effects of the intervention can be reproduced in a standardized manner, thereby paving the way for clinical trials.

Trial registration - Both studies described in this manuscript are registered at www.clinicaltrials.gov (breathing exercises study: NCT02417155; experimental human endotoxaemia study: NCT03240497).

Background

Previous work from our group revealed that healthy volunteers who followed a training program were able to voluntarily activate their sympathetic nervous system and attenuate the inflammatory response during experimental human endotoxaemia, a standardized, controlled, and reproducible model of systemic inflammation elicited by intravenous administration of lipopolysaccharide (LPS) [1]. The training program was devised by Dutch individual Wim Hof, who holds several world records with regard to withstanding extreme cold, and consisted of three elements, namely meditation, exposure to cold and breathing

exercises. Trained subjects, who practiced the breathing exercises during experimental endotoxaemia, exhibited high plasma concentrations of adrenaline, which were related to a rapid and profound increase of the anti-inflammatory cytokine IL-10 and subsequent attenuation of the pro-inflammatory response (e.g. plasma levels of TNF- α , IL-6, and IL-8) [1].

The anti-inflammatory effects of this intervention could represent a novel treatment modality that may empower patients with inflammatory conditions, such as auto-immune diseases. However, there are several unresolved questions that need to be addressed first. Most importantly, it needs to be established which (combination of) element(s) is/are responsible for the effects observed, as feasibility might increase if potential users of the intervention would have to learn and practice less elements, but still attain the same efficacy. The so-called “third eye meditation” is likely of limited relevance, as it was a very minor part of the training program and was not practiced during the endotoxaemia experiments [1]. The breathing exercises both involved cyclic hyperventilation [1]. In one exercise, each cycle of hyperventilation was followed by breath retention for up to several minutes, resulting in profound decreases in oxygen saturation, while in the other exercise, subjects only very shortly held their breath after each cycle of hyperventilation during which all body muscles were tightened, which was not associated with a decrease in oxygen saturation. Because both hyperventilation and hypoxia have been shown to result in adrenaline release [2–5], it is unknown which of these exercises is responsible for the effects observed. Furthermore, it is unclear whether it is necessary to be trained by Mr. Hof himself (‘guru-effect’ [6, 7]) and whether or not a short instruction instead of an extensive training would be sufficient to increase plasma adrenaline levels [1].

In the first part of the current study, we addressed these issues by investigating the effects of the two different breathing exercises and different training modalities (i.e. training by Mr. Hof vs. an independent trainer, and a short instruction vs. extensive training) on plasma adrenaline levels. In the second part of this study, we investigated the effects of the optimal breathing exercise established in the first part and of cold exposure, both independently and combined, on the inflammatory response during experimental human endotoxaemia.

Methods

Ethics

All procedures were approved by the local ethics committee of the Radboud university medical center (CMO Arnhem-Nijmegen, reference numbers are provided in the corresponding sections below) and were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines. All subjects provided written informed consent to participate in the study and were screened before the start of the experiment to confirm a normal physical examination, electrocardiography, and routine laboratory values. Exclusion criteria were: prior experience with any of the elements of the intervention developed by Mr. Hof or other breathing, meditation, or cold exposure exercises, including mindfulness, yoga and exposure to cold showers, frequent visits to sauna facilities

(more than once per month), use of any medication, smoking, previous spontaneous vagal collapse, use of recreational drugs within 21 days prior to the experiment day, surgery or trauma with significant blood loss or blood donation, hospital admission or surgery with general anaesthesia, or participation in another study within three months prior to the experimental day, or clinically significant acute illness (including infections) within four weeks before the experiment day.

Breathing exercises study

Study design

After ethical approval (reference number: 2014 – 1374/NL51237.091.14), 40 males provided written informed consent to participate in this prospective randomized study registered at clinicaltrials.gov (NCT02417155). A schematic overview of the study is depicted in Fig. 1. Participants were randomized to four different groups (n = 10 per group) by an independent research nurse using the sealed envelope method: extensive training by Mr. Hof, extensive training by an independent trainer, short training by Mr. Hof, and short training by an independent trainer. All subjects were trained in both breathing exercises, with and without the prolonged breath retention (detailed in section 'breathing exercises' below), in the week before the experiment day.

Training procedures

In the extensive training by Mr. Hof group, subjects were trained every morning for two hours during four days, and after these initial four days of training, subjects were instructed to practice the learned exercises at home, both analogous to our previous study [1]. In the short training by Mr. Hof group, subjects were trained for only two hours on the morning of the fourth day (see Fig. 1) and subjects were instructed not to practice the learned exercises at home. The training procedures in the other two groups were exactly the same, with the exception that Mr. Hof was substituted by an independent trainer from our research group (RvG), and that subjects also received a detailed written instruction of both breathing exercises, see Additional File 2 .

Breathing exercises

In the exercise with the prolonged retention of breath (henceforth designated as “with [+] retention”), subjects hyperventilated for an average of 30 breaths using deep and powerful breaths. Subsequently, the subjects exhaled and held their breath for approximately two minutes (“retention phase”). The duration of breath retention was entirely at the discretion of the subject. Breath retention was followed by a deep inhalation breath, that was held for 10 s. Subsequently a new cycle of hyper/hypoventilation began. In the exercise without retention of breath (henceforth designated as “without [-] retention”), subjects also hyperventilated for an average of 30 times using deep and powerful breaths. Subsequently, subjects held their breath for only 10 seconds, during which all body muscles were tightened, and then a new cycle of hyperventilation was initiated.

Procedures on the experiment day

The experiments were conducted at the research unit of the intensive care department of the Radboud university medical center and an overview of the procedures is depicted in Fig. 1. To allow comparison with our previous study [1], subjects refrained from caffeine and alcohol 24 hours before the experiment, and refrained from any intake of food and drinks 10 hours before the experiment. A cannula was placed in the antecubital vein of the non-dominant arm for hydration, and the radial artery of the same arm was cannulated under local anaesthesia (lidocaine HCl 20 mg.mL⁻¹) using a 20-gauge arterial catheter for continuous arterial blood pressure monitoring and blood withdrawal. After a one-hour rest period, participants were randomized to start with one of the breathing exercises at 09:00 am (morning session): half of the participants started with the exercise with retentions, whereas the other half started with the exercise without retentions. They performed the exercise for 1.5 hours, after which they rested for 1.5 hours, and the second breathing exercise was started at noon (afternoon session), which also lasted 1.5 hours. Serial blood samples were obtained throughout the experiment (see Fig. 1).

Experimental human endotoxaemia study

Study design

After ethical approval (reference number 2016–2312/NL56686.091.16), 48 males provided written informed consent to participate in this prospective randomized controlled study registered at clinicaltrials.gov (NCT03240497). A schematic overview of the study is depicted in Fig. 2. We employed a 2 by 2 design, in which 48 participants were randomized using the sealed envelope method to 4 different groups (n = 12 per group): cold exposure (CEX), breathing exercise without retention (BRT), cold exposure and the breathing exercise without retention (CBR), and a control group (CON). Subjects of all groups except the control group were trained in the week leading up to the endotoxaemia experiment day.

Training procedures

An impression of the training procedures is provided in Additional Video 1. All training procedures were provided by the same independent trainer (RvG). Mr. Hof was not involved in the training course. The study team, including an MD, was present during all training procedures. Subjects in the CEX group followed an intensive 4-day cold exposure training program similar to that of our previous study [1], consisting of standing in snow with bare feet for up to 30 minutes, lying in snow in shorts for up to 20 minutes, and sitting and swimming in ice-cold water for up to 3 minutes (Additional Video 1). Subjects were instructed to end their daily shower with a period of 60 seconds of cold water. Subjects in the BRT group were trained in the breathing exercise without retentions of breath as described in the section ‘breathing exercises’ above. Similar to the short training by an independent trainer group in the breathing exercises study (see section ‘training procedures’ above), the independent trainer provided an instruction course of 2 hours. Subjects were instructed not to practice the learned exercises at home. Subjects randomized to the CBR group participated in both training procedures and subjects in the control group did not receive any training.

Procedures on the endotoxaemia experiment day

Endotoxaemia experiments were conducted at the research unit of the intensive care department of the Radboud university medical center according to our standard protocol [8] also used in our previous study into this intervention [1], and an overview of the procedures is depicted in Fig. 2. Participants refrained from caffeine, alcohol and intake of food and drinks in the same way as the participants of the breathing exercises study did. A cannula was placed in the antecubital vein of the non-dominant arm for hydration, and the radial artery of the same arm was cannulated under local anaesthesia (lidocaine HCl 20 mg.mL⁻¹, Fresenius Kabi, Zeist, The Netherlands) using a 20-gauge arterial catheter for continuous arterial blood pressure monitoring, and blood withdrawal. Subjects received 1.5L of 2.5% glucose/0.45% saline solution for 1 hour (prehydration) before endotoxin administration, followed by 150 mL.h⁻¹ until the end of the experiment (8 hours after endotoxin administration). Subjects of BRT and CBR groups practiced the learned breathing exercise from 30 minutes before administration of endotoxin to 2.5 hours afterwards, identical to our previous study [1].

Purified LPS (derived from *Escherichia coli* O:113, Clinical Center Reference Endotoxin) obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA) and supplied as a lyophilized powder, was reconstituted in 5 mL saline 0.9% for injection and vortex-mixed for 20 minutes before being administered as an intravenous bolus at a dose of 2 ng.kg⁻¹ body weight over 1 minute at T = 0 hours at 09.30 AM. Blood samples were serially obtained throughout the experiment (see Fig. 2).

Adrenaline and blood gas analysis

For circulating adrenaline measurements, blood was collected into lithium heparin tubes and were immediately placed on ice and centrifuged at 2000 × g for 10 min at 4 °C, after which plasma was stored at – 80 °C until analysis. Plasma adrenaline concentrations were subsequently measured using HPLC with fluoro-metric detection [9]. Blood gas parameters were analysed in lithium heparin anticoagulated arterial blood using an i-STAT Blood Gas Analyzer (Abbot, Hoofddorp, The Netherlands) and CG4 + cartridges.

Plasma cytokines

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood was centrifuged immediately at 2000 × g for 10 min at 4 °C, after which plasma was stored at – 80 °C until analysis. Concentrations of TNF-α, IL-6, IL-8, IL-10, IP-10, MCP-1, MIP-1α, and MIP-1β were measured in one batch using a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex, Millipore, Billerica, USA).

Haemodynamic parameters, symptom score, and body temperature

Heart rate (three-lead electrocardiogram), blood pressure (intra-arterial cannula), respiratory rate, and oxygen saturation (pulse oximetry) data were recorded from a Philips MP50 patient monitor (Eindhoven, The Netherlands) every 30 seconds by a custom in-house-developed data recording system. Endotoxin-induced flu-like symptoms (headache, nausea, shivering, muscle, and back pain) were scored every 30

minutes on a six-point Likert scale (0 = no symptoms, 5 = worst ever experienced, in case of vomiting 3 points were added), forming an arbitrary total symptom score with a maximum of 28 points. Body temperature was determined every 30 minutes using an infrared tympanic thermometer (First-Temp Genius, Sherwood Medical, Norfolk, NE, USA).

Calculations and statistical analysis

Data are expressed as median and interquartile range [IQR] or mean $\pm$ SEM, based on their distribution calculated by Shapiro-Wilk tests. Except for demographic characteristics, all non-parametric data were log-transformed before statistical analysis. Comparisons were made using Kruskal Wallis tests, paired Student T-tests and repeated measures two-way ANOVA (time*group interaction term followed by Bonferroni post-hoc tests to evaluate differences at individual timepoints). A linear multivariate analysis was performed to assess the effects of cold exposure training and the breathing exercises on plasma levels of all measured cytokines (expressed as area under the concentration-time curves [AUC] to provide an integral measure of the responses). A p-value < 0.05 was considered statistically significant. Calculations and statistical analyses were performed using Graphpad Prism version 5.03 (Graphpad Software, San Diego, CA, USA) and SPSS v25.0.0.1 (IBM Corp, Armonk, New York, USA).

Results

Breathing exercises study

Demographic characteristics

Demographic characteristics of the participants are listed in Table 1 and were not different between the study groups.

Table 1
Demographic characteristics.

Breathing exercises study	All subjects (n = 40)	Short training by independent trainer (n = 10)	Extensive training by independent trainer (n = 10)	Short training by Mr. Hof (n = 10)	Extensive training by Mr. Hof (n = 10)	p-value
Age, years	21 [19–24]	20 [19–22]	22 [19–26]	21 [20–23]	23 [19–26]	p = 0.46
BMI, kg.m ⁻²	22.9 [21.4–24.2]	22.5 [21.5–24.8]	23.9 [21.1–25.0]	23.8 [22.2–24.6]	22.3 [20.1–23.6]	p = 0.60
Systolic blood pressure, mmHg	140 [128–145]	135 [123–148]	143 [128–147]	146 [137–150]	135 [131–140]	p = 0.26
Diastolic blood pressure, mmHg	71 [64–79]	71 [61–77]	77 [70–85]	69 [62–81]	69 [62–81]	p = 0.22
Heart rate, bpm	77 [60–88]	77 [69–84]	86 [59–103]	77 [53–89]	63 [54–82]	p = 0.27
Endotoxaemia study	All subjects (n = 48)	Control (CON) (n = 12)	Cold exposure (CEX) (n = 12)	Breathing exercise (BRT) (n = 12)	Cold exposure and breathing exercise (CBR) (n = 12)	
Age, years	22 [20–24]	22 [20–22]	23 [20–26]	22 [20–24]	23 [20–25]	p = 0.86
BMI, kg.m ⁻²	23.3 [22.2–24.6]	23.1 [22.2–23.9]	22.8 [21.0–24.2]	23.5 [22.7–24.7]	24.5 [22.5–25.6]	p = 0.37
Systolic blood pressure, mmHg	140 [136–152]	137 [122–156]	142 [137–155]	140 [136–152]	144 [136–152]	p = 0.88

Data were obtained using the screening visit and are presented as median [IQR]. kg: kilogram, m: meter, bpm: beats per minute, mmHg; millimeters mercury. P-values were calculated using Kruskal-Wallis tests.

Breathing exercises study	All subjects (n = 40)	Short training by independent trainer (n = 10)	Extensive training by independent trainer (n = 10)	Short training by Mr. Hof (n = 10)	Extensive training by Mr. Hof (n = 10)	p-value
Diastolic blood pressure, mmHg	72 [64–80]	73 [66–82]	72 [64–81]	70 [62–75]	77 [68–82]	p = 0.35
Heart rate, bpm	64 [56–71]	66 [59–75]	65 [56–73]	62 [56–66]	62 [51–67]	p = 0.33
Data were obtained using the screening visit and are presented as median [IQR]. kg: kilogram, m: meter, bpm: beats per minute, mmHg; millimeters mercury. P-values were calculated using Kruskal-Wallis tests.						

Plasma adrenaline levels and blood gas parameters

Changes in blood gas parameters (saturation, pO_2 , pH, and pCO_2) were identical during both the morning and afternoon sessions across all groups (Additional Fig. 1A-D). During the morning session, plasma adrenaline levels sharply increased upon initiation of the breathing exercises across all groups (from 0.51 nmol/L [0.33–0.72] at T = 0 to 1.01 nmol/L [0.64–1.48] at T = 0.5, Additional Fig. 1E). We previously showed that this initial increase in adrenaline levels is the main driving factor inducing the anti-inflammatory phenotype (see Fig. 5 in [1]). Adrenaline levels remained elevated for as long as the subjects practiced the exercises in the morning (0.87 nmol/L [0.51–1.24] and 0.99 nmol/L [0.56–1.68] at T = 1, and 1.5 hours, respectively, Additional Fig. 1E). In contrast, during the afternoon session plasma adrenaline levels failed to rapidly increase after commencing the breathing exercises, although concentrations were slightly elevated at later timepoints (T = 0: 0.48 nmol/L [0.33–0.65], T = 0.5: 0.44 nmol/L [0.30–0.73], T = 1: 0.54 nmol/L [0.38–1.12] and T = 1.5: 0.75 nmol/L [0.54–1.26], Additional Fig. 1E). Statistical comparison of plasma adrenaline levels over time between the morning and afternoon session yielded a highly significant difference ($p < 0.0001$, Additional Fig. 1E). Because of this, we restricted all further analyses to data obtained during the morning session.

A comparison of the breathing exercises with and without retention revealed that only the exercise with retention resulted in profound decreases in oxygen saturation levels at the end of each retention phase (from $98\% \pm 0.2\%$ at T = 0 to $67\% \pm 5\%$, $58 \pm 3\%$ and $73 \pm 4\%$, at T = 0.5, 1, and 1.5 hours, respectively, Fig. 3A). Accordingly, sharp decreases in pO_2 were observed in this group (Fig. 3B). pH and pCO_2 were largely comparable between the two breathing exercises, with only a slight, but statistically significant, difference at the last measured timepoint (Fig. 3C-D). The initial increase in plasma adrenaline levels was comparable between both breathing exercises (from 0.51 [0.38–0.75] nmol/L at T = 0 to 0.98 [0.67–1.78] nmol/L at T = 0.5 in the subjects performing the breathing exercise with breath retention, and from 0.51 [0.32–0.68] nmol/L at T = 0 to 1.01 [0.63–1.46] nmol/L at T = 0.5 in the participants performing the

breathing exercise without retention (Additional Fig. 2). However, the increase in plasma adrenaline concentrations was slightly more sustained in the subjects practicing the breathing exercise with retention, resulting in significantly higher levels at $T = 1.5$ compared to participants practicing the exercise without retention (Additional Fig. 2).

Blood gas parameters and plasma adrenaline levels were not statistically different between the participants trained by an independent trainer compared to participants trained by Mr. Hof (Additional Fig. 3). Additionally, no significant differences in these parameters were found between the participants that received the short training versus the long training (Additional Fig. 4).

Based on these results, we conclude that the magnitude of the initial increase in adrenaline levels, which we previously showed to be a main determinant of the anti-inflammatory phenotype [1], is not dependent on prolonged breath retention. Furthermore, neither training by Mr. Hof himself, nor a long training program are required to attain the pronounced adrenaline response. Hence, we utilized the short training by an independent trainer in only the breathing exercise without breath retention for the subsequent experimental human endotoxaemia study.

Experimental human endotoxaemia study

Demographic characteristics

Demographic characteristics of the participants are listed in Table 1 and were not different between groups.

Blood gas parameters and plasma adrenaline levels

No significant changes in any of the arterial blood gas parameters were observed over time in the groups that did not practice the breathing exercise (CEX and CON groups, Fig. 4A-C). In contrast, blood gas parameters were profoundly altered in the BRT and CBR groups upon initiation of the breathing exercise and normalized quickly after cessation. pH increased from 7.46 ± 0.04 (BRT) and 7.40 ± 0.004 (CBR) at baseline to 7.72 ± 0.02 (BRT) and 7.70 ± 0.01 (CBR) 15 minutes following the start of the breathing exercise (Fig. 4A). Oxygen saturation increased from 98% [98–99] (BRT) and 99% [98–100] (CBR) at baseline to 100% [100–100] (BRT) and 100% [100–100] (CBR) 15 minutes into practicing of the breathing exercise (Fig. 4B). $p\text{CO}_2$ dropped from 5.16 ± 0.1 kPa (BRT) and 5.17 ± 0.01 kPa (CBR) at baseline to 1.74 ± 0.06 kPa (BRT) and 1.99 ± 0.07 kPa (CBR) 15 minutes after the start of the breathing exercise (Fig. 4C).

Baseline plasma adrenaline levels were comparable between all four groups (Fig. 4D). Concentrations increased during human endotoxaemia in all groups, with peak values observed 1.5 hours after administration of LPS (Fig. 4D). There were no differences in plasma adrenaline levels over time between the CEX and CON groups. However, in both groups of subjects who practiced the breathing exercises, the increase in plasma adrenaline commenced much earlier and was significantly more pronounced than in the CEX and CON groups that did not exercise the breathing exercise.

Haemodynamic parameters, temperature, and symptoms

Experimental endotoxaemia resulted in a gradual increase in heart rate in the CEX and CON groups, with no differences between the groups (Fig. 5A). In the two groups that performed the breathing exercise, a sharp increase in heart rate was observed immediately following the start of the first hyperventilation cycle, and this effect ensued during the largest part of the breathing exercise period (Fig. 5A). After cessation of the breathing exercises, the heart rate data of the BRT and CBR groups was similar to that of the CEX and CON groups. Expectedly, mean arterial pressure (MAP) gradually decreased in all groups (Fig. 5B), and no clear differences between any of the groups were present. Although there was a statistically significant difference between the BRT and CON groups in MAP over time, post-hoc analysis did not reveal significance at any of the individual timepoints. An endotoxin-induced mean increase in body temperature of 1.8 ± 0.1 °C was observed across all groups (Fig. 5C). Although peak temperatures were similar between the CON (38.8 ± 0.1 °C) and the three intervention groups (BRT: 38.8 ± 0.2 °C, $p = 0.94$, CEX: 38.6 ± 0.2 °C, $p = 0.44$, CBR: 38.7 ± 0.2 °C, $p = 0.73$), these were attained significantly earlier in the BRT group (Fig. 5C). Administration of endotoxin resulted in flu-like symptoms in all groups (Fig. 5D). Peak symptom scores were comparable between the CON (9.3 ± 1.3) and the BRT (9.4 ± 1.5 , $p = 0.70$) and CBR (7.04 ± 1.2 , $p = 0.21$) groups, but significantly lower in the CEX group (5.5 ± 0.8 , $p = 0.02$). Symptoms resolved significantly more rapidly in all three intervention groups compared with the CON group (Fig. 5D).

Plasma cytokines

As expected, plasma concentrations of the anti-inflammatory cytokine IL-10 and the proinflammatory cytokines TNF- α , IL-6, IL-8, IP-10, MCP-1, MIP-1 α , and MIP-1 β increased following endotoxin administration in all groups (Fig. 6 and Additional Fig. 5). In the CBR group, IL-10 levels were significantly higher compared with the CON group (mean increase in area under the concentration-time curves [AUC] of +44%, Fig. 6A). Furthermore, concentrations of pro-inflammatory cytokines in this group were significantly attenuated compared with the CON group (mean decrease in AUC of TNF- α : -32%, IL-6 -35%, IL-8: -30%, IP-10: -48%, MCP-1: -29%, MIP-1 α : -35%, MIP-1 β : -30%, Fig. 6B-D and Additional Fig. 5). In the BRT group, similar, but less pronounced effects on plasma cytokines were observed, reaching statistical significance for IL-6, IL-8, IP-10, MCP-1, MIP-1 α , and MIP-1 β (-34%, -14%, -48%, -37%, and -28% compared to CON, respectively), but not for IL-10 (+17%, $p = 0.17$ vs. CON). In the CEX group, only levels of MCP-1 were significantly lower than in the CON group (-25%).

In accordance with the abovedescribed results, a multivariate analysis yielded a significant effect of the breathing exercises, as well as the interaction between cold exposure training and breathing exercises on the integral cytokine response (breathing exercises: $F(8, 37) = 3.804$, $p = 0.002$; Wilk's $\Lambda = 0.549$, partial $\eta^2 = 0.451$; cold exposure*breathing exercises: $F(8, 37) = 2.571$, $p = 0.024$; Wilk's $\Lambda = 0.649$, partial $\eta^2 = 0.357$). Cold exposure alone did not significantly affect the integral cytokine response: $F(8, 37) = 0.603$, $p = 0.769$, Wilk's $\Lambda = 0.885$, partial $\eta^2 = 0.115$.

Discussion

In this study, we thoroughly investigated the effects of different aspects of a training program which was previously shown to allow for voluntary activation of the sympathetic nervous system and attenuation of the inflammatory response. First, we showed that, although arterial blood saturation levels and pO_2 were significantly lower when subjects performed the breathing exercise with prolonged breath retention compared to that without, plasma adrenaline levels increased with a similar magnitude shortly after initiation of both breathing exercises. Second, we demonstrated that the previously observed physiological and immunological effects [1] are independent from either the length of training or the individual who provides it. Third, our data signify that the combination of the breathing exercise and cold exposure training is most effective in attenuating the inflammatory response during human endotoxaemia.

The magnitude of the initial increase in plasma adrenaline concentrations was similar for the breathing exercises with and without prolonged breath retention. The cyclic hypoxia caused by the exercise with prolonged breath retention is therefore unlikely to be an important factor in the observed adrenaline response. In accordance, hyperventilation itself and the subsequent shift in acid-base balance have been shown to increase plasma catecholamines in the absence of hypoxia, and an important role for bicarbonate has been implicated [5, 10]. Nevertheless, as catecholamine release from the adrenal chromaffin cells is dependent on a combination of neural, hormonal, redox, as well as immune signaling pathways [11, 12], the exact mechanism behind the adrenaline release induced by the breathing exercise remains elusive. The finding that neither the duration of the training, nor the trainer who provides it affected any of the measured parameters signifies that the breathing exercise is easy to learn within a time-frame of two hours. These findings may greatly facilitate uncomplicated implementation of the training program in clinical studies.

Our data clearly demonstrate that the breathing exercise plays a pivotal role in the anti-inflammatory effect of the training intervention. Nevertheless, although cold exposure training alone had minimal effects on the cytokine response, it significantly potentiated the breathing exercise-induced anti-inflammatory effects. As plasma adrenaline levels in our study were comparable between the groups practicing the breathing exercises with or without prior cold exposure training, other mechanisms are likely involved. Noteworthy, despite little effects on the cytokine response, subjects in the cold exposure training group reported remarkably less symptoms compared to the control group as well as to the other two groups. In accordance, other studies reporting symptoms during repeated exposures to cold found similar attenuation of symptoms such as discomfort and shivering [13, 14]. Symptoms, especially headache, were more pronounced during practicing of the breathing exercise, likely resulting from the hyperventilation-induced changes in pCO_2 and pH. After cessation of the breathing exercise, a sharp decrease of symptoms was observed and flu-like symptoms resolved more rapidly compared to the control group.

The increase in plasma adrenaline concentrations observed shortly after initiation of the breathing exercises in both the breathing exercises and endotoxemia studies described in the present work was similar to that in our previous study [1]. Nevertheless, adrenaline levels prior to the start of the breathing

exercises were higher in the past work, ultimately resulting in higher absolute plasma concentrations [1]. Effects on the cytokine response in the combined cold exposure and breathing group in the current study were largely comparable to our previous work, in which subjects were also trained in both elements [1], although the magnitude of the immunomodulatory effects was less pronounced, with the anti-inflammatory IL-10 response augmented by 44% instead of 194% in [1], and pro-inflammatory cytokines attenuated by approximately 30% as opposed to more than 50% in [1]. There are several possible explanations for this discrepancy. First, the previously mentioned higher absolute plasma adrenaline concentrations during practicing of the breathing exercises could be involved [1], which may in turn have triggered a more pronounced IL-10 release and subsequent stronger attenuation of the pro-inflammatory response. Second, the hypoxia induced by breath retention in our previous study may have directly (i.e. independently from adrenaline) modulated the inflammatory response, as our group has recently demonstrated that hypoxia enhances IL-10 release and attenuates the pro-inflammatory response via enhanced adenosine release [15]. In this light, future studies into the training intervention should still consider including the exercise with prolonged breath retention.

A striking finding from the breathing exercises study was that the profound increase in plasma adrenaline levels only occurred during the first session in the morning, not during the second session performed in the afternoon after a 1.5 hour resting period. Nevertheless, the saturation, pO_2 , pCO_2 , and pH were identical between the morning and the afternoon sessions. Therefore, the lack of a profound increase of plasma adrenaline levels during the afternoon session may be due to adaptation of the stress response, resulting in lower adrenaline release by the adrenal gland in response to repeated application of the same stressor, a phenomenon which has been described in animals [16]. Alternatively, because the synthesis and storage of catecholamines mainly takes place within chromaffin cells of the adrenal medulla, it may be speculated that the breathing exercises deplete the intravesicular stores in the cytoplasm of these cells [11, 12]. Although animal experiments have shown that fully depleted catecholamine stores can be replenished within 2 hours [17], this may take longer in humans. In any case, if stores are indeed depleted by the breathing exercise, replenishment must occur within a relatively short timeframe (< 24 hours), as participants of this study, as well as our previous study [1] practiced the breathing exercises daily in the week leading up to the experiment in which the plasma adrenaline concentrations were measured.

Several limitations of our work need to be addressed. First, we studied groups of healthy young male adults, not (older) patients with possible comorbidities, who represent the intended target group for this intervention. Nevertheless, this study provides essential information in terms of designing the most safe and optimal training protocol for use in future clinical studies. Second, the auto-immune response observed in patients with chronic inflammatory conditions clearly differs from that elicited by LPS administration, which models an acute inflammatory response to a bacterial infection. However, several drugs currently used in patients with inflammatory conditions such as rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis are aimed at reducing the release of several pro-inflammatory cytokines [18], on which the studied intervention has a major suppressive effect. Furthermore, in vivo human

efficacy of many biologics used in the treatment of auto-inflammatory disorders was first established in the experimental human endotoxaemia model [19, 20], illustrating that it has value for these diseases.

Conclusions

The present study corroborates previous findings that voluntary activation of the sympathetic nervous system, attenuation of the pro-inflammatory response, and alleviation of symptoms during experimental human endotoxaemia is possible using a combination of exposure to cold and a breathing exercise. Furthermore, these interventions can be provided by an independent trainer and acquired within a short time-frame. Future studies should focus on these elements of the intervention and establish whether or not these can reduce the burden of inflammatory diseases in patients.

List Of Abbreviations

ANOVA analysis of variance

AUC area under the curve

BRT breathing exercise without retention group

CBR cold exposure and the breathing exercise without retention group

CEX cold exposure group

CMO [Dutch] commissie mensgebonden onderzoek - human research ethics committee

CON control group

EDTA ethylenediaminetetraacetic acid

IL interleukin

IP interferon gamma-induced protein

IQR interquartile range

LPS lipopolysaccharide

MAP mean arterial pressure

MCP monocyte chemoattractant protein

MIP macrophage inflammatory protein

SEM standard error of the mean

TNF tumor necrosis factor

Declarations

Ethics approval and consent to participate

For both studies, ethics approval was granted by the local human research ethics committee (CMO Arnhem-Nijmegen) and are registered at www.clinicaltrials.gov. Reference numbers are as follows: Breathing exercises study: 2014-1374/NL51237.091.14 and NCT02417155. Experimental human endotoxaemia study: 2016-2312/NL56686.091.16 and NCT03240497).

Consent for publication

All participants that feature in the additional video material (Additional Video 1) provided individual consent for publication through an institutional consent form. As described in the authors instructions, we will not send these forms on submission but can provide these at any stage when requested.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

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All authors read and approved the final manuscript.

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Additional Figure Legends

Additional Figure 1: arterial blood gas parameters and plasma adrenaline levels during the breathing exercises study: morning vs. afternoon session. **A.** Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma adrenaline concentrations. Morning session: data from participants during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1). Afternoon session: data from participants during the second breathing exercise on the experiment day (breathing exercise 2, Figure 1). Panels A-D: data are presented as mean $\pm$ SEM of 40 subjects per group and p-values depicted in the graphs represent the between-group comparison calculated using repeated measures two way ANOVA (time*group interaction term); significant p-values are shown in bold. Panel E: data are presented as median and IQR of 20 subjects per group, and the p-value depicted in the graph represents the between-group comparison calculated using repeated measurements two way ANOVA on log-transformed data (time*group interaction term); significant p-values are shown in bold. * indicates $p < 0.05$ calculated using Bonferroni post-hoc tests.

Additional Figure 2: plasma adrenaline levels during the breathing exercises study: influence of breath retention. - retention: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants performing the breathing exercise without prolonged retention of breath. + retention: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants performing the breathing exercise with prolonged retention of breath. Data are presented as median and IQR of 20 subjects per group, and the p-value depicted in the graph represents the between-group comparison calculated using repeated measurements two way ANOVA on log-transformed data (time*group interaction term); significant p-values are shown in bold. * indicates $p < 0.05$ calculated using Bonferroni post-hoc tests.

Additional Figure 3: arterial blood gas parameters and plasma adrenaline levels during the breathing exercises study: influence of trainer. **A.** Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma adrenaline concentrations. Training by Mr. Hof: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from

participants were trained by Mr. Hof. Training by independent trainer: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants were trained by the independent trainer. Panels A-D: data are presented as mean $\pm$ SEM of 20 subjects per group and p-values depicted in the graphs represent the between-group comparison calculated using repeated measures two way ANOVA (time*group interaction term). Panel E: data are presented as median and IQR of 20 subjects per group, and the p-value depicted in the graph represents the between-group comparison calculated using repeated measurements two way ANOVA on log-transformed data (time*group interaction term).

Additional Figure 4: arterial blood gas parameters and plasma adrenaline levels during the breathing exercises study: influence of length of training. **A.** Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma adrenaline concentrations. Long training: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants that received four days of training. Short training: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants that received 2 hours of training. Panels A-D: data are presented as mean $\pm$ SEM of 20 subjects per group and p-values depicted in the graphs represent the between-group comparison calculated using repeated measures two way ANOVA (time*group interaction term). Panel E: data are presented as median and IQR of 20 subjects per group, and the p-value depicted in the graph represents the between-group comparison calculated using repeated measurements two way ANOVA on log-transformed data (time*group interaction term).

Additional Figure 5: Plasma concentrations of inflammatory cytokines during human endotoxaemia. **A.** Interferon Gamma-Induced Protein 10 (IP-10). **B.** Monocyte chemoattractant protein 1 (MCP-1). **C.** Macrophage Inflammatory Protein 1 α (MIP-1 α). **D.** Macrophage Inflammatory Protein 1 β (MIP-1 β). The grey box indicates the period during which the trained subjects practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean $\pm$ SEM of 12 subjects per group. p-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using repeated measures two-way ANOVA (time*group interaction term); significant p-values are shown in bold. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

Figures

Figure 1: Schematic overview of the procedures of the breathing exercises study.

Figure 1

Schematic overview of the procedures of the breathing exercises study.

Figure 2: Schematic overview of the procedures of the human endotoxaemia study.

Figure 2

Schematic overview of the procedures of the human endotoxaemia study. LPS: lipopolysaccharide.

Figure 3: Arterial blood gas parameters during the breathing exercises study.

Figure 3

Arterial blood gas parameters during the breathing exercises study. A. Oxygen saturation. B. Oxygen partial pressure (pO₂). C. pH. D. Carbon dioxide partial pressure (pCO₂). - retention: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants performing the breathing exercise without prolonged retention of breath. + retention: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants performing the breathing exercise with prolonged retention of breath. Data are presented as mean $\pm$ SEM of 20 subjects per group. p-values depicted in the graphs represent the between-group comparison calculated using repeated measures two way ANOVA (time*group interaction term); significant p-values are shown in bold. * indicates p<0.05 calculated using Bonferroni post-hoc tests.

Figure 4: Arterial blood gas parameters and plasma adrenaline levels during human endotoxaemia.

Figure 4

Arterial blood gas parameters and plasma adrenaline levels during human endotoxaemia. A. pH. B. Oxygen saturation. C. Carbon dioxide partial pressure (pCO₂). D. Plasma adrenaline concentrations. The grey box indicates the period during which the trained subjects practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean $\pm$ SEM of 12 subjects per groups. p-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using repeated measures two-way ANOVA (time*group interaction term); significant p-values are shown in bold. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

Figure 5: Cardiorespiratory parameters, temperature, and symptoms during human endotoxaemia.

Figure 5

Cardiorespiratory parameters, temperature, and symptoms during human endotoxaemia. A. Heart rate. B. Mean arterial pressure (MAP). C. Temperature. D. Score of self-reported symptoms. The grey box indicates the period during which the trained subjects practiced the breathing exercise (BRT and CBR groups only). Data are expressed as mean $\pm$ SEM of 12 subjects per group. p-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using repeated measures two-way ANOVA (time*group interaction term); significant p-values are shown in bold. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

Figure 6: Plasma concentrations of inflammatory cytokines during human endotoxaemia.

Figure 6

Plasma concentrations of inflammatory cytokines during human endotoxaemia. A. Tumor necrosis factor (TNF)- α . B. Interleukin (IL)-6. C. IL-8. D. IL-10. The grey box indicates the period during which the trained subjects practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean $\pm$ SEM of 12 subjects per group. p-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using repeated measures two-way ANOVA (time*group interaction term); significant p-values are shown in bold. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

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