Non-pharmacological immunomodulation

Jelle Zwaag

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Radboud Dissertation Series

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Title: Non-pharmacological immunomodulation

Radboud Dissertations Series
ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Annelies Lips
Cover: Proefschrift AIO | Guntra Laivacuma
Printing: DPN Rikken/Pumbo

ISBN: 9789465150017
DOI: 10.54195/9789465150017
Free download at: www.boekenbestellen.nl/radboud-university-press/dissertations

RADBOUD
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PRESS

Non-pharmacological immunomodulation

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 2 december 2024
om 14.30 uur precies

door

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geboren op 7 augustus 1986
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Dissertation to obtain the degree of doctor
from Radboud Universiteit Nijmegen
on the authority of the Rector Magnificus prof. dr. J.M. Sanders,
according to the decision of the Doctorate Board
to be defended in public on

Monday, December 2, 2024
at 2.30 pm

by

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Table of contents

Chapter 1

General introduction, aim, and outline of this thesis

The inflammatory response in health

There is a constant interaction between man and pathogens. Humans not only host large populations of microorganisms, such as bacteria, viruses, fungi and yeasts [1], but also continuously evolve with them. The microorganisms present in, for instance, the gut [2], the skin [3] and the respiratory tract [4] live in symbiosis with the host and only abnormalities in these microbiomes are associated with several diseases [5, 6]. This relationship between (non-pathogenic) microorganism and the host is ancient and mostly profitable for both parties [2]. However, dysregulation of this symbiosis or invasion of disease-inducing pathogens into other parts of the body are sterile may result in infection [7].

Our body has evolved several defense mechanisms against this continuous threat of pathogens. This ranges from relatively simple mechanical barriers like the skin [8], to the intricate and highly sophisticated immune system [9, 10]. The immune system can generate an inflammatory response towards invading pathogens aimed to eradicate them. Furthermore, this response can also be mounted following sterile injury, for instance as a result of (surgical) trauma or burns, where it plays a key role in tissue healing and remodeling. Key elements and orchestrators of the inflammatory response are cytokines: small messenger molecules that play either a pro- or anti-inflammatory role in the elaborate network of signals that are sent within and between tissues [11]. Even before the classic symptoms of inflammation (fever, redness, swelling and impaired function) emerge, cytokine concentrations increase to propagate, balance, and focus the inflammatory response [12]. As long as this response is balanced and controlled, and abates when the danger has passed, it is a beneficial process crucial to our survival [12]. However, this is not always the case.

The inflammatory response in disease

Apart from the paramount importance of the immune system in infectious diseases, there is also a downside to its potent ability to 'search and destroy'. The immune system obviously needs to leave the `self` alone, but sometimes fails in making the correct distinction between `self` and `foreign`. A large global burden of disease is caused by the pathophysiological phenomenon of the immune system attacking the body's own tissues. These diseases are collectively known as auto-immune diseases, with rheumatoid arthritis, systemic lupus and inflammatory bowel disease, such as Crohn`s disease, as prime examples [13-15]. The inflammatory response caused by auto-immune disease cause the classic signs of inflammation (calor, rubor, tumor, dolor, functo laesa) [16], but also systemic symptoms like fatigue [17] and depression [18]. Furthermore, next to being an important primary cause of

death [19], persistent inflammatory activity caused by auto-immune diseases are also associated with a higher risk of cardiovascular death with hazard ratio`s up to 1.5 [20] and predispose towards infections [18]. Pro-inflammatory cytokines play a central role in the pathophysiology of auto-immune diseases [21, 22]. Overproduction of these cytokines is also critically involved in complications encountered in patients undergoing major surgery, which causes significant tissue injury [23]. For instance, overzealous inflammation is recognized as one of the major drivers in the development of one of the most dreaded complications of surgery: peri-operative renal damage leading to acute kidney injury (AKI) [24, 25]. AKI represents a major health care burden, especially in high risk patient undergoing high risk surgery [24, 26]. A global meta-analysis in 320.000 patients reported an incidence of 22.3% for AKI and 2.3% for patients on renal replacement therapy after cardiac surgery, with long-term mortality up to 30% [27].

Taken together, the immune system is essential in our fight against infectious diseases, but a dysregulated response may result in collateral damage to the host and thereby it is also an important cause of morbidity in many conditions. Therefore, therapies that modulate the immune response and prevent overproduction of cytokines are warranted in e.g. both auto-immune diseases and in patients undergoing major surgery.

Modulating the inflammatory response

Over the last decades, a large number of pharmacological therapies have been developed to attenuate the inflammatory response in auto-immune diseases [28] or during major surgery, such as cardiac surgery [29-31]. A large number of these interventions are directed against the above described cytokines or their receptors. Medication such as anti-TNF, soluble TNF-receptor, anti-IL-6 receptor, and IL-1 receptor antagonists have proven to be very effective treatments for auto-immune diseases [32, 33]. However, they are expensive and can have serious side effects [34, 35]. Furthermore, in surgical patients, of all available immunomodulatory therapies, only dexamethasone may be effective in modulating the pro-inflammatory immune response [36, 37] as it might reduce the need for renal replacement therapy after cardiac surgery [38, 39]. However, the side-effects of corticosteroids are numerous and severe (e.g. insulin resistance, adrenal suppression, hypertension) [40]. In this light, it may come as no surprise that research efforts have also been directed towards non-pharmacological ways to modulate the immune system.

Aim of this thesis

The aim of this thesis was to explore non-pharmacological immunomodulatory interventions. We studied two interventions: First, we investigated the immunomodulatory effects of a training program developed by mr. Wim Hof, a Dutch individual also known as `The Iceman`. In addition, we explored the effects of (elements of) the 'Iceman intervention' on several non-immunological outcome parameters, namely pain perception, metabolic changes, and long-term cortisol levels. Second, we investigated the immunomodulatory and renoprotective properties of remote ischemic preconditioning (RIPC). Figure 1 provides an overview of the studied interventions and outcome parameters. In our studies, we made extensive use of the experimental human endotoxemia model (administration of bacterial lipopolysaccharide [LPS] to healthy volunteers) to elicit a standardized, controlled, and reproducible immune response. Details of this model are described in detail further below.

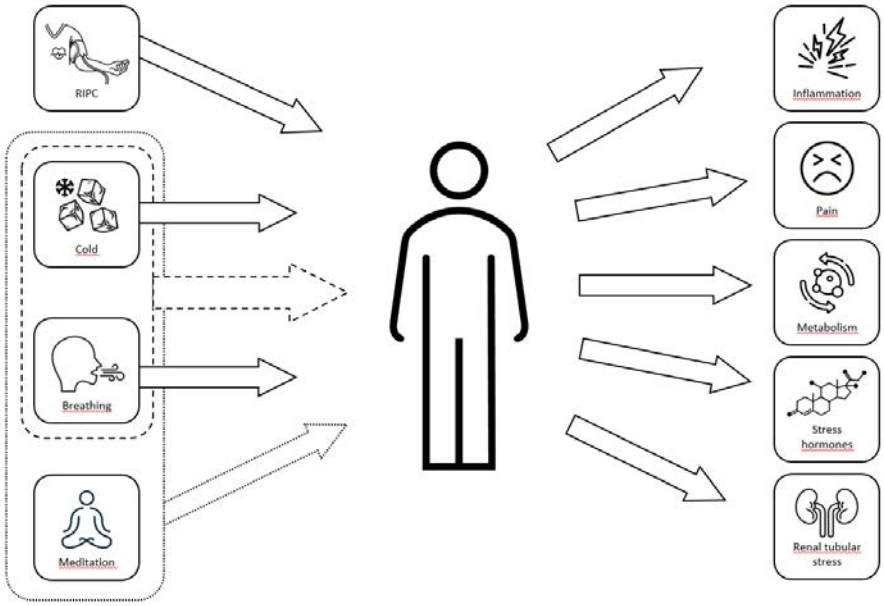

Figure 1. Overview of interventions and outcome parameters described in this thesis.

Iceman Wim Hof

The main starting point for the research described in this thesis is a case study by our group on autonomic nervous system activity and the immune response of Wim Hof [41]. Mr. Hof holds several world records with regard to withstanding extreme cold, including running a half marathon above the arctic circle wearing only shorts and swimming

underneath ice for 66 meters and climbing mount Kilimanjaro in his shorts [42]. He claims he is be able to achieve these remarkable feats by following a special training program that he developed himself. The training program consists of three elements: meditation, repeated exposure to cold and breathing exercises involving cyclic hyperventilation combined with retention of breath and tightening of all body muscles. He claims that, through this technique, coined the Wim Hof Method (WHM) [43], he can influence his autonomic nervous system, as well as his immune system [44].

In the case study, Hof was subjected to experimental endotoxemia, during which he performed his meditation and breathing exercises [41]. Plasma norepinephrine and epinephrine levels increased sharply shortly after start of the breathing exercises. Furthermore, plasma cortisol levels increased to much higher levels compared with a cohort of 112 volunteers who were previously subjected to experimental endotoxemia. Most importantly, compared with the abovementioned control group, endotoxin-induced increase in plasma levels of pro-inflammatory cytokines were remarkably attenuated in Hof, and he reported very few flu-like symptoms. These results suggested that the training program developed and practiced by Hof results in a consciously controlled and short-lived stress response, characterized by activation of the sympathetic nervous system, exemplified by catecholamine and cortisol release. In turn, this response appeared to be associated with an attenuated inflammatory response and remarkably mild subjective symptoms following endotoxemia. However, this case study can naturally not serve as scientific evidence. For instance, no proper statistical analysis can be performed on a n=1 study and individual factors not related to exercising the WHM, such as genetics and age of Mr. Hof are important potential confounders.

This thesis takes the next step in exploring if the training program can indeed attenuate the inflammatory response and therefore could represent a potential new adjuvant therapy for the treatment of auto-immune diseases.

To further investigate the underlying processes and consequences, in addition to the immunological analyses, we explored several other parameters as part of a more general approach to investigate the effects of the training program. First, we focused on metabolic changes. Activation of the sympathetic nervous system, as described above, is known to impact cellular metabolism [45]. Furthermore, metabolic reprogramming of innate immune cells has been shown to plays a critical role in regulation of the immune response [46, 47]. Therefore, changes in metabolism may contribute to the immunomodulatory effects of the training program. A relatively novel way to study the metabolism is represented by unsupervised metabolic profiling

of plasma samples using liquid chromatography–mass spectrometry (LC-MS). This technique provides information on a large number of circulating metabolites simultaneously, thereby providing an indication of the "metabolic status" [48]. Second, we evaluated whether (elements of) the training program may alter pain perception. This is highly relevant in the context of auto-immune diseases as subjective `patient-reported outcome measurement` (PROM) are increasingly used in clinical practice [49, 50]. A specific form of quantitative sensory testing (QST), the Nijmegen-Aalborg screening QST (NASQ) is a test battery is developed to map pain sensitivity at multiple body locations by non-invasive stimuli in an objective, controlled, and reproducible manner. Third, we investigated changes in the acid-base balance caused by the breathing exercise which is part of the training program devised by Wim Hof. In the case study performed in Mr. Hof, we observed that the breathing exercises led to extreme shifts in acid-base balance in arterial blood. However, the underlying mechanisms remained unknown, as a systematic analyses was not feasible in this n=1 study. Acid-base changes can be assessed by the traditional Henderson-Hasselbalch approach, including parameters such as pH, $PaCO_2$ and $HCO3^-$. However, an alternative analysis, the so-called Stewart approach, can also be employed and may be of additional value. This approach takes into account ionic shifts of Cl^-, K^+, Na^+, and PO_4^{3-}, and the buffering capacity of albumin, thereby providing a more detailed analysis of the underlying mechanisms involved [51]. Finally, we turned our attention to a long-term effect of the intervention, namely longitudinal changes in cortisol. As part of the body`s natural reaction to different stressors, cortisol is an important hormone, not only to initiate a stress response when necessary, but also to terminate it afterwards [52]. However, long-term exposure to high levels of cortisol can be detrimental and have been linked to, for instance, major depressive disorder [53, 54]. Traditional methods of cortisol analyses, in blood serum or saliva, only represent circulating cortisol levels in the preceding minutes to hours [55]. An emerging technique to evaluate long-term changes in cortisol is represented by analysis of scalp hair [56]. This technique can be used to construct retrospective timelines of cortisol exposure by separating hairs into segments and analyzing them for cortisol content individually [57, 58].

Remote ischemic preconditioning

The second non-pharmacological intervention explored in this thesis is remote ischemic preconditioning (RIPC). The precursor to RIPC is ischemic preconditioning (IPC), a concept first described in the 1980`s, where a protective effect of preconditioning the heart with 4 cycles of 5-minute long ischemia on the extent of myocardial infarction in dog hearts was demonstrated [59]. Follow-up animal studies showed the same protective effects on the heart by introducing the cycles of

ischemia to distant, or `remote`, organs like the kidney or the gut [60]. Furthermore, this principle of RIPC was also shown to prevent endothelial dysfunction in the arm measured with venous occlusion plethysmography [61]. As such, RIPC might represent an cheap, safe and easy-to-implement intervention to protect patients who undergo elective cardiac surgery from perioperative myocardial ischemic damage [62] and are at risk for acute kidney injury (AKI) [63]. With regard to the latter, urinary concentrations of TIMP2*IGFBP7 (an established biomarker for pre-clinical AKI [64, 65]) sharply increased directly following RIPC in patients undergoing cardiac surgery, after which tolerance was induced, as the post-operative increase in TIMP2*IGFBP7 related to the occurrence of acute kidney injury was attenuated in patients who received RIPC [63]. However, other studies did not reproduce these nephroprotective effects of RIPC [66, 67].

Two different timeframes in which RIPC potentially exerts its protective effects have been identified. The classical or `early window of protection' protects in the 1-2 hour after the RIPC stimulus [68] while a `second window of protection` is evident 12-24 hours after RIPC and may last for 48-72 hours [69]. Multiple-dose RIPC may be of additional value, as 7 daily doses of RIPC in humans resulted in protection against endothelial dysfunction measured using flow-mediated dilation and cutaneous vascular conductance [70]. Both the local and remote beneficial effects persisted for up to 8 days after the last RIPC dose [70, 71]. This could be due to additive or synergistic effects of combining the first and second windows of protection. These hypotheses, however, remain highly speculative as the mechanism of action by which RIPC exerts its protective effects is far from clear [72]. Next to neuronal pathways and humoral signal transfer, immunologic effects are likely of relevance. Several pro- and anti-inflammatory cytokines have been suggested to play role in the effects induced by RIPC in animals [73-75], healthy volunteers [76], and patients [77]. An open question is whether RIPC exerts direct immunomodulatory effects, or if RIPC-mediated attenuation of inflammation is secondary to its mitigating effect on tissue damage. We hypothesize that the underlying mechanism behind direct immunomodulatory effects is the release of danger associated molecular patterns (DAMPs) which induce immunological tolerance and thereby attenuated subsequent immune responses and tissue damage. DAMPs are released by cells under (ischemic) stress [78]. In the context of ischemia-reperfusion, this has for instance been described for high-mobility group box 1 (HMGB1) in mice [79].

Investigating immunomodulatory interventions: The experimental human endotoxemia model

Before a clinical trial in patients for new drugs or other therapeutic regimens can be justified, a strong foundation of basic, pre-clinical and translational research is required. The experimental human endotoxemia model is a prime example of a translational model which enables evaluation of the safety and efficacy of immunomodulatory interventions in humans *in vivo* in a controlled, transient and reproducible manner [80]. Thereby filling a niche between animal and patient experiments. In this model, *E. Coli* derived lipopolysaccharide (LPS) is intravenously administered to humans, either in a bolus or continuously over several hours [81-83]. In the hours after LPS administration, a systemic inflammatory response develops, characterized by increased blood concentrations of pro- and anti-inflammatory mediators such as TNF, IL-6 and IL-10, hemodynamic alterations such as tachycardia, tachypnoea, and a decrease in mean arterial blood pressure (MAP). Furthermore, participants experience flu-like symptoms for 2-4 hours and an increase in core body temperature of 1.5-2 °C.

Originally, this model was developed in the early 20th century as a therapeutic tool by which the induction of fever was thought to support the immune system in a fight against infection and cancer [84]. Later, during the 1950`s and 1960`s, endotoxemia was increasingly used as a model to study the pathophysiology of inflammation and to evaluate possible therapies for sepsis [83]. The reason for this lies in the interaction of the lipid-A part of LPS with the Toll Like receptor (TLR)-4. This interaction starts the cascade of pro- and anti-inflammatory mediators that mimics the first steps in the inflammatory cascade observed during Gram-negative sepsis [84, 85]. In the last decades of the 20th century, TNF-blocking drugs were developed with the aim to treat sepsis, with the human endotoxemia model as a pre-clinical, translational tool to provide proof-of-principle that these compounds can attenuate the immune response [86]. Unfortunately, anti-TNF drugs did not confer a benefit on clinical outcomes of sepsis patients in clinical trials [87, 88], plausibly related to patient selection and kinetics of TNF in sepsis patients. Nevertheless, anti-cytokine therapies were found to be very effective in auto-immune diseases [89, 90].

Taken together, in the experimental human endotoxemia model, the carefully selected and homogenous study population in combination with the highly standardized and reproducible manner in which these experiments are conducted allows to evaluate interventions that aimed at influencing systemic inflammation in humans *in vivo* [91]. Therefore, this model was used to evaluate the non-pharmacological interventions in this thesis.

Outline of this thesis

In *chapter 2,* the effects of the training program devised and provided by Iceman Wim Hof on sympathetic nervous system parameters and the systemic inflammatory response in healthy volunteers during experimental endotoxemia are compared to the responses in untrained volunteers. In a follow up study described in *chapter 3*, the contribution of the different training modalities and elements of the aforementioned training program is established, again using the experimental human endotoxemia model. To evaluate the 'guru-effect', it was also investigated whether use of an independent trainer instead of Wim Hof influenced the results. Therefore, this study aimed to shed light on the underlying mechanisms responsible for the previously observed anti-inflammatory effects and to aid future clinical development of the training intervention.

Chapter 4 details the effects of single and repeated RIPC on the systemic inflammatory response during experimental human endotoxemia. Furthermore, the effects of RIPC on subclinical renal injury, reflected by urinary levels of IGFBP7*TIMP-2 levels, are detailed.

In *chapter 5,* results of metabolomic profiling of plasma samples obtained in the study described in chapter 2 are presented. This study aimed to determine changes in the plasma metabolome of trained and untrained participants, and to assess if differences between groups could play a role in the observed immunomodulatory effects of the intervention.

Chapter 6 describes pain threshold measurements (using the NASQ test battery) performed during the conduct of the study described in chapter 3. Previous data from our group revealed that the systemic inflammatory response during experimental endotoxemia causes decreased pain thresholds, and we explored whether the different training modalities influenced this effect.

In *chapter 7,* changes in acid-base balance in healthy volunteers practicing the hyperventilation exercise devised by Wim Hof are demonstrated using the Stewart approach. Furthermore, Stewart-based results are compared to those obtained using the conventional Henderson-Hasselbalch analysis.

Finally, *chapter 8* entails a short report examining whether the training program described in chapter 2 affects long-term cortisol levels in scalp hair.

Finally this thesis is concluded by a summary in *chapter 9* and a general discussion of the findings and future perspectives in *chapter 10*.

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Chapter 2

Voluntary activation of the sympathetic nervous system and attenuation of the innate immune response in humans

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Abstract

Excessive or persistent pro-inflammatory cytokine production plays a central role in autoimmune diseases. Acute activation of the sympathetic nervous system attenuates the innate immune response. However, both the autonomic nervous system and innate immune system are regarded as systems that cannot be voluntarily influenced. Herein, we evaluated the effects of a training program on the autonomic nervous system and innate immune response. Healthy volunteers were randomized to either the intervention (n=12) or control group (n=12). Subjects in the intervention group were trained for 10 days (including breathing techniques [cyclic hyperventilation followed by breath retention] and exposure to cold [i.a. immersions in ice cold water]). The control group was not trained. Subsequently, all subjects underwent experimental endotoxemia (i.v. administration of 2 ng/kg *E. Coli* endotoxin). In the intervention group, practicing the learned techniques resulted in intermittent respiratory alkalosis and hypoxia resulting in profoundly increased plasma epinephrine levels. In the intervention group, plasma levels of the anti-inflammatory cytokine IL-10 increased more rapidly after endotoxin administration, correlated strongly with preceding epinephrine levels, and were higher. Levels of pro-inflammatory mediators TNF-α, IL-6, and IL-8 were lower in the intervention group and correlated negatively with IL-10 levels. Finally, flu-like symptoms were lower in the intervention group. In conclusion, we demonstrate that voluntary activation of the sympathetic nervous system results in epinephrine release and subsequent suppression of the innate immune response in humans *in vivo*. These results could have important implications for the treatment of conditions associated with excessive or persistent inflammation, such as autoimmune diseases.

Significance statement

Hitherto, both the autonomic nervous system and innate immune system were regarded as systems that can not be voluntarily influenced. The present study demonstrates that, through practicing techniques learned in a short-term training program, the sympathetic nervous system and immune system can indeed be voluntarily influenced. Healthy volunteers practicing the learned techniques exhibited profound increases in the release of epinephrine which in turn led to increased production of anti-inflammatory mediators and subsequent dampening of the pro-inflammatory cytokine response elicited by intravenous administration of bacterial endotoxin. This study could have important implications for the treatment of a variety of conditions associated with excessive or persistent inflammation, especially auto-immune diseases in which therapies that antagonize pro-inflammatory cytokines have shown great benefit.

Introduction

The innate immune system is crucial to our survival, but excessive or persistent pro-inflammatory cytokine production can result in tissue damage and organ injury, such as in autoimmune diseases. Biological therapies that antagonize pro-inflammatory cytokines or their receptors are very effective and have revolutionized the treatment of auto-immune diseases such as rheumatoid arthritis and inflammatory bowel disease (1, 2). However, these drugs are expensive and have serious side effects (3, 4). Therefore, innovative therapies aimed at limiting inflammatory cytokine production in a more physiological manner are warranted.

Acute activation of the sympathetic nervous system attenuates inflammation via activation of β2-adrenoceptors by catecholamines, exemplified by the fact that (nor) epinephrine attenuates lipopolysaccharide(LPS)-induced TNF-α release *in vitro (5, 6)* and short-term infusion of epinephrine limits production of pro-inflammatory cytokines *in vivo* during experimental endotoxemia (intravenous administration of LPS in healthy volunteers) (7). In addition, as part of a stress response, increased levels of catecholamines are often accompanied by elevations of the well-known immunosuppressive hormone cortisol (via activation of the hypothalamic-pituitary-adrenal (HPA) axis) (8, 9).

Next to exogenous (i.e. pharmacological or electrical) modulation of the autonomic nervous system (ANS), endogenous stimulation of ANS activity may also limit the inflammatory response, but the ANS is generally regarded as a system that cannot be voluntarily influenced. However, results from a recently performed case study on a Dutch individual, who holds several world records with regard to withstanding extreme cold, suggest otherwise (10). It was shown that this individual was able to voluntarily activate the sympathetic nervous system through a self-developed method involving meditation, exposure to cold, and breathing techniques. This resulted in increased catecholamine- and cortisol release and a remarkably mild innate immune response during experimental endotoxemia compared with a more than 100 subjects who previously underwent experimental endotoxemia.

In the present study, we investigated the effects of his training program (see Video 1 for an impression) on sympathetic nervous system parameters and the innate immune response in healthy male volunteers during experimental endotoxemia in a randomized controlled fashion.

Materials and Methods

Subjects

This parallel randomized controlled study was registered at ClinicalTrials.gov as NCT01835457. After approval by the local ethics committee of the Radboud University Nijmegen Medical Centre (CMO 2012/455), 30 Dutch healthy, non-smoking, male volunteers were included in the trial. All subjects provided written informed consent and experiments were in accordance with the Declaration of Helsinki, including current revisions, and Good Clinical Practice guidelines. Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values. Exclusion criteria were: febrile illness during the 2 weeks before the endotoxemia experiment, taking any prescription medication, history of spontaneous vagal collapse, practicing or experience with any kind of meditation, or participation in a previous trial where LPS was administered. The subjects were randomly allocated to the trained group (n=18) or the control group (n=12) by the opening of a sealed envelope prepared by a research nurse not involved in the study. After having fulfilled the training program, 12 of the 18 trained subjects were randomly assigned to participate in the experimental endotoxemia experiments (further explained in section "study design and training procedure").

Three subjects in the control group that underwent endotoxemia on the same day and received LPS from the same ampoule were excluded from the trial and replaced. Their symptoms, temperature rise, hemodynamic response and cytokine response were inconsistent with having received an adequate dose of 2 ng/kg LPS. Batchwise determination of cytokine levels revealed exceptionally levels in all three subjects: Their peak cytokine response (TNF-α and IL-6) was less than half of that of the lowest recorded in a cohort of 112 healthy male subjects that previously underwent experimental endotoxemia (10) and peaked at atypical time-points (subject 1: TNF-α: 39 pg/mL at 4 hours after LPS administration and IL-6: 27 pg/mL at 4 hours after LPS administration; subject 2: TNF-α: 32 pg/mL at 3 hours after LPS administration and IL-6: 31 pg/mL at 3 hours after LPS administration; subject 3: TNF-α: 9 pg/mL at 2 hours after LPS administration and IL-6: 7 pg/mL at 3 hours after LPS administration). Therefore, a endotoxin dose administration error was assumed and the subjects were replaced.

Study design and training procedure

The study was sequentially conducted in two identical blocks, each consisting of 9 subjects in the trained group (of which 6 finally participated in the endotoxemia experiments, further explained below) and 6 subjects in the control group. This

design was chosen to minimize the bias due to differences in the interval between the end of the training period and the endotoxemia experiments. As the aim of our study was to investigate the effects of the training intervention on the innate immune response in a standardized model of systemic inflammation, we did not assess the effects of the training intervention on immune system parameters in the absence of endotoxemia. A schematic overview of the study design (1 block) is depicted in Figure S3 (Supporting Information). The trained group was trained by Dutch individual Wim Hof and three trainers who previously received an instructor course by Wim Hof to become a trainer. A medical doctor of the study team (LvE) and the principal investigator (MK) were present during all training sessions (in Poland and in the Netherlands), and during the experimental endotoxemia experiments. The first 4 days of the training program took place in Poland and were most intensive. The program consisted of three main elements: meditation, exposure to cold, and breathing techniques (see Video 1 for an impression of the training program).

1) Meditation, so-called "third eye meditation", a form of meditation including visualizations aimed at total relaxation.

2) During the training, subjects voluntarily exposed themselves to cold in several ways:

a) standing in the snow bare foot for up to 30 minutes and lying bare chested in the snow for 20 minutes, b) daily dipping/swimming in ice-cold water (0 - 1 °C) for up to several minutes (including complete submersions) and c) hiking up a snowy mountain (elevation: 1590 m) bare chested, wearing nothing but shorts and shoes at temperatures ranging from -5 to -12°C (wind chill: -12 to -27°C).

3) Breathing techniques, consisting of two exercises. In the first exercise subjects were asked to hyperventilate for an average of 30 breaths. Subsequently, the subjects exhaled and held their breath for approximately 2-3 minutes ("retention phase"). The duration of breath retention was entirely at the discretion of the subject himself. Breath retention was followed by a deep inhalation breath, that was held for 10 seconds. Subsequently a new cycle of hyper/hypoventilation began. The second exercise consisted of deep inhalations and exhalations in which every in- and exhalation was followed by breath holding for 10 seconds, during which the subject tightened all his body muscles. These two breathing exercises were also performed during the endotoxemia experiments. Additional element of the training program consisted of strength exercises (e.g. push-ups and yoga balance techniques).

After returning from Poland, the subjects practiced the techniques they learned daily by themselves at home (2-3 hours per day, cold exposure was achieved through taking cold showers), until the endotoxemia experiment day (5 to 9 days later). In addition, a final group training took place and at the end of this day, 6 out of 9 trained subjects (in each block) were randomly selected for participation in the endotoxemia experiments, using the sealed envelope method. This selection was performed to allow for subject replacement in case of an adverse event or illness in one of the trained subjects selected for the endotoxemia experiments. The selected subjects practiced in a final training session led by Wim Hof on the day before the endotoxemia experiment day. Wim Hof was present to coach the subjects during the endotoxemia experiment days during the three hours that the subjects in the trained group practiced the learned techniques. The control group did not undergo any training procedures throughout the study period.

Experimental human endotoxemia

Subjects refrained from caffeine- or alcohol containing substances 24 hours before the start of the experiment, and food 10 hours before the start of the endotoxemia experiment. The experiments were performed at the research unit of the intensive care department. The procedures on the endotoxemia experiment day are depicted in Figure S4 (Supporting Information). Purified lipopolysaccharide (LPS, US Standard Reference Endotoxin Escherichia Coli O:113) obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD), supplied as a lyophilized powder, was reconstituted in 5 ml saline 0.9% for injection and vortex-mixed for at least 20 minutes after reconstitution. The LPS solution was administered as an intravenous bolus injection at a dose of 2 ng/kg body weight in one minute at T = 0 hours. A cannula was placed in an antecubital vein to permit infusion of 0.9% NaCl solution; the subjects received 1.5 L 0.9% NaCl during one hour starting one hour before endotoxin infusion (pre-hydration) as part of our standard endotoxemia protocol (29), followed by 150 ml/h until 6 hours after endotoxin infusion and 75 ml/h until the end of the experiment. The radial artery was cannulated using a 20-gauge arterial catheter (Angiocath, Becton Dickinson, Sandy, Utah) and connected to an arterial pressure monitoring set (Edwards Lifesciences LLC, Irvine, CA, USA) to allow the continuous monitoring of blood pressure and blood sampling. Heart rate (3-lead electrocardiogram), blood pressure, respiratory rate, and oxygen saturation (pulse oximetry) data were recorded from a Philips MP50 patient monitor (Philips, Eindhoven, The Netherlands) every 30 seconds by a custom in-house developed data recording system, starting 1 hour before administration of LPS until discharge from the intensive care unit 8 hours after LPS administration. Body temperature was measured using an infrared

tympanic thermometer (FirstTemp Genius 2, Sherwood Medical, Crawley/Sussex, UK). LPS-induced flu-like symptoms (headache, nausea, shivering, muscle- and back pain) was scored every 30 minutes on a 6-point Likert scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total score of 0 to 25.

Thirty minutes before LPS administration (T=-0.5 hrs), subjects in the trained group started the first breathing technique (hyper/hypoventilation cycles, see Video 2) until T=1 hr, followed by the second breathing technique (deep in- and exhalation in combination with tightening muscles) until T=2.5 hrs. Afterwards, the subjects stopped practicing all the techniques. The control group did not practice any techniques throughout the endotoxemia experiment day.

Blood gas parameters

Blood gas parameters were analyzed in lithium heparin anti-coagulated arterial blood using CG4+ cartridges and a point-of-care i-STAT Blood Gas Analyzer (Abbot, Hoofddorp, The Netherlands).

Catecholamines

Blood was collected into chilled lithium-heparin tubes and were immediately placed on ice and centrifuged at 2000g for 10 minutes at 4°C after which plasma was stored at -80°C until analysis. Plasma norepinephrine, epinephrine, and dopamine concentrations were measured using routine analysis methods also used for patient samples (high-performance liquid chromatography with fluorometric detection, as described previously (30)).

Cortisol

Blood was collected in serum-separating tubes and was allowed to clot at room temperature for a minimum of 30 minutes. Subsequently, samples were centrifuged at 2000g for 10 minutes at 4°C, after which serum was stored at -80°C until analysis. Cortisol levels were determined using a routine analysis method also used for patient samples (Electrochemiluminescent Immunoassay (ECLIA) on a Modular Analytics E170 (Roche Diagnostics, Mannheim, Germany)).

Leukocyte counts and differentiation

Analysis of leukocyte counts and differentiation in were performed in EDTA anticoagulated blood using routine analysis methods also used for patient samples (flow cytometric analysis on a Sysmex XE-5000 (Etten-Leur, The Netherlands)).

Plasma cytokines

EDTA anticoagulated blood was centrifuged immediately at 2000g for 10 minutes at 4°C after which plasma was stored at -80°C until analysis. Concentrations of TNF-α, IL-6, IL-8, and IL-10 were measured using a simultaneous Luminex Assay according to the manufacturer's instructions (Milliplex, Millipore, Billerica, MA, USA). IL-1β, TGF-β, and leptin were measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (IL-1β and TGF-β: Quantikine, R&D systems, Minneapolis, MN USA. Leptin: Duoset, R&D systems, Minneapolis, MN USA).

Calculations and statistical analysis

Data are represented as median and interquartile range/range or mean and SEM based on their distribution (calculated by the Shapiro-Wilk test). Statistical tests used are indicated in the figure/table legends or text. Spearman's correlation was used. A p-value of < 0.05 was considered statistically significant. Statistical calculations were performed using Graphpad Prism version 5.0 (Graphpad Software, San Diego, CA, USA).

Results

Baseline characteristics of subjects that underwent experimental endotoxemia in both groups were similar (Table 1).

Cardiorespiratory parameters, temperature, and symptoms

In the control group, arterial blood gas parameters pCO_2, pO_2, pH, bicarbonate, lactate, and oxygen saturation were normal and did not substantially change during endotoxemia (Figure 1, A-F).

In contrast, in trained individuals, practicing the learned breathing techniques resulted in an immediate and profound decrease of pCO_2 and bicarbonate, and an increase in pH (reaching up to 7.75 in individual subjects, see Figure 2 and Video 2), indicating acute respiratory alkalosis which normalized quickly after cessation of the breathing techniques.

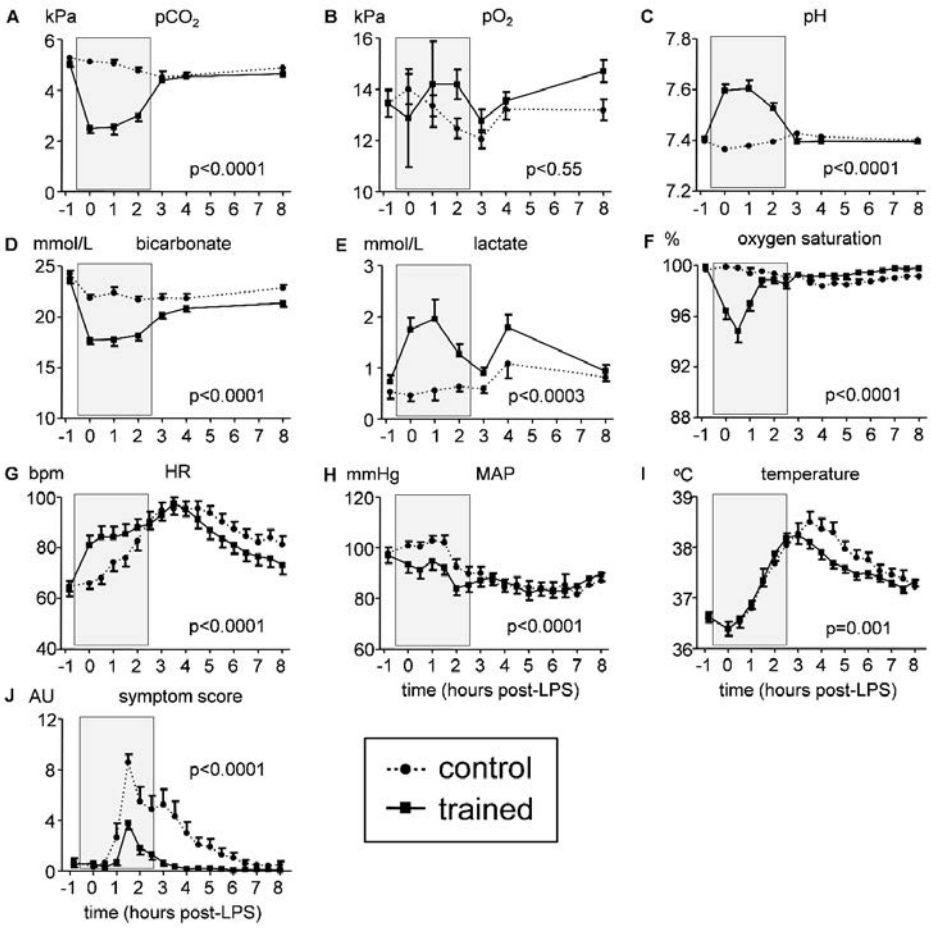
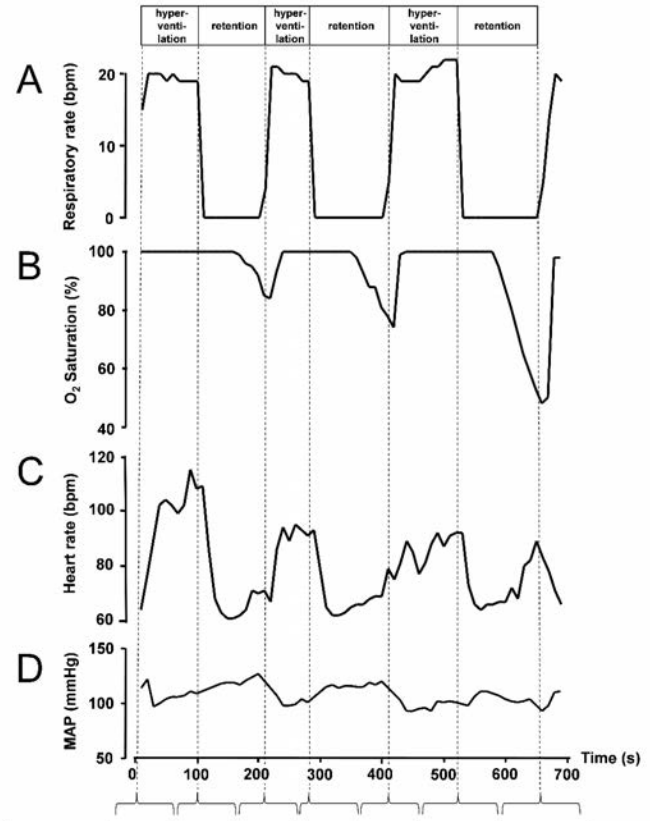


Figure 1. Cardiorespiratory parameters, temperature, and symptoms during experimental endotoxemia in control and trained subjects. (A) Carbon dioxide partial pressure (pCO_2) in arterial blood. **(B)** Oxygen partial pressure (pO_2) in arterial blood. **(C)** pH in arterial blood. **(D)** Bicarbonate ($HCO3^-$) in arterial blood. **(E)** Lactate in arterial blood. **(F)** Oxygen **s**aturation measured by pulse oximetry. **(G)** Heart rate (HR). **(H)** Mean arterial pressure (MAP). **(I)** Temperature. **(J)** Score of self-reported symptoms. Data are expressed as mean±SEM of 12 subjects per group. Grey box indicates period in which the trained subjects practiced their learned breathing techniques. P values between groups calculated using repeated measures two-way analysis of variance (ANOVA, interaction term). bpm: beats/min. AU: arbitrary units.



	Start	End hyperventilation	End retention	End hyperventilation	End retention	End hyperventilation	End retention
pH	7.40	7.66	7.44	7.67	7.46	7.75	7.50
pC02 (kPa)	4.49	2.11	4.01	2.03	3.76	1.69	3.48
pO2 (kPa)	16.5	22.0	5.6	22.9	4.8	22.6	3.4
HCO3- (mmol/l)	20.9	18.0	20.3	17.6	20.2	17.4	20.4
Lactate (mmol/l)	0.69	0.86	0.69	1.03	0.77	1.16	0.91

Figure 2: Cardiorespiratory and biochemical changes during cyclic hyperventilation and breath retention in a representative subject of the trained group. (A) The respiratory rate alternately increased to around twenty breaths per minute (bpm) for several minutes, and then dropped to zero during voluntary breath retention. These cyclic changes in respiration resulted in profound changes in **(B)** oxygen saturation, **(C)** heart rate, and **(D)** mean arterial pressure. The data depicted in these panels were sampled from the monitor every 10 seconds. At the end of each hyperventilation phase and breath retention phase, an arterial blood sample was drawn for arterial blood gas analysis, of which the results are listed in the table below panel D. The cycles of hyper/hypoventilation in this particular subject can be viewed in Video 2.

Mean pO_2 remained virtually unaltered in trained subjects, while lactate levels were significantly elevated, but not to clinically relevant levels. A significant decrease in oxygen saturation was observed in the trained group during practicing of the breathing techniques (Figure 1F). Minimum oxygen saturation levels in each cycle of hyper/hypoventilation (after cessation of breathing for several minutes) typically dropped to around 50% in trained individuals for a short period of time (approximately 10 seconds, see Figure 2 and Video 2). Heart rate and mean arterial blood pressure (MAP) showed a pattern typical for endotoxemia in the control group: a gradual decrease in MAP and a compensatory rise in heart rate after LPS administration (Figure 1, G and H). In the trained group, heart rate increased after commencing the breathing techniques and normalized earlier compared with the control group while MAP decreased during the breathing techniques and thereafter followed the same pattern as in the control group. LPS administration resulted in fever, with a maximum temperature increase in the control group of 1.9±0.2 °C (mean±SEM), while this increase was less pronounced and normalized earlier in the trained group (Figure 1I). Self-reported symptoms (nausea, headache, shivering, muscle, and back pain on a 6-point Likert scale) peaked 1.5 hours after LPS administration in both groups, but were attenuated in the trained individuals compared with the control group (reduction of 56% in peak levels, Figure 1J).

Catecholamine and cortisol levels

Plasma epinephrine levels (Figure 3A) increased sharply 1 hour after LPS administration and peaked at T=1.5 hours in the control group.

In trained subjects, baseline epinephrine levels were significantly higher compared with the control group (mean±SEM: 1.02±0.22 vs. 0.35±0.06 nmol/L, p=0.007 (unpaired Student t-test)). After starting practicing the learned breathing techniques, epinephrine levels further increased in this group and peaked just before administration of LPS (mean±SEM: 2.08±0.37 nmol/L at T=0 hours, with individual subjects reaching up to 5.3 nmol/L) and remained elevated until cessation of the breathing techniques. In contrast to epinephrine, norepinephrine and dopamine levels remained within the reference range throughout the experiment (Figure 3, B and C). Norepinephrine levels were similar between groups during the breathing period, although trained subjects displayed higher levels at baseline and after cessation of the breathing techniques. In contrast, dopamine levels were slightly lower in trained individuals during the breathing techniques but were similar between groups before and afterwards. There were no differences in serum levels of the stress hormone cortisol between the groups before or during the period in which the trained group practiced their techniques, however, levels normalized more quickly in trained individuals (Figure 3D).

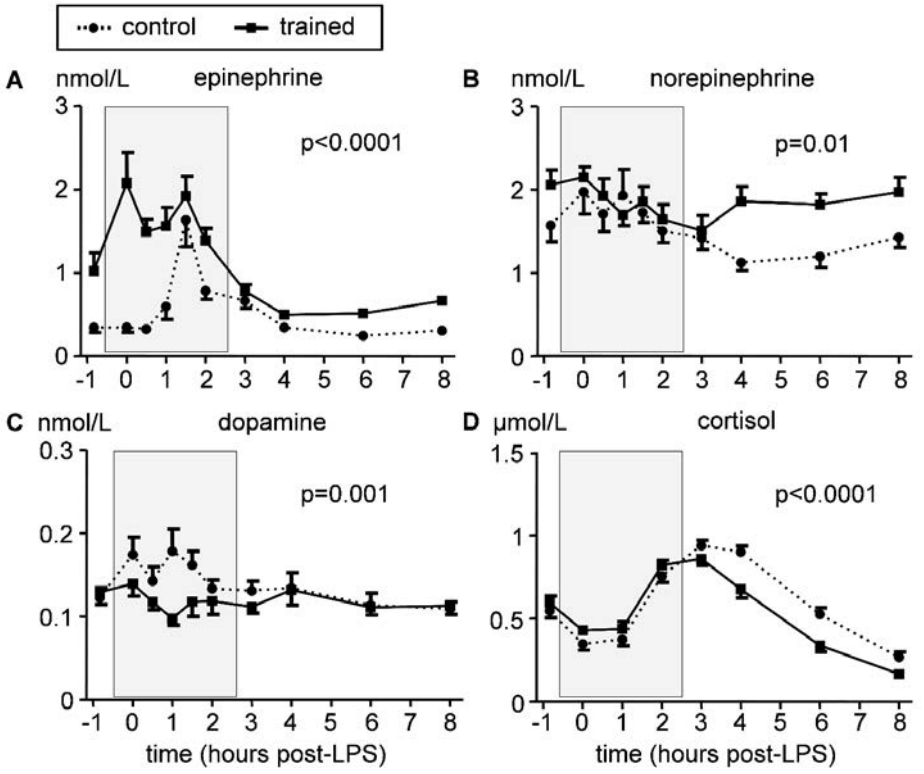


Figure 3. Plasma catecholamines concentrations and serum cortisol concentrations during experimental endotoxemia in control and trained subjects. (A) Plasma epinephrine. **(B)** Plasma norepinephrine. **(C)** Plasma dopamine. **(D)** Serum cortisol. Data are expressed as mean±SEM of 12 subjects per group. Grey box indicates period in which the trained subjects practiced their learned breathing techniques. P values between groups calculated using repeated measures two-way analysis of variance (ANOVA, interaction term).

Leukocyte counts

Total leukocyte counts in both groups showed the typical endotoxemia-induced biphasic pattern with an initial leukopenia followed by leukocytosis (Figure S1A, Supporting Information). Leukocyte concentrations were markedly higher in trained individuals. 30 minutes after start of the breathing techniques (T=0 hours), an increase in lymphocytes was observed in trained individuals which was not present in the control group (Figure S1B, Supporting Information). Concentrations of neutrophils and monocytes were similar between groups at this early time-point, but were distinctly higher in the trained group at later time-points (Figure S1, C and D, Supporting Information).

Plasma cytokines

Plasma concentrations of pro-inflammatory cytokines TNF-α, IL-6, and IL-8, and the anti-inflammatory cytokine IL-10 all markedly increased after LPS administration in both groups (Figure 4).

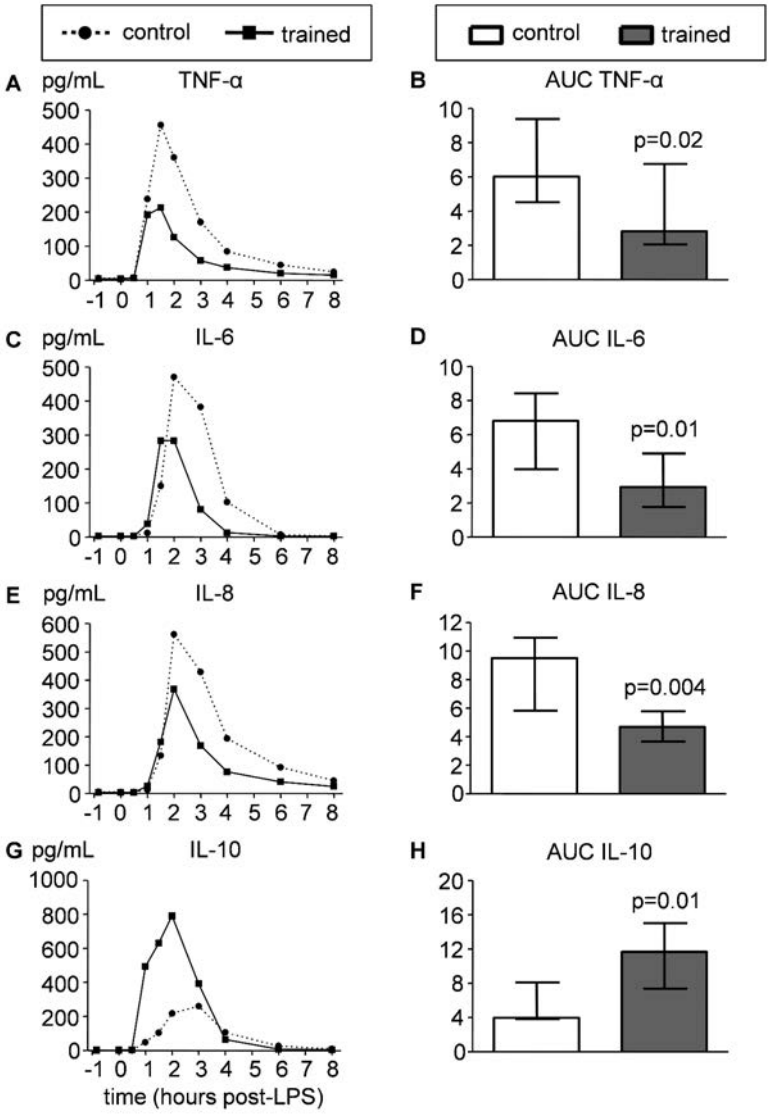


Figure 4. Plasma cytokine concentrations during endotoxemia in control and trained subjects. Panels **A, C, E, and G** depict median values of pro- (TNF-α, IL-6, IL-8) and anti-inflammatory (IL-10) cytokines (n=12 per group). Panels **B, D, F, and H** depict median±interquartile range of area under curve (AUC) of pro- (TNF-α, IL-6, IL-8) and anti-inflammatory (IL-10) cytokines (n=12 per group; unit: $x10^4$ pg/ml.hour). P values calculated using Mann-Whitney U-tests.

However, in trained individuals, TNF-α, IL-6, and IL-8 levels were significantly attenuated, while the IL-10 response was greatly augmented compared with the control group (TNF-α, IL-6, and IL-8 levels 53%, 57%, and 51% lower; IL-10 levels 194% higher). Furthermore, IL-10 levels in the trained group increased sharply early after LPS administration (at T=1 hr) and peaked one hour prior to the peak observed in the control group. In line with previous reports (11), plasma levels of the pro-inflammatory cytokine IL-1β were barely detectable during human endotoxemia. Concentrations were below the detection limit (3.9 pg/mL) in all but four subjects (two in each group, showing very low concentrations [4-6 pg/mL] at 1-3 time-points with no apparent kinetics over time). Concentrations of the anti-inflammatory cytokine TGF-β showed no kinetics after administration of LPS and were not different between groups (Figure S2A, Supporting Information). We also measured plasma concentrations of leptin, an adipokine that exerts pro-inflammatory activity. At baseline (T=-1 hour), there was a trend towards lower levels of leptin in the trained group compared with the control group (mean±SEM: 3.36±0.55 vs. 4.99±0.74 ng/mL, p=0.09, unpaired Student T-test), which remained apparent at all subsequent time-points (Figure S2B, Supporting Information). Leptin kinetics showed a biphasic pattern with an initial modest decrease followed by a gradual increase in both groups. However, there were no differences between groups over time.

Correlation analyses

As depicted In Figure 5A, there was a strong positive correlation (r_s=0.82, p=0.001) between epinephrine levels in the trained group at T=0 hours (30 minutes after commencing the breathing techniques) and the early increase in IL-10 levels at T=1 hour, which was not present in the control group (r_s=0.18, p=0.571).

Furthermore, there were significant inverse correlations between levels of the anti-inflammatory cytokine IL-10 at T=1 hour and peak levels of the pro-inflammatory mediators TNF-α (at T=1.5 hours), IL-6 (at T=2 hours), and IL-8 (at T=2 hours) in the trained group (Figure 5, B-D). In the control group, no such inverse correlations between IL-10 and pro-inflammatory cytokines were observed. In fact, we found significant positive correlations between preceding TNF-α and IL-6 levels on the one hand and IL-10 levels at later time-points (TNF-$\alpha^{T=1}$ vs. IL-$10^{T=2}$: r_s=0.59, p=0.045; IL-$6^{T=1.5}$ vs. IL-$10^{T=2}$: r_s=0.60, p=0.039).

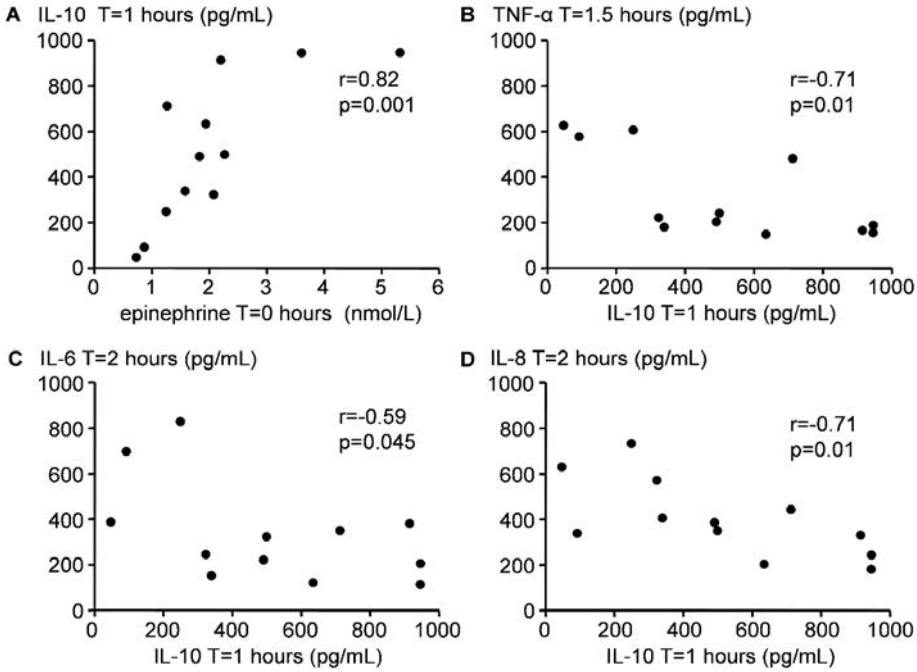


Figure 5. Correlations in trained individuals. (A) Correlation between peak plasma levels of epinephrine (at T=0 hrs) and plasma levels of the anti-inflammatory cytokine IL-10 at T=1 hr. **(B)** Correlation between plasma levels of the anti-inflammatory cytokine IL-10 at T=1 hr and peak plasma levels of the pro-inflammatory cytokine TNF-α (at T=1.5 hrs). **(C)** Correlation between plasma levels of the anti-inflammatory cytokine IL-10 at T=1 hr and peak plasma levels of the pro-inflammatory cytokine IL-6 (at T=2 hrs). **(D)** Correlation between plasma levels of the anti-inflammatory cytokine IL-10 at T=1 hr and peak plasma levels of the pro-inflammatory cytokine IL-8 (at T=2 hrs). R and p values calculated using Spearman correlation.

Discussion

Herein, we show that a short-term training program and practicing breathing techniques learned during this training program results in release of epinephrine, induction of early anti-inflammatory IL-10 production, and consequently attenuation of the pro-inflammatory innate immune response during experimental human endotoxemia. Also, trained individuals experienced fewer endotoxemia-associated flu-like symptoms, and a more swift normalization of fever and cortisol levels, which are likely the result of the attenuated pro-inflammatory response.

This study demonstrates that the *in vivo* innate immune response can be voluntarily influenced in a non-pharmacological manner through voluntary activation of the sympathetic nervous system. In accordance with the data of our control group, human endotoxemia in itself has been shown previously to result in increased levels of epinephrine (12). However, in trained individuals epinephrine levels were already profoundly increased 30 minutes after start of practicing the breathing techniques, before LPS administration. Epinephrine levels in trained individuals were even higher than those reported in a recent study in which acute stress elicited by a bungee jump was found to suppress cytokine production by leukocytes *ex vivo* stimulated with LPS (13). As norepinephrine, dopamine, and cortisol levels were not increased in the training group, it appears that the techniques predominantly result in stimulation of the sympathetic input to the adrenal medulla, because this is the most abundant source of epinephrine in the body and epinephrine-producing chromaffin cells in the adrenal medulla are much more abundant than those producing norepinephrine (14).

The observed potentiating effects on anti-inflammatory IL-10 production as well as the attenuation of pro-inflammatory cytokine levels are in agreement with a previously performed study where epinephrine was intravenously administered before LPS in healthy volunteers and resulted in early and increased IL-10 production (7), and with studies showing that pretreatment with IL-10 results in attenuation of the pro-inflammatory response in healthy volunteers (15, 16). In the training group, strong inverse correlations between IL-10 levels at an early time-point and later-occurring peak levels of the pro-inflammatory mediators were found, while in the control group the opposite was found: positive correlations between preceding levels of pro-inflammatory mediators with the later-occurring peak levels of IL-10. These findings indicate that the pro-inflammatory response drives IL-10 production in the control group, while the epinephrine-induced early increase in IL-10 production inhibits pro-inflammation in the trained group. The early increases in lymphocytes and subsequent higher concentrations of circulating neutrophils in the training group compared with the control group can also be attributed to the elevated epinephrine levels found in trained individuals, as catecholamines induce leukocytosis characterized by an initial lymphocytosis followed by an increase of other subpopulations (17). Furthermore, similar changes in leukocyte counts were previously observed during voluntary hyperventilation (18). Our study is limited by the fact that we did not measure specific leukocyte subtypes such as CD3, CD4, and CD8 numbers as well as B-cells, dendritic cells, and NK cells, some of which have been shown to be specifically altered by catecholamines and/or stress (19, 20).

It appears that mainly the breathing techniques employed by the trained individuals account for the increase in epinephrine and subsequent attenuation of the inflammatory response. A limitation of our study design is that it does not allow to identify the particular component of the practiced breathing exercises that results in increased epinephrine levels. Furthermore, the effect of the length of the training and the length of propensity for altered responses after training has yet to be determined. However, the effects on epinephrine are likely a consequence of both the hyperventilation phase and hypoxia due to breath retention, as both have been demonstrated to increase epinephrine levels (18, 21-24). The hyperventilation-induced increase in epinephrine was shown to be dependent on decreased levels of bicarbonate, as hyperventilation combined with bicarbonate infusion (resulting in hypocapnia and alkalosis, but normal bicarbonate levels) nullified epinephrine increase (24). In concordance, in the present study, bicarbonate levels were significantly lower in the trained subjects during practicing of the breathing techniques compared with control subjects. The attenuated cytokine response is unlikely to be a direct result from low pCO_2 and high pH levels since hypocapnic alkalosis, as opposed to hypercapnic acidosis (25), is not associated with anti-inflammatory effects. Therefore, epinephrine is the most probable intermediate factor (7). Nevertheless, it cannot be ruled out that other elements of the training, apart from practicing the breathing exercises, ultimately affected the LPS-induced innate immune response. For instance, the exposition to extreme cold and subsequent rewarming during the training sessions might have resulted ischemic preconditioning and/or release of danger associated molecular patterns (DAMPs), which could result in a tolerant state towards a subsequent LPS challenge.

It remains to be determined whether the results of this study using an acute model of inflammation in healthy volunteers can be extrapolated to patients with chronic auto-immune diseases. For instance, chronic stress might be harmful in these conditions due to induction of pro-inflammatory mediators (26), whereas bouts of short-term stress, similar to the effects of the training intervention described in this study, may be beneficial due to immunosuppressive effects (26). Of interest, the *in vivo* anti-inflammatory potential in humans of biologics currently used in the treatment of rheumatoid arthritis was first established in proof-of-principle human endotoxemia studies (27, 28), illustrating the relevance of the model to investigate novel therapies for this type of disease.

In conclusion, the present proof-of-principle study demonstrates that the sympathetic nervous system and immune system can be voluntarily influenced through practicing techniques that are relatively easy to learn within a short time frame. It therefore could

have important implications for the treatment of a variety of conditions associated with excessive or persistent inflammation, especially auto-immune diseases.

Acknowledgements

The authors thank K. Mostard, K. Pijper, D. Bernard, and E. Hof for help during the training sessions, the Radboud University Nijmegen Sports Centre for providing space for training days in the Netherlands, and the research nurses of the ICU department for help during the endotoxemia experiments. This study was supported by a Serendipity Grant from Reumafonds (reumafonds.nl). Reumafonds was in no way involved in collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The authors have no financial or other relationship with Reumafonds and declare no other competing financial interests.

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Supporting information

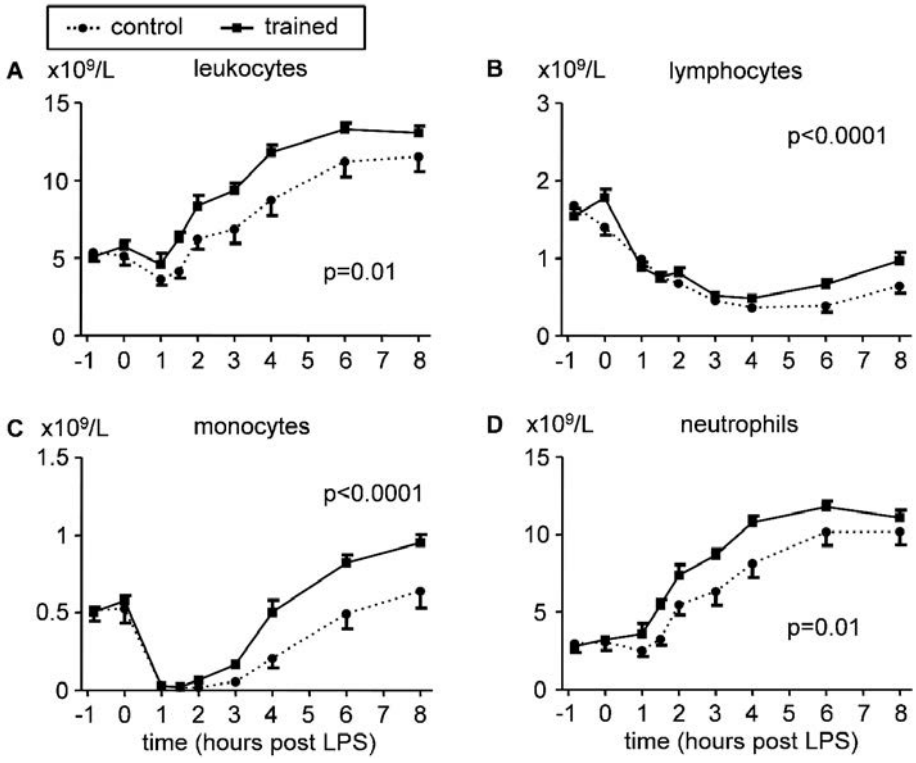

Figure S1. Leukocyte and subpopulation concentrations during endotoxemia in control and trained subjects.

(A) Total leukocytes. **(B)** Lymphocytes. **(C)** Monocytes. **(D)** Neutrophils. Data are expressed as mean±SEM of 12 subjects per group. P values between groups calculated using repeated measures two-way analysis of variance (ANOVA, interaction term).

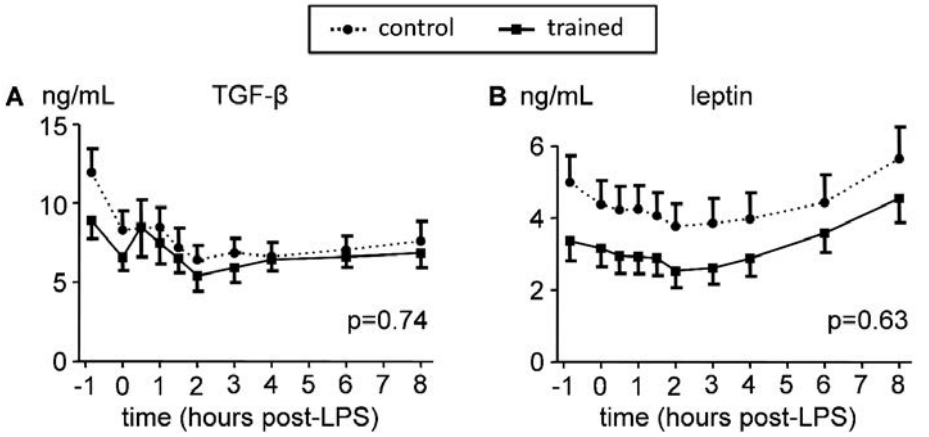

Figure S2. Plasma TGF-β (A) and leptin (B) concentrations during endotoxemia in control and trained subjects.

Data are expressed as mean±SEM of 12 subjects per group. P values between groups calculated using repeated measures two-way analysis of variance (ANOVA, interaction term).

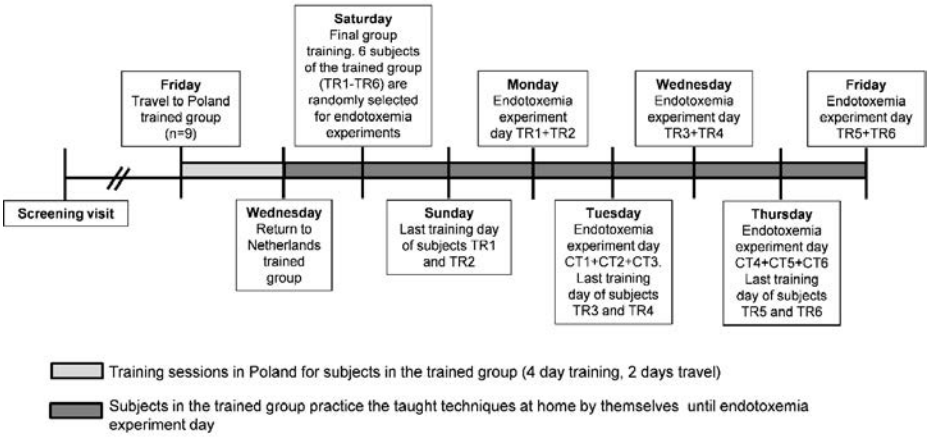

Figure S3. Study design.

This block was carried out twice in identical fashion, resulting in 12 subjects in both groups that underwent experimental endotoxemia. TR: trained subject. CT: control subject.

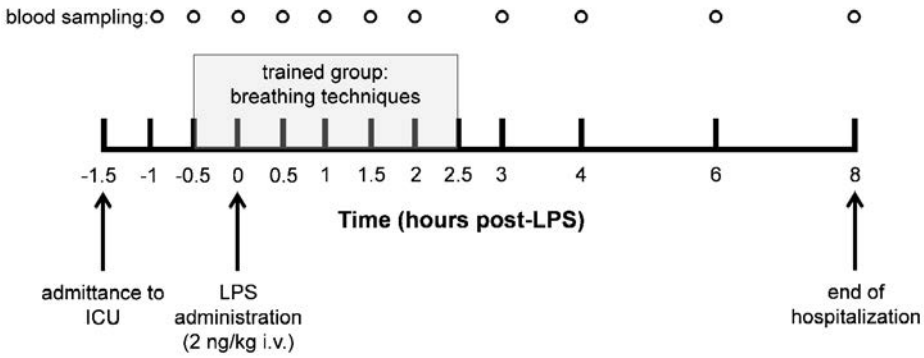

Figure S4. Experimental procedures on endotoxemia experiment day.

Chapter 3

The effects of cold exposure training and a breathing exercise on the inflammatory response in humans, a pilot study

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Abstract

Objective: A training intervention based on a combination of meditation, exposure to cold and breathing exercises has been shown to activate the sympathetic nervous system, reflected by profoundly increased plasma epinephrine levels, and subsequent attenuation of the lipopolysaccharide (LPS)-induced inflammatory response. Several elements of the intervention may contribute to these effects, namely two different breathing exercises (either with or without prolonged breath retention) and exposure to cold. We determined the contribution of these different elements to the observed effects.

Methods: Forty healthy male volunteers were randomized to either a short or an extensive training in both breathing exercises by either the creator of the training intervention or an independent trainer. The primary outcome was plasma epinephrine levels. 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. These 48 participants were subsequently intravenously challenged with 2 ng/kg LPS. The primary outcome was plasma cytokine levels.

Results: Both breathing exercises were associated with an increase in plasma epinephrine levels, which did not vary as a function of length of training or the trainer ($F(4,152)=0.53$, $p=0.71$ and $F(4,152)=0.92$, $p=0.46$, respectively). In the second study, the breathing exercise also resulted in increased plasma epinephrine levels. Cold exposure training alone did not relevantly modulate the LPS-induced inflammatory response ($F(8, 37)=0.60$, $p=0.77$), whereas the breathing exercise led to significantly enhanced anti-inflammatory and attenuated pro-inflammatory cytokine levels ($F(8, 37)=3.80$, $p=0.002$). Cold exposure training significantly enhanced the immunomodulatory effects of the breathing exercise ($F(8, 37)=2.57$, $p=0.02$).

Conclusion: The combination of cold exposure training and a breathing exercise potently attenuates the *in vivo* inflammatory response in healthy young males. Our study demonstrates that the immunomodulatory effects of the intervention can be reproduced in a standardized manner, thereby paving the way for clinical trials.

Introduction

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 their inflammatory response during experimental human endotoxemia, a standardized, controlled, and reproducible model of systemic inflammation elicited by intravenous administration of bacterial lipopolysaccharide (LPS) [1]. The training program was devised by a Dutch individual, who holds several world records with regard to withstanding extreme cold, in whom initial indications for the above described effects of the intervention were observed [2]. The training consists of three elements, namely meditation, exposure to cold and breathing exercises. Trained participants, who practiced the breathing exercises during experimental endotoxemia, exhibited high plasma concentrations of epinephrine, which were related to a rapid and profound increase of the anti-inflammatory cytokine Interleukin (IL)-10 and subsequent attenuation of the pro-inflammatory response (e.g. plasma levels of Tumor Necrosis Factor [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 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 may increase if potential users of the intervention would have to learn and practice less elements, but still attain the same efficacy. The meditation exercise is likely of limited relevance, as it was a very minor part of the training program and was not practiced during the endotoxemia 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, participants 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 epinephrine release [3-6], it is unknown which of these exercises is responsible for the observed effects. Furthermore, it is unclear whether it is necessary to be trained by the creator of the intervention (with regard to the so-called 'guru-effect', in which the mere presence of an authoritarian figure influences symptomatology [7, 8]) and whether or not a short instruction instead of an extensive training would be sufficient to increase plasma epinephrine 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 the creator of the intervention vs. an independent trainer, and a short instruction vs. extensive training) on plasma epinephrine levels, as these are implicated to be the main determinant of the anti-inflammatory effects of the intervention [1]. 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 endotoxemia. In this highly controlled and reproducible model, a systemic inflammatory response is elicited by intravenous administration of bacterial lipopolysaccharide (LPS) to healthy volunteers [9]. This model is used to investigate the inflammatory response and possible therapeutics in sepsis, but also offers possibilities to study mechanisms underlying cytokine-induced behavioural changes and to characterize potential targets of therapies against inflammation-associated depression [10]. Cold exposure may influence the inflammatory response through either a direct, epinephrine-independent effect, or by enhancing epinephrine levels elicited by the breathing exercise.

By identifying efficacy of the different training modalities and elements, this study aims to shed light on the underlying mechanisms responsible for the previously observed anti-inflammatory effects and will aid future clinical development of the training intervention.

Methods

Ethical approval and participants

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. Data were collected between December 2014 and June 2016. Participants were community volunteers, mostly students, who were recruited through paper leaflets, posters and online communities within the campus of the Radboud University in Nijmegen, The Netherlands. All participants 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 the creator of the intervention

or other breathing, meditation, or cold exposure exercises (including mindfulness, yoga, exposure to cold showers, and frequent visits to sauna facilities (more than once per month)). Additional exclusion criteria were 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 anesthesia, 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 clincialtrials.gov (NCT02417155). A schematic overview of the study is depicted in Figure 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 the creator of the intervention, extensive training by an independent trainer, short training by the creator of the intervention, and short training by an independent trainer. All participants 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 group who received the extensive training by the creator of the intervention, participants were trained every morning for two hours during four days, and after these initial four days of training, participants were instructed to practice the learned exercises at home, both analogous to our previous study [1]. In the group who received the short training by the creator of the intervention, participants were trained for only two hours on the morning of the fourth day (see Figure 1) and participants were instructed not to practice the learned exercises at home.

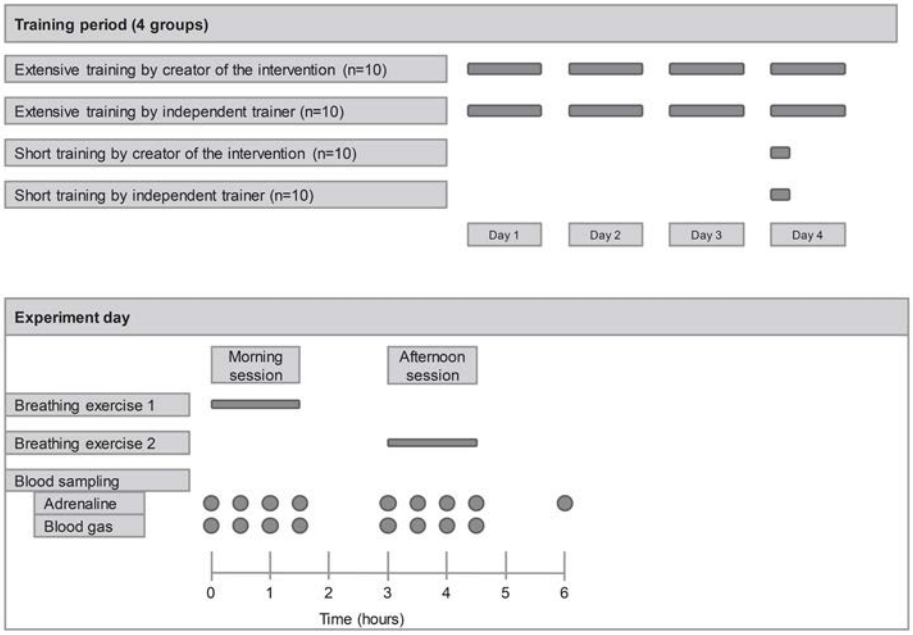

Figure 1. Schematic overview of the procedures of the breathing exercises study. Dots indicate blood sampling from the arterial catheter at the corresponding time points.

The training procedures in the other two groups were exactly the same, with the exception that the creator was substituted by an independent trainer from our research group (RvG), and that participants also received a detailed written instruction of both breathing exercises (see Appendix 1 "Written instruction of breathing exercises").

Breathing exercises

In the exercise with the prolonged retention of breath (henceforth designated as "with [+] retention"), participants hyperventilated for an average of 30 breaths using deep and powerful breaths. Subsequently, the participants exhaled and held their breath for approximately two minutes ("retention phase"). The duration of breath retention was entirely at the discretion of the participant. Breath retention was followed by a deep inhalation breath, that was held for 10s. Subsequently a new cycle of hyper/hypoventilation began. In the exercise without retention of breath (henceforth designated as "without [-] retention"), participants also hyperventilated for an average of 30 times using deep and powerful breaths. Subsequently, participants 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 Figure 1. To allow comparison with our previous study [1], participants refrained from caffeine and alcohol 24 hours before the experiment, and refrained from any intake of food and drinks 10 hours before the experiment. Fasting was maintained throughout the two breathing exercise sessions. 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 anesthesia (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. Adherence was assured by a member of the research team that was present in the room during the entire experiment. Serial blood samples were obtained throughout the experiment (see Figure 1).

Experimental human endotoxemia 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 clincialtrials.gov (NCT03240497). A schematic overview of the study is depicted in Figure 2.

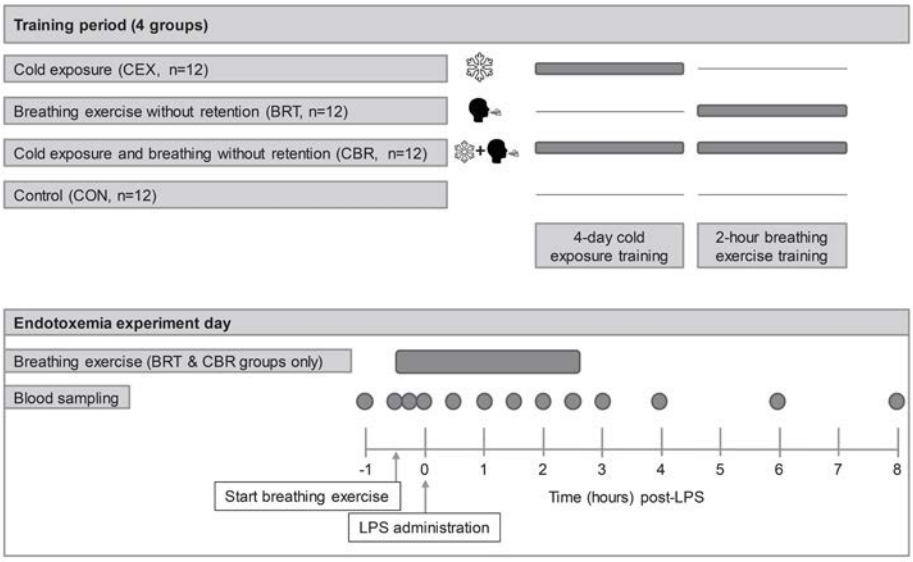

Figure 2. Schematic overview of the procedures of the human endotoxemia study. LPS: lipopolysaccharide. Dots indicate blood sampling from the arterial catheter at the corresponding time points.

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). Participants of all groups except the control group were trained in the week leading up to the endotoxemia experiment day.

Training procedures

An impression of the training procedures is provided (see Video, Supplemental Digital Content 1). All training procedures were provided by the same independent trainer (RvG). The creator of the intervention was not involved in the training course. The study team, including an MD, was present during all training procedures. Participants 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 (see Video, Supplemental Digital Content 1). Participants were instructed to end their daily shower with a period of 60 seconds of cold water. Participants 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. Participants were instructed not to practice the learned exercises at home. Participants randomized to the CBR group participated in both training procedures and participants in the control group did not receive any training.

Procedures on the endotoxemia experiment day

Endotoxemia experiments were conducted at the research unit of the intensive care department of the Radboud university medical center according to our standard protocol [11] also used in our previous study into this intervention [1], and an overview of the procedures is depicted in Figure 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 anesthesia (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. Participants received 1.5L of 2.5% glucose/0.45% saline solution for 1 hour (prehydration) before LPS administration, followed by 150 $mL.h^{-1}$ until the end of the experiment (8 hours after LPS administration). Participants of BRT and CBR groups practiced the learned breathing exercise from 30 minutes before administration of LPS to 2.5 hours afterwards, identical to our previous study [1]. Adherence was assured by a member of the research team that was present in the room during the entire experiment. 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 body weight over 1 minute at T = 0 hours at 09.30 AM. Blood samples were serially obtained throughout the experiment (see Figure 2).

Epinephrine and blood gas analysis

For circulating epinephrine 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 epinephrine concentrations were subsequently measured using HPLC with fluorometric detection [12]. 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 one assay with a simultaneous Luminex assay according to the manufacturer's instructions (Milliplex, Merck-Millipore, Billerica, USA) on a Magpix instrument (Luminex Corporation, Austin, USA). Intra-assay %CVs of the measured cytokines as provided by the manufacturer range from 1.5 to 2.6, whereas inter-assay %CVs ranged from 3.5 to 18.3. The detection range was 3.2-10,000 pg/mL for cytokines TNF-α, IL-10, IP-10, MCP1, MIP1a, MIP1b and 1.4-10,000 pg/mL for IL-6 and IL-8. Samples below the detection limit were imputed by 3.2 pg/mL and 1.4 respectively; no samples were above the upper detection limit.

Hemodynamic parameters, symptom score, and 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. LPS-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 and 95% confidence interval (CI), based on their distribution calculated by Shapiro-Wilk tests. For the sample size of the endotoxemia study, we wished to remain in line with our previous published endotoxemia study on this intervention [1], in which 12 participants per group were included. We calculated the achieved power using previous endotoxemia data of our group on the archetypal pro-inflammatory cytokine TNF-α. The mean of the TNF-α response (area under time-concentration curve [AUC]) was 970 arbitrary units with an standard deviation of 300 arbitrary units (31% of the mean). Using these values, a detectable contrast (effect size) of 40% (388 arbitrary units), a two-sided alpha of 0.05, and 12 participants per group in an unpaired t-test design, a power of 86% is achieved. There were no outliers that needed to be removed from any analysis. In the breathing exercises study, of the total of 280 sample moments, there were three missing values in the blood gas parameters due to technical issues. In the endotoxemia study, there was one

missing value in the blood gas parameters (out of a total of 312 sample moments) and 5 missing values in the epinephrine data (out of a total of 432 sample moments), all due to technical issues. Serial data were analyzed using linear mixed model analysis (p-values of `time x column factors` are depicted in the figures, whereas the results of post-hoc Sidak`s multiple comparison tests [only performed in case time x column p-value was <0.05] to evaluate differences at individual timepoints are provided in the supplemental tables). Area under the concentration-time curves (AUCs) were calculated on a per-participant basis using the "Area under Curve" function in Graphpad Prism 8.0 (Graphpad Software, San Diego, CA, USA) to provide an integral measure of the cytokine responses. Multivariate multiple linear regression, entering AUC cytokine responses as dependent variables and the different groups as independent variables, was performed to assess the effects of cold exposure training and the breathing exercises on plasma levels of all measured cytokines. Demographic characteristics were analyzed using Kruskal-Wallis tests. A p-value < 0.05 was considered statistically significant. Calculations and statistical analysis were performed using Graphpad Prism version 8.3.0 and SPSS v25.0.0.1 (IBM Corp, Armonk, New York, USA).

Table 1: Demographic characteristics.

Breathing exercises study	**All participants (n=40)**	**Short training by independent trainer (n=10)**	**Extensive training by independent trainer (n=10)**	**Short training by creator (n=10)**	**Extensive training by creator (n=10)**	**KW statistic**	**p-value**
Age, years	21 [19-24]	20 [19-22]	22 [19-26]	21 [20-23]	23 [19-26]	2.61	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]	1.88	p=0·60
Systolic blood pressure, mmHg	140 [128-145]	135 [123-148]	143 [128-147]	146 [137-150]	135 [131-140]	4.02	p=0·26
Diastolic blood pressure, mmHg	71 [64-79]	71 [61-77]	77 [70-85]	69 [62-81]	69 [62-81]	4.46	p=0·22
Heart rate, bpm	77 [60-88]	77 [69-84]	86 [59-103]	77 [53-89]	63 [54-82]	3.96	p=0·27
Endotoxemia study	**All participants (n=48)**	**Control (CON)(n=12)**	**Cold exposure (CEX)(n=12)**	**Breathing exercise (BRT)(n=12)**	**Cold exposure and breathing exercise (CBR)(n=12)**	**KW**	**p-value**
Age, years	22 [20-24]	22 [20-22]	23 [20-26]	22 [20-24]	23 [20-25]	0.74	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]	3.17	p=0·37
Systolic blood pressure, mmHg	140 [136-152]	137 [122-156]	142 [137-155]	140 [136-152]	144 [136-152]	0.69	p=0·88
Diastolic blood pressure, mmHg	72 [64-80]	73 [66-82]	72 [64-81]	70 [62-75]	77 [68-82]	3.28	p=0·35
Heart rate, bpm	64 [56-71]	66 [59-75]	65 [56-73]	62 [56-66]	62 [51-67]	3.40	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. Kruskal-Wallis tests were used to calculate the Kruskal-Wallis (KW) statistic and p-values across the different groups.

Results

Breathing exercises study

Demographic characteristics

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

Plasma epinephrine levels and blood gas parameters

Changes in blood gas parameters were identical during both the morning and afternoon sessions accross all groups (saturation: $F(6,462)=0.74$, $p=0.62$; $pO2$: $F(6,463)=0.98$, $p=0.44$; pH: $F(6,463)=0.56$, $p=0.76$ and $pCO2$: $F(6,463)=1.26$, $p=0.28$; Supplemental Figure 1A-D and Supplemental Table 5). During the morning session, plasma epinephrine levels sharply increased upon initiation of the breathing exercises accross 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, $p<0.0001$, Supplemental Figure 1E and Supplemental Table 5). We previously hypothesized that this initial increase in epinephrine levels is the main driving factor inducing the anti-inflammatory phenotype (see Supplemental Figure 5 in [1]). Epinephrine levels remained elevated for as long as the participants 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, Supplemental Figure 1E and Supplemental Table 5). In contrast, during the afternoon session plasma epinephrine 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], Supplemental Figure 1E and Supplemental Table 5). Statistical comparison of plasma epinephrine levels over time between the morning and afternoon session yielded a highly significant difference ($F(4,312)=6.42$, $p<0.001$, Supplemental Figure 1E and Supplemental Table 5). Because of these findings, 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%±0.2% at T=0 to 67%±5%, 58±3% and 73±4%, at T=0.5, 1, and 1.5 hours, respectively, $F(6,224)=31.50$, $p<0.001$, Figure 3A and Supplementary Table 1).

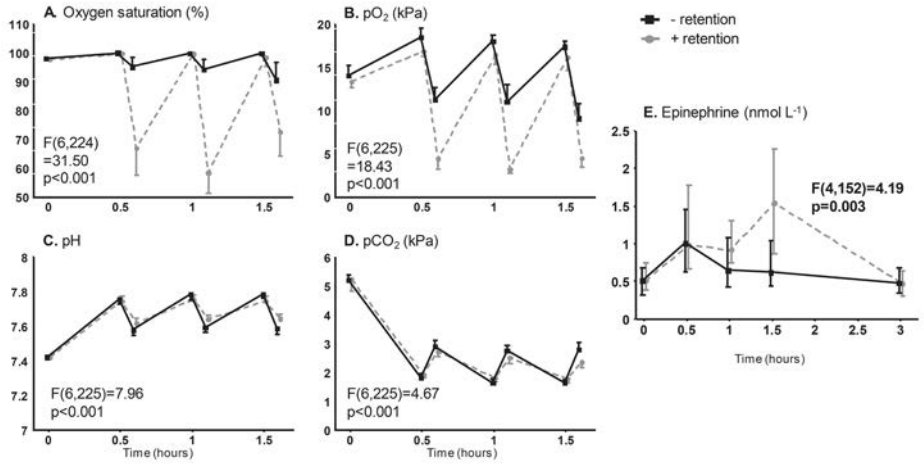

Figure 3: Arterial blood gas parameters and plasma epinephrine levels during the breathing exercises study: influence of breathing exercise. A. Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). - retention: data obtained during the first 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 ± 95% confidence interval (panels A-D) or median and interquartile range (panel E) of 20 participants per group and p-values depicted in the graphs represent the between-group comparison calculated using linear mixed models analysis (time*column factor). Epinephrine data were log-transformed before analysis. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 1.

Accordingly, sharp decreases in pO2 were observed in this group (F(6,225)=18.43, p<0.001, Figure 3B and Supplementary Table 1). 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 (pH: F(6,225)=7.96, p<0.001; pCO_2: F(6,225)=4.67, p<0.001, Figure 3C-D and Supplementary Table 1). The initial increase in plasma epinephrine 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 participants 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, p>0.99, Figure 3E and Supplementary Table 1). However, the increase in plasma epinephrine concentrations was slightly more sustained in the participants practicing the breathing exercise with retention, resulting in significantly higher levels at T=1.5 compared to participants practicing the exercise without retention (F(4,152)=4.19, p=0.003, Figure 3E and Supplementary Table 1).

Blood gas parameters and plasma epinephrine levels were not statisically different between the participants trained by an independent trainer compared to participants trained by the creator of the intervention (saturation: $F(6,224)=0.55$, $p=0.76$; pO_2: $F(6,225)=0.26$, $p=0.90$; pH: $F(6,225)=0.59$, $p=0.86$; pCO_2: $F(6,225)=1.57$, $p=0.24$, Supplemental Figure 2). Additionally, no significant differences in these parameters were found between the participants that received the short training versus the long training (saturation: $F(6,224)=0.28$, $p=0.95$; pO_2: $F(6,225)=0.59$, $p=0.74$; pH: $F(6,225)=1.30$, $p=0.26$; pCO_2: $F(6,210)=0.83$, $p=0.55$, Supplemental Figure 3).

Based on these results, we conclude that the magnitude of the initial increase in epinephrine 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 the creator of the intervention, nor a long training program are required to attain the pronounced epinephrine response. Hence, we utilized the training modality consisting of a short training by an independent trainer in only the breathing exercise without breath retention for the subsequent experimental human endotoxemia study.

Experimental human endotoxemia study

Demographic characteristics

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

Blood gas parameters and plasma epinephrine levels

pCO_2 levels slighly decreased over time in the groups that did not practice the breathing exercise (CEX and CON groups, Figure 4A-C and Supplementary Table 2), which may indicate a small increase in breathing frequency following LPS administration.

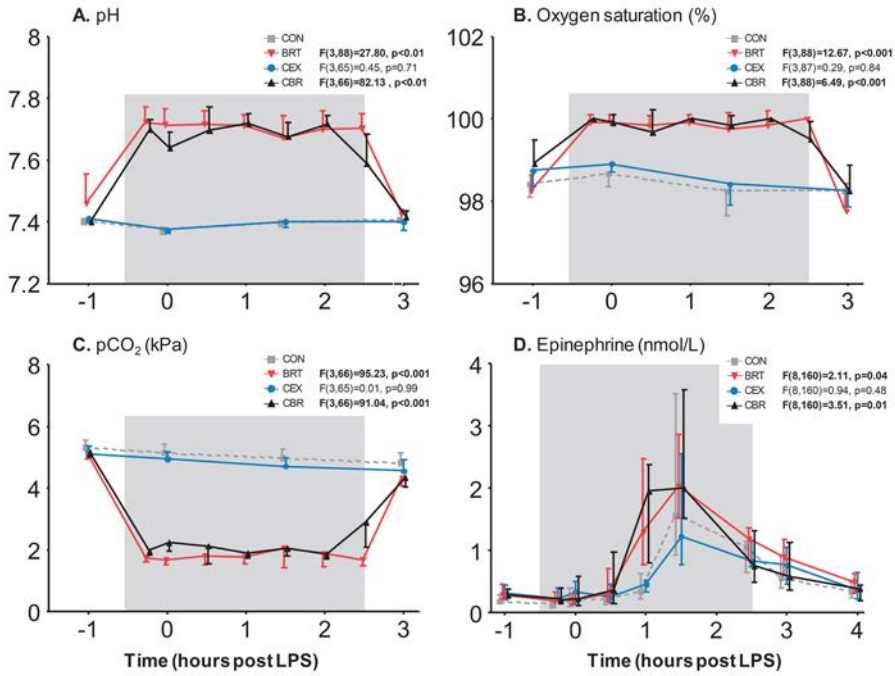

Figure 4: Arterial blood gas parameters and plasma epinephrine levels during human endotoxemia. A. pH. **B.** Oxygen saturation. **C.** Carbon dioxide partial pressure (pCO_2). **D.** Plasma epinephrine concentrations. The grey box indicates the period during which the trained participants practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean ± 95% confidence interval (panels A-C) or median and interquartile range of 12 participants per groups. P-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using linear mixed models analysis on log-transformed data (time*column factor). Epinephrine data were log-transformed before analysis. Significant p-values are shown in bold. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 2. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

However, no significant changes in this, or any of the other arterial blood gas parameters were observed over time in these groups, and all values remained within the reference ranges (CEX and CON groups, Figure 4A-C and Supplementary Table 2). 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, resulting in significant higher pH during the execution of the breathing exercises in both groups (BRT: $F(3,88)=27.80$, $p<0.01$; CBR: $F(3,66)=82.13$, $p<0.01$, Figure 4A and Supplementary Table 2). 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 (BRT: $F(3,88)=12.67$, $p<0.001$; CBR: $F(3,88)=6.49$), $p<0.001$, Figure 4B and Supplementary Table 2). pCO_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 (BRT: $F(3,66)=95.23$, $p<0.001$; CBR: $F(3,66)=91.04$, $p<0.001$, Figure 4C and Supplementary Table 2).

Baseline plasma epinephrine levels were comparable between all four groups (all p-values >0.05, Figure 4D and Supplementary Table 2). Concentrations increased during human endotoxemia in all groups, with peak values observed 1.5 hours after administration of LPS (Figure 4D and Supplementary Table 2). There were no differences in plasma epinephrine levels over time between the CEX and CON groups ($F(8,160)=0.94$, $p=0.48$). However, in both groups of participants who practiced the breathing exercises, the increase in plasma epinephrine commenced much earlier and was significantly more pronounced than in the CEX and CON groups that did not exercise the breathing exercise (BRT $F(8,160)=2.11$, $p=0.04$; CBR: $F(8,160)=3.51$ $p=0.01$, Figure 4D and Supplementary Table 2).

Hemodynamic parameters, temperature, and symptoms

Experimental endotoxemia resulted in a gradual increase in heart rate in the CEX and CON groups, with no differences between these two groups ($F(18,396)=0.61$, $p=0.89$, Figure 5A and Supplemental Table 3).

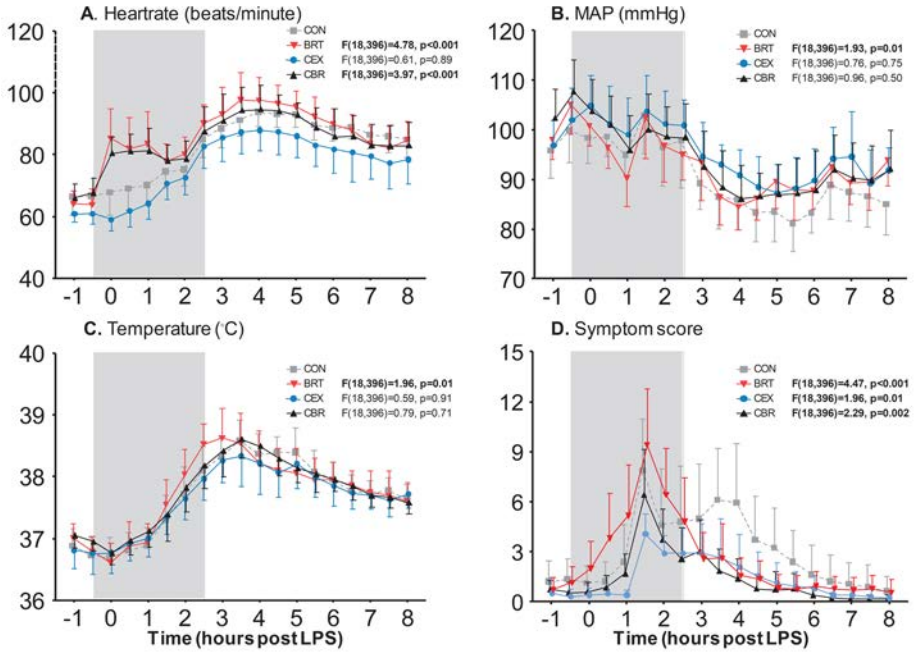

Figure 5: Cardiorespiratory parameters, tympanic temperature, and symptoms during human endotoxemia. A. Heart rate. **B.** Mean arterial pressure (MAP). **C.** Tympanic temperature. **D.** Score of self-reported symptoms. The grey box indicates the period during which the trained participants practiced the breathing exercise (BRT and CBR groups only). Data are expressed as mean ± 95% confidence interval of 12 participants per group. P-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using linear mixed models analysis (time*column factor). Significant p-values are shown in bold. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 3. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

In the two groups that performed the breathing exercise (BRT and CBR groups), a sharp increase in heart rate was observed immediately following the start of the first hyperventilation cycle, and this effect ensued during most of the period that the participants practiced the exercise, resulting in a significant higher heartrate during the experiment compared to the control group (BRT: $F(18,396)=4.78$, $p<0.001$; CBR: $F(18,396)=3.97$, $p<0.001$, Figure 5A and Supplemental Table 3). After cessation of the breathing exercises, the heart rate data of the BRT and CBR groups were similar to that of the CEX and CON groups. Expectedly, mean arterial pressure (MAP) gradually decreased in all groups (Figure 5B and Supplemental Table 3), 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 (F(18,396)=1.93, p=0.01, Figure 5B), post-hoc analysis did not reveal significance at any of the individual timepoints (Supplemental Table 3). An LPS-induced mean increase in tympanic temperature of 1.8±0.1 °C was observed accross all groups (Figure 5C and Supplemental Table 3). 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 sigificantly earlier in the BRT group (F(18,396)=1.96, p=0.01, Figure 5C and Supplemental Table 3). Administration of LPS resulted in flu-like symptoms in all groups (Figure 5D and Supplemental Table 3). Peak symptom scores were comparable between the CON (9.3±1.3), 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=0.017). Symptoms resolved significantly more rapidly in all three intervention groups compared with the CON group (BRT: F(18,396)=4.47, p<0.001; CEX: F(18,396)=1.96, p=0.01; CBR: F(18,396)=2.29, p=0.002, Figure 5D and Supplemental Table 3).

Plasma cytokines

Due to the absence of an inflammatory response before LPS administration and waning of this response multiple hours after the LPS challenge, 349 out of a total of 4224 cytokine measurements (8%) fell below the lower limit of detection. As expected, plasma concentrations of the anti-inflammatory cytokine IL-10 and the proinflammatory cytokines TNF-, IL-6, IL-8, IP-10, MCP-1, MIP-1α, and MIP-1β increased following LPS administration in all groups (Figure 6 and Supplemental Table 4, Supplemental Figure 4 and Supplemental Table 6).

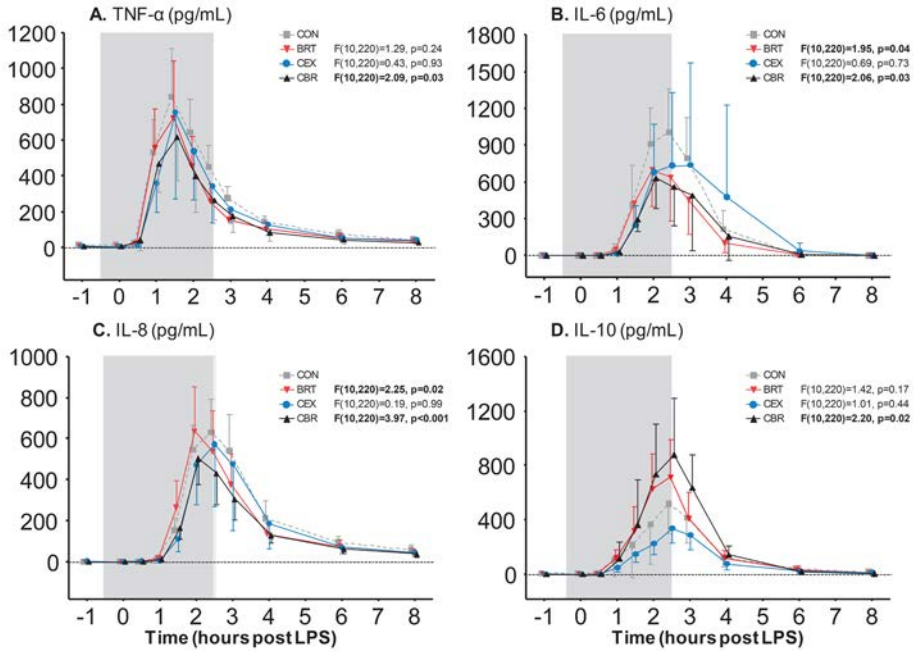

Figure 6: Plasma concentrations of inflammatory cytokines during human endotoxemia. A. Tumor necrosis factor (TNF)-α. **B.** Interleukin (IL)-6. **C.** IL-8. **D**. IL-10. The grey box indicates the period during which the trained participants practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean ± 95% confidence interval of 12 participants per group. P-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using linear mixed models analysis (time*column factor). Significant p-values are shown in bold. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 4. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

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%, F(10,220)=2.20, p=0.02 vs CON, Figure 6D and Supplemental Table 4). 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%, F(10,220)=2.09, p=0.03; IL-6 -35%, F(10,220)=2.06, p=0.03; IL-8: -30%, F(10,220)=3.97, p<0.001; IP-10: -48%, F(10,220)=7.99, p<0.001; MCP-1:-29%, F(10,220)=4.64, p<0.001; MIP-1α: -35%, F(10,220)=6.25, p<0.001; MIP-1β: -30%, F(10,220), p<0.001, Figure 6B-D and Supplemental Table 4; Supplemental Figure 4 and Supplemental Table 6). When comparing the BRT group with the control group, similar, but less pronounced effects on plasma cytokines were observed, reaching statistical significance for pro-inflammatory cytokines IL-6 (-34%, F(10,220)=1.95, p=0.04), IL-8 (-14%, F(10,220)=2.25, p=0.02), IP-10 (-48%, F(10,220)=9.42, p<0.001),

MCP-1 (-37%, $F(10,220)=6.07$, $p<0.001$), MIP-1α (-37%, $F(10,220)=6.59$, $p<0.001$, and MIP-1β (-28%, $F(10,220)=2.24$, $p=0.02$), but not for IL-10 (+17%, $F(10,220)=1.42$, $p=0.17$ vs. CON). In the CEX group, only levels of MCP-1 were significantly lower than in the CON group (-25%, $F(10,220)=4.75$, $p<0.001$).

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 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 participants performed the breathing exercise with prolonged breath retention compared to that without, plasma epinephrine 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 endotoxemia.

The magnitude of the initial increase in plasma epinephrine 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 epinephrine 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 [6, 13]. Nevertheless, as catecholamine release from the adrenal chromaffin cells is dependent on a combination of neural, hormonal, redox, as well as immune signaling pathways [14, 15], the exact mechanism behind the epinephrine 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 epinephrine 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, participants 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 [16, 17]. This is possibly part of a stress-induced analgetic response to cold [18] 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.

In the endotoxemia study, the increase in plasma epinephrine concentrations observed after initiation of the breathing exercises described in the present work was similar in magnitude to that in our previous endotoxemia study [1]. Nevertheless, epinephrine levels prior to the start of the breathing exercises were higher in the past work [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 participants were also trained in both exercises [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 baseline plasma epinephrine concentrations could play a role [1], which may in turn have triggered a more pronounced early IL-10 release and subsequent stronger attenuation of the pro-inflammatory response. Second, our data from the breathing exercises study show

that, although the initial increase in epinephrine levels was similar in response to the exercise with and without breath retention, it was more prolonged in the former. Despite that fact that we previously showed that the initial epinephrine increase is a main determinant of the anti-inflammatory phenotype(1), we cannot exclude the possibility that a more prolonged increase has a more pronounced effect. Third, the hypoxia induced by breath retention in our previous study may have directly (i.e. independently from epinephrine) 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 [19]. In this light, future studies into the training intervention should still consider including the exercise with prolonged breath retention. This exercise may nevertheless be less preferable from a safety perspective, as the profound cyclic decreases in oxygen saturation may present risks for patients with for instance cardiovascular conditions.

A striking finding from the breathing exercises study was that the profound increase in plasma epinephrine 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 epinephrine levels during the afternoon session may be due to adaptation of the stress response, resulting in lower epinephrine release by the adrenal gland in response to repeated application of the same stressor, a phenomenon which has been described in animals [20]. 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 intravesiculair stores in the cytoplasm of these cells [14, 15]. Although animal experiments have shown that fully depleted catecholamine stores can be replenished within 2 hours [21], 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 exercise daily in the week leading up to the experiment in which the plasma epinephrine concentrations were measured.

Several limitations of our work need to be adressed. First, we studied groups of healthy young male adults, not (older) patients with possible comorbidities, who represent the intended target group for this intervention. We only included male participants because there are considerable differences in the cytokine response to LPS between the sexes [22]. This could be due to menstrual cycle-

related hormonal variations that affect immunity. As human endotoxemia studies are very labor-intensive and expensive, and for ethical reasons (to expose as few volunteers as possible to endotoxemia), nearly all of our LPS studies are restricted to males to increase homogeneity and reduce sample size. Of note, there are no data available regarding the influence of sex differences on training-induced modulation of the immune response. Furthermore, the creator of the intervention has trained both men and women, with no apparent differences in competence regarding completion of the training exercises (observational data). Although upcoming studies into this intervention should also include females, this study provides essential information in terms of designing the most safe and optimal training protocol for use in these future investigations. 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 [23], on which the studied intervention has a substantial suppressive effect. Furthermore, *in vivo* human efficacy of many biologics used in the treatment of auto-inflammatory disorders, such as anakinra and infliximab, was first established in the experimental human endotoxemia model, [24, 25] illustrating that it has value for these diseases. Third, in the breathing exercises study, we did not include a control group that was not trained. We chose not to, because the breathing exercises study was designed to primarily investigate what specific exercise caused the increase in epinephrine during the combination of breathing exercises performed by the participants in our original study [1], and whether training by the creator of the intervention is required. Therefore, we compared different breathing exercises and training modalities head-to-head. Furthermore, the participants acted as their own controls by measuring epinephrine levels before start of the breathing exercise on the experiment day. Fourth, although psychological, social, or behavioral factors may also be influenced by this intervention, the focus of the present study was on physical outcomes. As the intervention is ultimately aimed at alleviating symptoms among individuals with chronic disease, future studies should also evaluate whether the beneficial physiological effects of the intervention are offset, for example, by anxiety. Fifth, the reliability of the cytokine assay could not be confirmed because samples were not run in duplicate. Finally, the control group in the current study did not undergo any form of training. It must be acknowledged that a training program or other intervention guaranteed not to influence the sympathetic nervous system and immune response, but that does result in matching expectations compared to the

intervention groups would represent a more optimal control condition. Possibilities may entail mindfulness training, self-affirmation, educational materials, and placebo injection of a purported beneficial substance. Furthermore, in future studies a dismanteling design could be considered in which the component conditions are compared to the full protocol. Another point related to the control conditions is that we did not record the frequency of spontaneous (deep) breaths in the endotoxemia study. As endotoxemia has been shown to increase deep breath frequency [26], this may have introduced bias in the control and cold exposure training groups. However, our pCO_2 data indicate that the LPS-induced increase in (deep) breathing frequency was limited at most. Furthermore, if anything, it would have led to an underestimation of the effect of the learned breathing exercises and therefore does not compromise the validity of our findings.

In conclusion, 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 endotoxemia is possible after following a training program consisting of cold exposure and a breathing exercise. Furthermore, these interventions can be provided by an independent trainer and acquired within a short time-frame. Although these results provide an important next step in the clinical development of this intervention, they will need to be replicated and generalized before this intervention can be considered appropriate for application in clinical populations with chronic disease.

Acknowledgements

We thank the independent trainer Rogier van Groenendael for his contribution to this study and Remi Beunders for editing of the video illustrating the training procedures.

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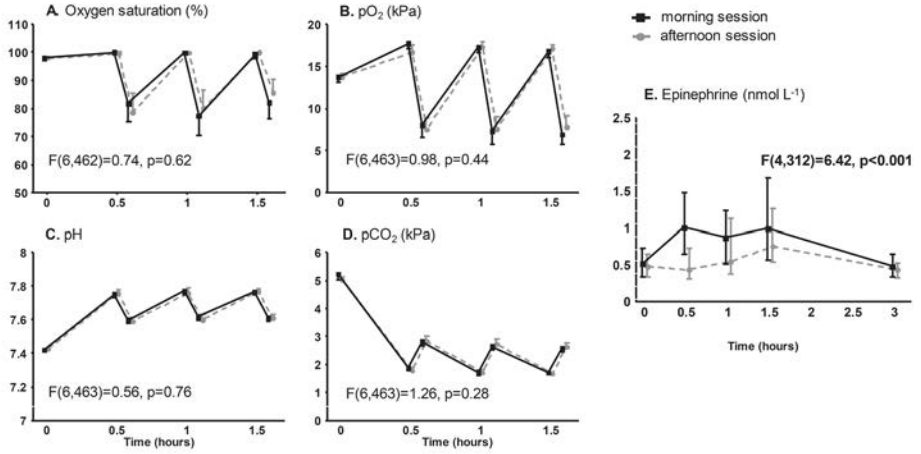
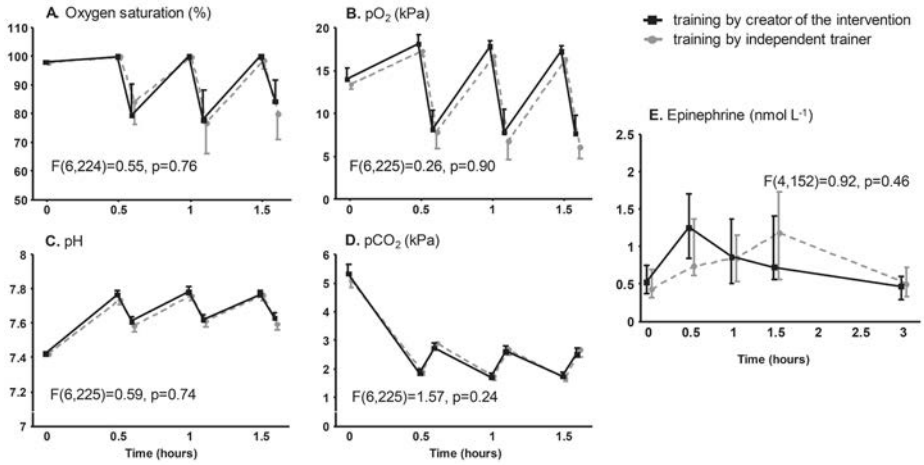
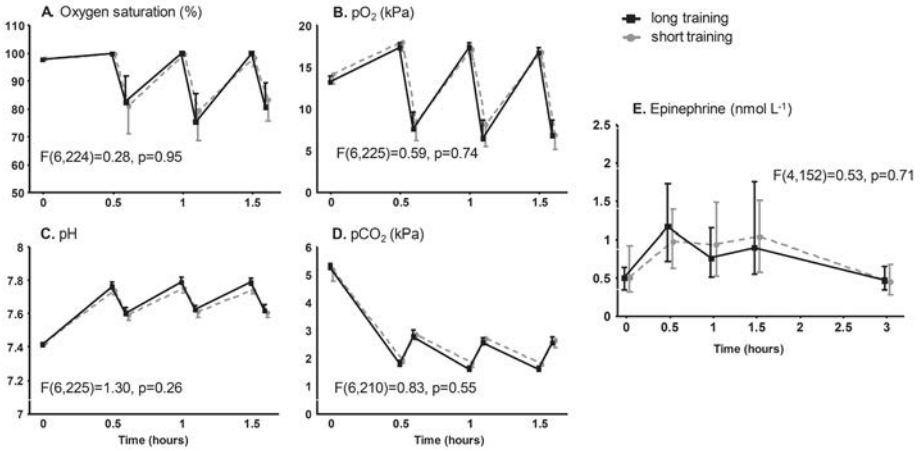
Supplemental Figures and legends



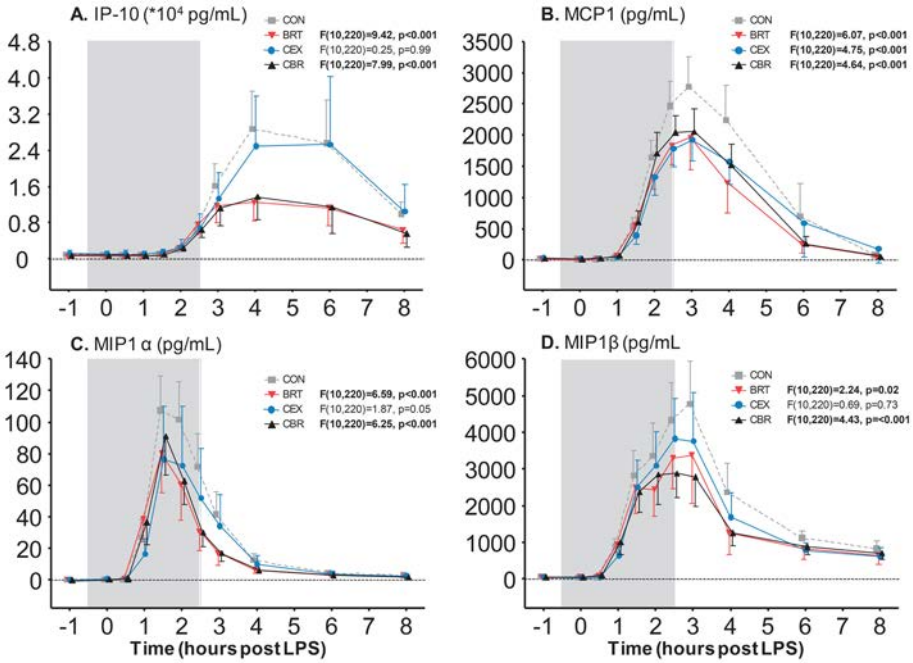
Supplemental Figure 1: Arterial blood gas parameters and plasma epinephrine levels during the breathing exercises study: morning vs. afternoon session. A. Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma epinephrine concentrations. 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). Data are presented as mean ± 95% confidence interval (panels A-D) or median and interquartile range (panel E) of 20 participants per group and p-values depicted in the graphs represent the between-group comparison calculated using linear mixed models analysis (time*column factor). Epinephrine data were log-transformed before analysis. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 5**.**



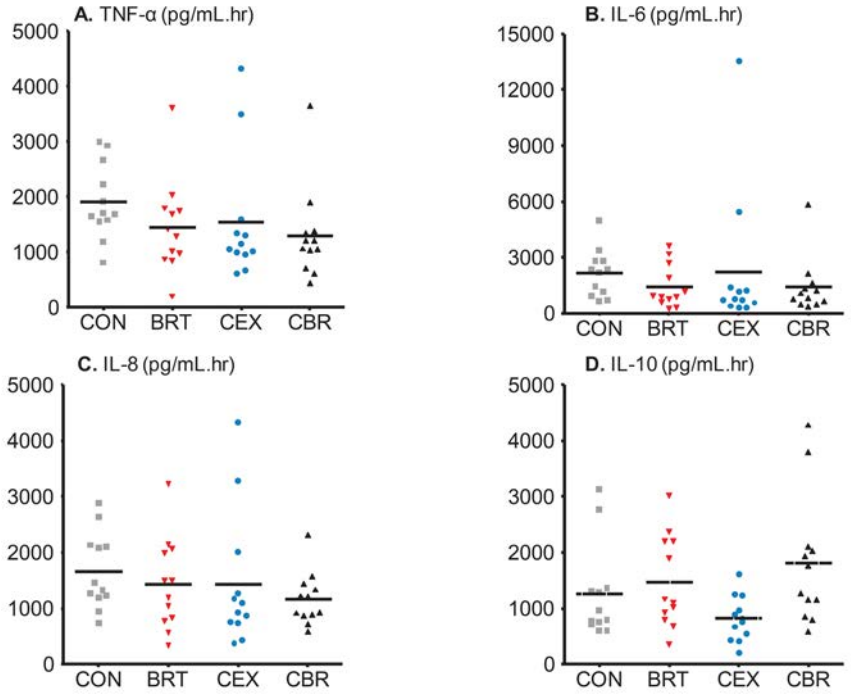
Supplemental Figure 2: arterial blood gas parameters and plasma epinephrine levels during the breathing exercise study: influence of trainer. A. Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma epinephrine concentrations. Training by the creator of the intervention: data obtained during the first breathing exercise on the experiment day (breathing exercise 1, Figure 1) from participants were trained by the creator of the intervention. 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. Data are presented as mean ± 95% confidence interval (panels A-D) or median and interquartile range (panel E) of 20 participants per group and p-values depicted in the graphs represent the between-group comparison calculated using linear mixed models analysis (time*column factor). Epinephrine data were log-transformed before analysis.



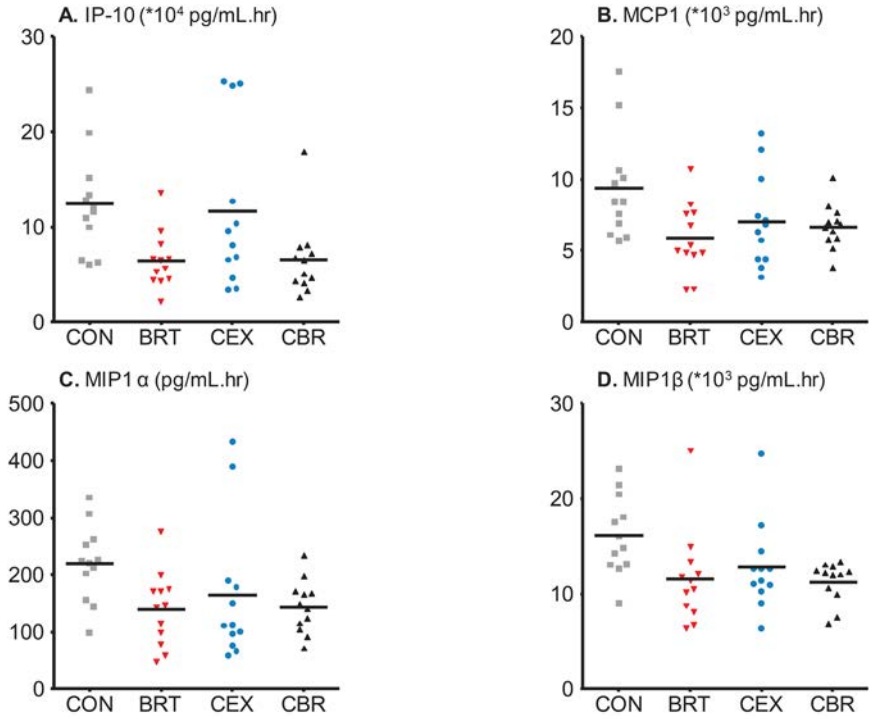
Supplemental Figure 3: Arterial blood gas parameters and plasma epinephrine levels during the breathing exercise study: influence of length of training. A. Oxygen saturation. **B.** Oxygen partial pressure (pO_2). **C.** pH. **D.** Carbon dioxide partial pressure (pCO_2). **E.** Plasma epinephrine concentrations. 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. Data are presented as mean ± 95% confidence interval (panels A-D) or median and interquartile range (panel E) of 20 participants per group and p-values depicted in the graphs represent the between-group comparison calculated using linear mixed models analysis (time*column factor). Epinephrine data were log-transformed before analysis.



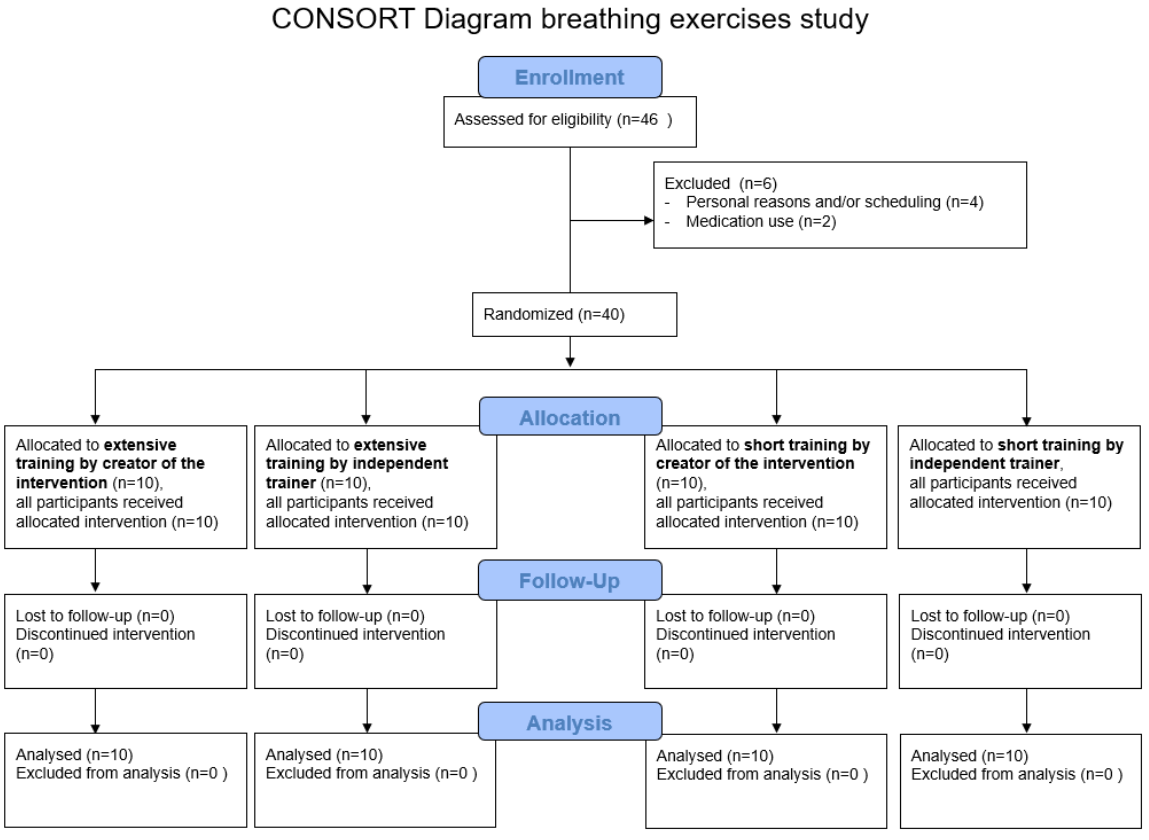
Supplemental Figure 4: Plasma concentrations of inflammatory cytokines during human endotoxemia. A. Interferon Gamma-Induced Protein 10 (IP-10). **B.** Monocyte chemoattractant protein 1 (MCP-1). **C.** Macrophage Inflammatory Protein 1α (MIP-1α). **D**. Macrophage Inflammatory Protein 1β (MIP-1β). The grey box indicates the period during which the trained participants practiced the breathing exercise (BRT and CBR groups only). Data are presented as mean ± 95% CI of 12 participants per group. P-values depicted next to the legend represent the comparison of that group with the control group over time, calculated using linear mixed models analysis (time*column factor). 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. For comparisons that yielded time*column factor p-values <0.05, results of post-hoc analyses performed using Sidak`s multiple comparison test are reported in Supplemental Table 6.



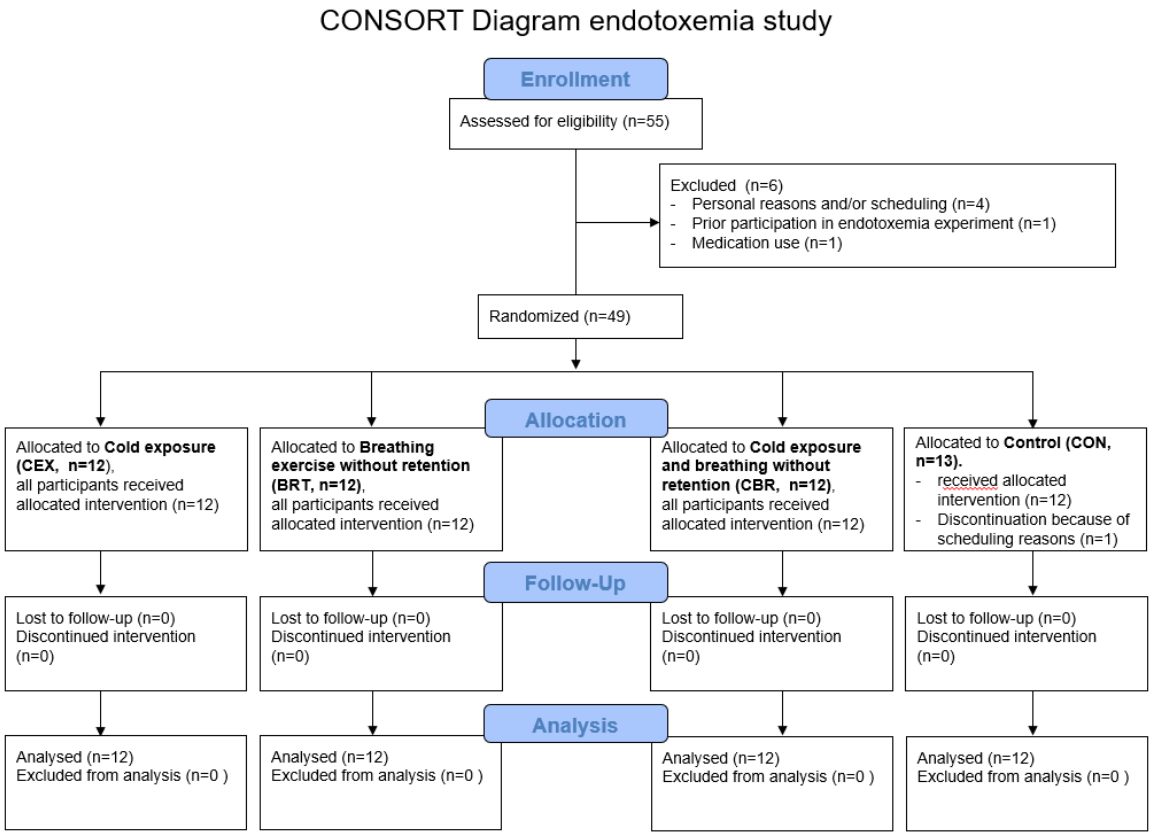
Supplemental Figure 5: Area under the curve (AUC) of cytokine responses during human endotoxemia. A. Tumor necrosis factor (TNF)-α. **B.** Interleukin (IL)-6. **C.** IL-8. **D**. IL-10. Data are presented as scatterplot with bars representing the mean value of 12 participants per group. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.



Supplemental Figure 6: Area under the curve (AUC) of cytokine responses during human endotoxemia. A. Interferon Gamma-Induced Protein 10 (IP-10). **B.** Monocyte chemoattractant protein 1 (MCP-1). **C.** Macrophage Inflammatory Protein 1α (MIP-1α). **D**. Macrophage Inflammatory Protein 1β (MIP-1β). Data are presented as scatterplot with bars representing the mean value of 12 participants per group. CON: control group. BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.



Supplemental Figure 7: CONSORT diagram of the breathing exercises study.



Supplemental Figure 8: CONSORT diagram of the human endotoxemia study.

Appendix 1: written instruction of breathing exercises

1. Exercise with prolonged retention of breath

Preparation:

1. Sit down comfortably in a place where you are not disturbed.
2. It is permitted to drink water, coffee, or tea, but do not add sugar or milk.

Exercise:

1. Breathe in and out approximately 30 times. Do not force anything. Breathe preferably in through the nose and out through the mouth. Use your abdomen, diaphragm and chest. Try to find a rhythm, bring your attention to breathing in-and-out, in-and-out, as if you are inflating a balloon. During this phase you can start to feel tingling in the neck, finger, legs, etc. You have to end with a deep exhalation (empty lungs) and holding of your breath.
2. Breathe deeply in and out once and hold your breath after exhalation. Relax, do not force anything. Hold your breath until you feel the urge to breathe again, do not force anything.
3. Finally, take a deep breath and hold it for 10 seconds. Do not force anything. Now your lungs are filled with oxygen. Close your eyes and then breathe out.

This is the end of the first cycle; now start immediately with a new cycle (point 1). Do not force anything. Perform a total of 3 or 4 cycles, whatever you feel comfortable with.

2. Breathing exercise without retention of breath

Preparation:

1. Sit down comfortably in a place where you are not disturbed.
2. It is permitted to drink water, coffee, or tea, but do not add sugar or milk.

Exercise:

1. Breathe in and out approximately 30 times. Do not force anything. Breathe preferably in through the nose and out through the mouth. Use your abdomen, diaphragm and chest. Try to find a rhythm, bring your attention to breathing in-and-out, in-and-out, as if you are inflating a balloon. During this phase you can start to feel tingling in the neck, finger, legs, etc.
2. Exhale and inhale vigorously once, and firmly tighten all your muscles for approximately 10 seconds.

This is the end of the first cycle; now start immediately with a new cycle (point 1). Perform a total of 3 or 4 cycles, whatever you feel comfortable with.

Supplemental table 1. Accompanying statistics to Figure 3: Arterial blood gas parameters and plasma epinephrine levels during the breathing exercises study: influence of breathing exercise.

	Panel A. Oxygen saturation	**Panel B. pO_2**	**Panel C. pH**	**Panel D. CO_2**	**Panel E. Epinephrine**
Time x Column factor	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**0.003**
F(DFn,DFd)	F(6,224) =31.50	F(6,225) =18.43	F(6,225) =7.96	F(6,225) =4.67	F(4,152) =4.19
Time					
0	>0.9999	0.9909	>0.9999	>0.9999	0.9805
0.5 EH	>0.9999	0.235	0.9979	0.9552	>0.9999
0.5 ER	**<0.0001**	**<0.0001**	0.228	0.774	
1.0 EH	>0.9999	0.2545	0.4551	0.7556	0.652
1.0 ER	**<0.0001**	**<0.0001**	0.0584	0.3722	
1.5 EH	0.9983	0.5801	0.1328	0.9225	**0.0006**
1.5 ER	**<0.0001**	**<0.0001**	**0.0113**	**0.0057**	
3.0					0.9983

EH - end of hyperventilation. ER - end of retention of breath.
p-values of Time x Column factor were calculated using linear mixed models analysis comparing the with retention group (+retention) vs. the without retention group (-retention).
p-values of individual timepoints represent adjusted p-values comparing the with retention group (+retention) vs. the without retention group (-retention) calculated using Sidak`s post-hoc tests.

Supplemental table 2. Accompanying statistics to Figure 4: Arterial blood gas parameters and plasma epinephrine levels during human endotoxemia.

	Panel A. pH		Panel B. Oxygen saturation		Panel C. pCO_2		Panel D. Epinephrine	
Group	**BRT**	**CBR**	**BRT**	**CBR**	**BRT**	**CBR**	**BRT**	**CBR**
Time x Column factor	**<0.0001**	**<0.0001**	**<0.0001**	**0.0005**	**<0.0001**	**<0.0001**	**0.0378**	**0.0009**
F(DFn,DFd)	F(3,88) =27.80	F(3,66) =82.13	F(3,88) =12.67	F(3,88) =6.49	F(3,66) =95.23	F(3,66) =91.04	F(8,160) =2.11	F(8,160) =3.51
Time								
-1	0.6763	0.98	0.9281	0.3802	0.8801	0.6244	0.7062	0.6356
-0.25							0.5793	0.3439
0	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	<0.0001	>0.9999	0.9998
0.5							0.7874	0.6968
1							**0.0346**	**0.0006**
1.5	**<0.0001**	**<0.0001**	**0.0009**	**0.0004**	**<0.0001**	<0.0001	0.8164	0.849
2.5							0.9706	0.9983
3	0.1456	0.8799	0.5357	>0.9999	0.0933	0.1306	0.3644	0.9958
4							0.8192	>0.9999

p-values of Time x Column factor were calculated using linear mixed models analysis comparing the indicated group (e.g. BRT or CBR) vs. the control group (CON).
p-values of individual timepoints represent adjusted p-values calculated using Sidak`s post-hoc tests.
BRT: breathing exercise group. CBR: cold exposure and breathing exercise group.

Supplemental table 3. Accompanying statistics to Figure 5: Cardiorespiratory parameters, tympanic temperature, and symptoms during human endotoxemia.

	Panel A. Heartrate		Panel B. MAP	Panel C. Temp	Panel D. Symptom score		
Group	**BRT**	**CBR**	**BRT**	**BRT**	**BRT**	**CEX**	**CBR**
Time.CF	**<0.0001**	**<0.0001**	**0.0126**	**0.0112**	**<0.0001**	**0.0111**	**0.0021**
F(DFn,DFd)	F(18,396) =4.78	F(18,396) =3.97	F(18,396) =1.93	F(18,396) =1.96	F(18,396) =4.47	F(18,396) =1.96	F(18,396) =2.29
Time							
-1	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.9993	>0.9999
-0.5	>0.9999	>0.9999	0.989	>0.9999	>0.9999	0.836	0.9896
0	0.0738	0.1062	>0.9999	>0.9999	0.9998	0.9566	0.9993
0.5	0.4048	0.1159	>0.9999	>0.9999	0.744	0.9907	>0.9999
1	0.4446	0.3435	0.9644	>0.9999	0.9203	0.7643	>0.9999
1.5	0.9997	0.9985	>0.9999	0.9997	>0.9999	0.3527	>0.9999
2	0.9754	0.9999	>0.9999	0.9959	0.9999	0.999	>0.9999
2.5	0.9911	>0.9999	>0.9999	0.7718	>0.9999	0.997	0.989
3	0.9998	>0.9999	0.9995	0.9973	0.9637	0.9936	0.9974
3.5	0.9823	>0.9999	>0.9999	>0.9999	0.6507	0.7147	0.238
4	0.9998	>0.9999	>0.9999	>0.9999	0.3383	0.6272	0.2923
4.5	0.9985	>0.9999	>0.9999	0.966	0.809	0.9166	0.4748
5	>0.9999	>0.9999	0.9028	0.9862	0.6643	0.8812	0.6546
5.5	>0.9999	>0.9999	0.8134	>0.9999	0.857	0.9466	0.8936
6	>0.9999	>0.9999	0.8417	>0.9999	>0.9999	0.9997	0.9546
6.5	>0.9999	>0.9999	0.9995	>0.9999	>0.9999	0.9979	0.9821
7	0.9998	0.9998	>0.9999	>0.9999	>0.9999	0.9984	0.9443
7.5	0.9998	>0.9999	>0.9999	>0.9999	>0.9999	0.9999	0.9957
8	>0.9999	>0.9999	0.4134	>0.9999	>0.9999	0.9997	0.9953

p-values of Time x Column factor were calculated using linear mixed models analysis comparing the indicated group (e.g. BRT, CEX or CBR) vs. the control group (CON).

p-values of individual timepoints represent adjusted p-values vs. the CON group calculated using Sidak`s post-hoc tests.

BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

Supplemental table 4. Accompanying statistics to Figure 6: Plasma concentrations of inflammatory cytokines during human endotoxemia.

	Panel A. TNF-α	Panel B. IL-6		Panel C. IL-8		Panel D. IL-10
Group	**CBR**	**BRT**	**CBR**	**BRT**	**CBR**	**CBR**
Time x Column factor	**0.0263**	**0.0402**	**0.0291**	**0.0161**	<0.0001	0.0190
F(DFn,DFd)	F(10,220) =2.09	F(10,220) =1.95	F(10,220) =2.06	F(10,220) =2.25	F(10,220) =3.97	F(10,220) =2.20
Time						
-1	0.7778	>0.9999	>0.9999	>0.9999	0.9812	>0.9999
0	0.8051	>0.9999	>0.9999	0.9491	0.8476	>0.9999
0.5	0.9999	0.9894	0.9998	0.8715	0.7003	>0.9999
1	0.9999	>0.9999	>0.9999	0.6979	0.8908	>0.9999
1.5	0.906	>0.9999	0.9986	0.7169	>0.9999	0.9254
2	0.4979	0.9747	0.7848	0.998	>0.9999	**0.0238**
2.5	0.1871	0.7648	0.4367	0.9979	0.4874	**0.0291**
3	0.4659	0.6841	0.9558	0.7793	0.1891	0.5524
4	0.313	0.8405	>0.9999	0.7155	0.4983	>0.9999
6	0.3456	0.7306	0.9998	0.9826	0.4558	>0.9999
8	0.556	0.9879	0.9431	0.9694	0.7952	>0.9999

p-values of Time x Column factor were calculated using linear mixed models analysis comparing the indicated group (e.g. BRT or CBR) vs. the control group (CON).
p-values of individual timepoints represent adjusted p-values vs. the CON group calculated using Sidak`s post-hoc tests.
BRT: breathing exercise group. CBR: cold exposure and breathing exercise group.

Supplemental table 5. Accompanying statistics to Supplemental figure 1: Arterial blood gas parameters and plasma epinephrine levels during the breathing exercises study: morning vs. afternoon session.

	Panel D. Epinephrine
Time x Column factor	**<0.0001**
F (DFn, DFd)	F (4,312)=6.42
Timepoint	
0	0.9977
0.5	**<0.0001**
1.0	0.2473
1.5	0.8104
3.0	0.9240

p-value of Time x Column factor was calculated using linear mixed models analysis comparing the with morning session group (morning) vs. the afternoon session group (afternoon).
p-values of individual timepoints represent adjusted p-values comparing the morning session group (morning) vs. the afternoon session group (afternoon) calculated using Sidak`s post-hoc tests.

Supplemental table 6. Accompanying statistics to Supplemental figure 4: Plasma concentrations of inflammatory cytokines during human endotoxemia.

	Panel A. IP-10		Panel B. MCP1			Panel C. MIP1α		Panel D. MIP1β	
Group	**BRT**	**CBR**	**BRT**	**CEX**	**CBR**	**BRT**	**CBR**	**BRT**	**CBR**
Time x Column factor	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0165	<0.0001
F(DFn,DFd)	F(10,220) =9.42	F(10,220) =7.99	F(10,220) =6.07	F(10,220) =4.75	F(10,220) =4.64	F(10,220) =6.59	F(10,220) =6.25	F(10,220) =2.24	F(10,220) =4.43
Time									
-1	0.3636	0.9768	0.9479	0.9981	>0.9999	>0.9999	0.9894	0.9835	0.9582
0	0.3869	0.9966	0.9929	0.9082	0.8822	>0.9999	0.9986	0.9283	0.9741
0.5	0.2833	0.9768	>0.9999	0.7094	0.1006	0.9831	0.9983	0.5339	0.9316
1	0.2993	0.966	0.6302	0.9881	0.0726	0.8953	0.9667	>0.9999	0.9999
1.5	0.9031	0.9989	>0.9999	0.7848	0.9997	0.6022	0.976	0.9988	0.9706
2	0.9549	0.9978	0.4732	0.6536	>0.9999	0.1366	0.0835	0.6774	0.9925
2.5	0.9869	>0.9999	0.105	0.0605	0.5088	**0.0157**	**0.0111**	0.6882	0.167
3	0.8111	0.7109	0.1681	**0.0425**	0.1422	**0.0466**	**0.0353**	0.6845	0.0651
4	**0.0153**	**0.0355**	0.0654	0.3481	0.2218	0.0591	**0.0486**	0.2352	0.1395
6	0.0926	0.1219	0.6838	>0.9999	0.6735	0.1894	0.1791	0.6955	0.6542
8	0.4848	0.2467	0.9966	0.9894	0.979	0.6916	0.1518	0.9708	0.9895

p-values of Time x Column factor were calculated using linear mixed models analysis comparing the indicated group (e.g. BRT, CEX or CBR) vs. the control group (CON).
p-values of individual timepoints represent adjusted p-values vs. the CON group calculated using Sidak`s post-hoc tests.
BRT: breathing exercise group. CEX: cold exposure group. CBR: cold exposure and breathing exercise group.

Chapter 4

Remote ischaemic preconditioning does not modulate the systemic inflammatory response or biomarkers for renal tubular stress following endotoxaemia in healthy human volunteers: a single-centre, mechanistic, randomised controlled trial in healthy volunteers

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Abstract

Background: Remote ischaemic preconditioning (RIPC) consists of repeated cycles of limb ischaemia and reperfusion, which may reduce perioperative myocardial ischaemic damage and kidney injury. We hypothesised that RIPC may be beneficial by attenuating the systemic inflammatory response. We investigated whether RIPC affects the response in humans to bacterial endotoxin (lipopolysaccharide [LPS]) by measuring plasma cytokines and renal cell-cycle arrest mediators, which reflect renal tubular stress.

Methods: Healthy male volunteers were randomized to receive either daily RIPC for six consecutive days ($RIPC_{multiple}$, n=10) plus RIPC during the 40 minutes preceding intravenous LPS (2 ng kg^{-1}), RIPC only during the 40 minutes before LPS ($RIPC_{single}$, n=10), or no RIPC preceding LPS (control, n=10). As a surrogate marker of renal tubular stress, the product of urinary levels of two cell-cycle arrest markers was calculated (Tissue Inhibitor of Metalloproteinases-2 [TIMP2] * Insulin Like Growth Factor Binding Protein-7 [IGFBP7]).

Results: In both RIPC groups, RIPC alone increased [TIMP2]*[IGFBP7]. LPS administration resulted in fever, flu-like symptoms and haemodynamic alterations. Plasma cytokine levels increased profoundly during endotoxaemia (control group: TNF-α from 14 [9-16] $pg.mL^{-1}$ at baseline to 480 [284-709] $pg.mL^{-1}$ at 1.5 hours following LPS; IL-6 from 4 [4-4] $pg.mL^{-1}$ at baseline to 659 [505-1018] at 2 hours following LPS). LPS administration also increased urinary [TIMP2]*[IGFBP7]. RIPC had no effect on LPS-induced cytokine release or [TIMP2]*[IGFBP7].

Conclusions: RIPC neither modulated systemic cytokine release nor attenuated inflammation-induced tubular stress after LPS. However, RIPC alone induced renal markers of cell-cycle arrest.

Introduction

Multiple animal studies have shown cardioprotective effects following cycles of ischaemia and reperfusion (IR) in a distant organ, a phenomenon termed `remote ischaemic preconditioning` (RIPC). [1, 2] Preclinical studies also demonstrate protective effects of RIPC on extra-cardiac organs, including kidneys and lungs. [3, 4] In humans, RIPC is achieved by using a tourniquet to temporary cut off blood supply to a limb, typically the forearm, and was shown to prevent IR-induced endothelial dysfunction in healthy volunteers. [5] However, RIPC in clinical trials has generated mixed results, [6, 7] particularly in patients undergoing coronary artery bypass grafting (CABG). [8-10]

The mechanism of action by which RIPC exerts its putative protective effects is far from clear and several candidate mechanisms are implicated, [11] including immunological effects. Murine and human studies suggest that remote ischaemic conditioning attenuates systemic inflammatory response in a range of experimental settings. RIPC reduces plasma pro-inflammatory cytokine levels [7, 12-14] and downregulates Toll-like receptor-4 and tumour necrosis factor signalling pathways that are integral for the innate immune response in circulating leukocytes of healthy volunteers. [15] Nevertheless, it remains to be determined whether RIPC exerts direct immunomodulatory effects, or if the attenuated inflammation observed after RIPC is secondary to reduced tissue damage. Intrinsic anti-inflammatory effects of RIPC in humans *in vivo* would provide an important explanation for the beneficial effects observed in various studies over the past decades, and would render RIPC a safe and easy non-pharmacological anti-inflammatory therapy that could readily be applied preoperatively.

The primary aim of the present randomized controlled proof-of-principle study was to investigate the effects of RIPC on the systemic inflammatory response in humans *in vivo* during experimental endotoxaemia, a standardized controlled model of systemic inflammation elicited by intravenous administration of bacterial lipopolysaccharide (LPS). [16] We also assessed whether urinary levels of Insulin-like growth factor–binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinase 2 (TIMP-2), which are biomarkers of G1 cell cycle arrest indicative of renal tubular stress and/or impending AKI, [17, 18] were altered by RIPC as has been reported after cardiac surgery. [6]

Methods

A detailed description of the laboratory techniques used to determine plasma cytokines, *ex vivo* cytokine production, and urinary TIMP2*IGFBP7 levels is provided in the Supplemental Methods.

Participants

After approval from the ethics committee of the Radboud University Medical Center (reference no. 2015-1796; NL53584.091.15), 30 healthy non-smoking male volunteers provided written informed consent to participate in this study (Clinicaltrials.gov NCT02602977). All study procedures were conducted in accordance with the declaration of Helsinki including current revisions and Good Clinical Practice guidelines.

Inclusion criteria

Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values.

Exclusion criteria

Potential participants were excluded if they reported having a febrile illness during the 2 weeks before start of the study, use of prescription drugs, or history of vaso-vagal collapse. In addition, we excluded individuals with hypertension (systolic blood pressure of >160 mmHg or diastolic blood pressure of >90 mmHg), since ischaemia of the forearm was attained by inflating the cuff to 200 mmHg during the RIPC procedure.

Randomisation

Subjects were randomly assigned to either the single RIPC group, multiple RIPC group, or the control group (n=10 per group) using the sealed envelop method. The codelist was generated using the website www.random.org.

RIPC procedures

A PROBE (prospective randomized open label blinded endpoint) design was employed (Figure 1).

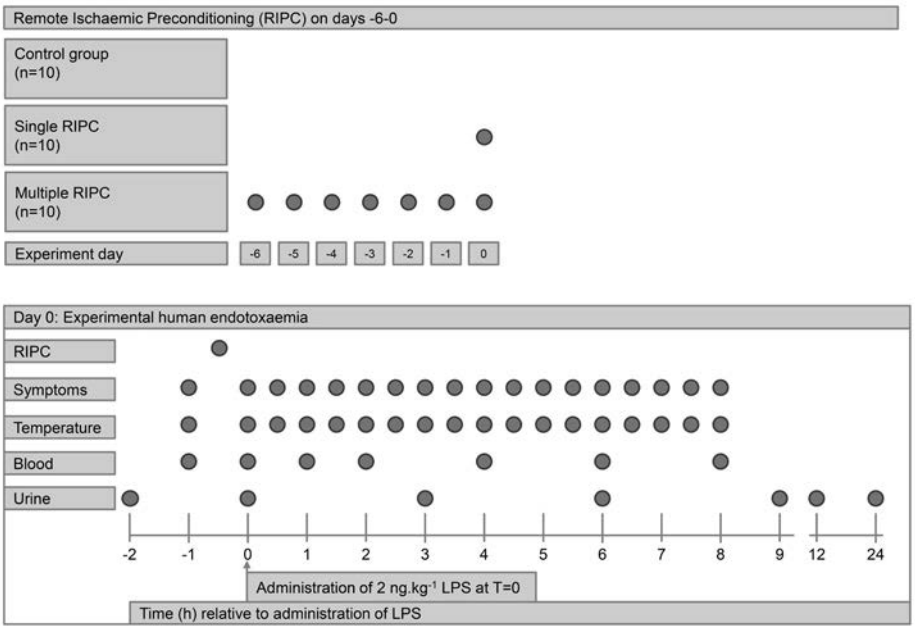

Figure 1: Schematic overview of the study procedures. LPS: lipopolysaccharide

One bout of RIPC consisted of 4 cycles of 5-minute forearm ischaemia followed by 5 minutes of reperfusion, as previously described. [9, 19, 20] RIPC was accomplished by inflating a blood-pressure cuff on the dominant arm to 250 mmHg and releasing the pressure after 5 minutes. [9, 19, 20] After 5 minutes of reperfusion, the cuff was inflated for the next ischaemia-reperfusion cycle, until four cycles were completed. [9, 19, 20]

We employed both single and multiple RIPC, because multiple RIPC combines the two different timeframes in which (R)IPC's protective effects have hitherto been identified: the classical or `early window of protection' that protects in the 1-2 hour after the IPC stimulus [21] and the `second window of protection` which is evident 12-24 hours after the IPC stimulus and lasts for 48-72 hours. [22] These windows employ different signaling mechanisms and may therefore work synergistically. Recent findings in humans indicate that 7 daily bouts of RIPC of the arm resulted in improved endothelial function and skin microcirculation, both locally and remotely, which may represent evidence of enhanced protective effects of multiple RIPC. [23]

The single RIPC group received no interventions on days -6 to -1, and 1 bout of RIPC on day 0 (endotoxaemia experiment day), starting 40 minutes before administration of LPS. The multiple RIPC group received one bout of RIPC per day

on the 6 days before the endotoxaemia experiment day, and also 1 bout of RIPC just prior to LPS administration. Every cycle of RIPC was supervised by a member of the research team. Subjects of the control group received no RIPC leading up to the endotoxaemia experiment.

Preparation of LPS for in vivo administration

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.

Endotoxaemia protocol

Endotoxaemia experiments were conducted at the research unit of the intensive care department of the Radboud university medical center according to our standard protocol. [24] 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 catheter 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 anesthesia (lidocaine HCl 20 $mg.mL^{-1}$) using a 20-gauge arterial catheter for continuous arterial blood pressure monitoring, and blood withdrawal. Subjects received 1.5 L of 2.5 % glucose/0.45 % saline solution for 1 hour (prehydration) before LPS administration, followed by 150 $mL.h^{-1}$ until the end of the experiment (8 hours after LPS administration). Body temperature was measured every 30 minutes using an infrared tympanic thermometer (First-Temp Genius, Sherwood Medical, Crawley/Sussex, UK). Heart rate (three-lead electrocardiogram), blood pressure, respiratory rate, and oxygen saturation (pulse oximetry) data were recorded from a Philips MP50 patient monitor every 30 seconds by a custom in-hous-developed data recording system. LPS-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.

Ex vivo whole blood LPS stimulation

Cytokine production by whole blood *ex vivo* stimulated with LPS was determined in the multiple RIPC group just before and immediately after the first bout of RIPC on day -6. On the endotoxemia experiment day (day 0), LPS-induced *ex vivo* cytokine production was determined before RIPC (T=-1) and immediately after RIPC

(T = 0, just before LPS administration) in both RIPC groups, and at the corresponding timepoints in the control group. Details are available in the Supplemental Methods.

Statistics

Data are expressed as median and interquartile range [IQR] or mean (SEM), based on their distribution (calculated by Shapiro-Wilk tests). For parametric data, the Grubb's test (extreme studentized deviate method) was used to identify significant outliers (maximum of one outlier per group per timepoint), which were excluded from subsequent analyses. All non-parametric data were log-transformed before statistical analysis. Comparisons were made using Student T-tests (within-group comparisons between two timepoints), one-way ANOVA (within-group comparisons over time), and two-way ANOVA (interaction term, between-group comparisons over time). 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).

Sample size calculation

In previous human endotoxaemia experiments performed by our group, the standard deviation of area under curve plasma TNF-α concentrations was 31% of the mean. Using an two-sided α of 0.05, a power of 80% (β of 0.2), and an expected detectable contrast (effect size) of 40% in an unpaired t-test design, 10 subjects per group were required. The effect size was based on a murine endotoxaemia study in which RIPC attenuated TNF-α plasma levels by approximately 50%. [14]

Results

Participant characteristics

Demographic characteristics of the subjects are listed in Table 1 and were not different between groups. No serious adverse events occurred during the conduct of the study. RIPC was well tolerated in all 20 subjects.

Physiological response to intravenous LPS administration

After LPS administration, an increase in body temperature and heart rate, accompanied by flu-like symptoms, was observed in all groups ($p<0.0001$; Figure 2). Endotoxaemia resulted in a decrease in mean arterial pressure in all groups ($p<0.0001$; Figure 2).

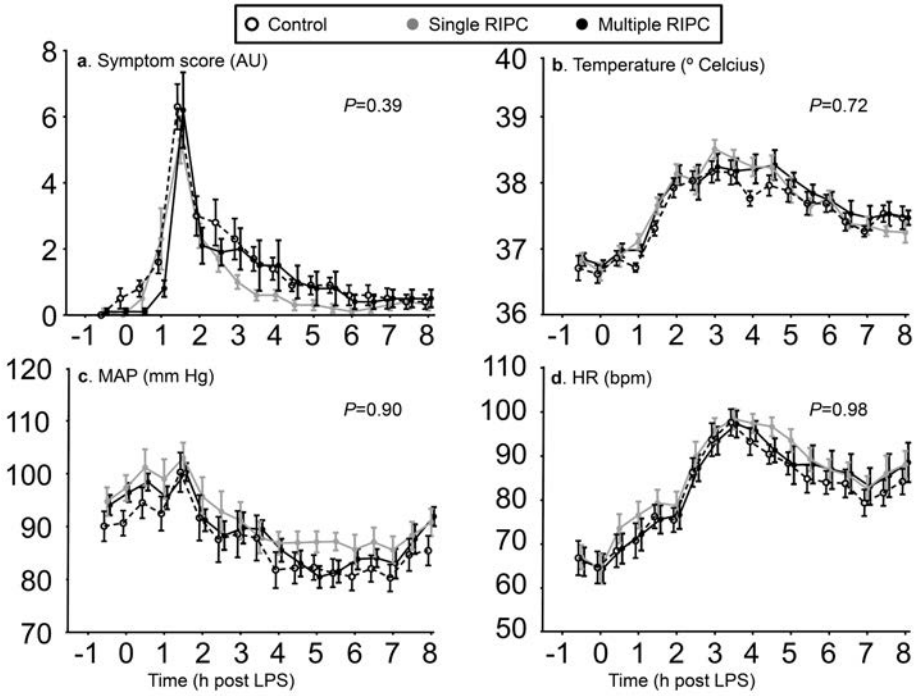

Figure 2: Symptoms, temperature and haemodynamic parameters during experimental endotoxaemia.

a. Aggregated score of self-reported symptoms. **b.** Temperature. **c.** Mean arterial pressure. **d.** Heart rate. Data are presented as mean and SEM. Within-group changes over time were significant for all parameters within all groups ($p < 0.0001$, calculated using one-way ANOVA). p-values depicted in the graphs represent the three-group comparison over time calculated using two-way ANOVA (interaction term). RIPC: remote ischaemic preconditioning. AU: arbitrary units. bpm: beats per minute.

Plasma cytokines and leukocyte numbers

Administration of LPS increased plasma levels of the pro-inflammatory cytokines TNF-α, IL-6, and IL-8 ($p<0.0001$; Figure 3) and chemokines MCP-1, MIP-1α, and MIP-1β in all groups ($p<0.0001$; Supplemental Figure 1).

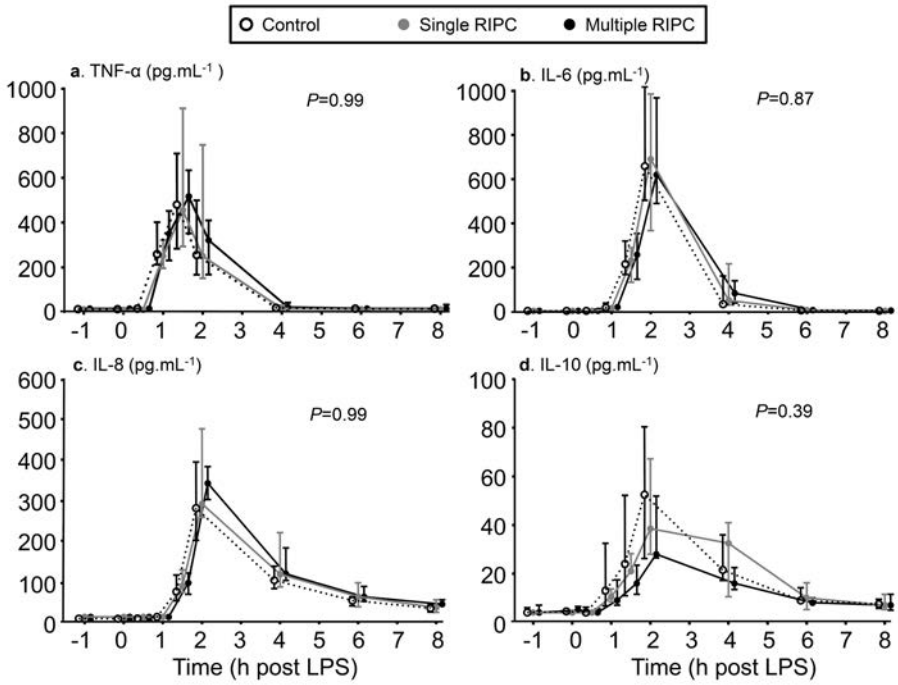

Figure 3: Plasma concentrations of inflammatory cytokines during experimental endotoxaemia. **a.** Tumor necrosis factor (TNF)-α. **b.** Interleukin (IL)-6. **c.** IL-8. **d**. IL-10. Data are presented as median and IQR. Within-group changes over time were significant for all cytokines within all groups ($p < 0.0001$, calculated using one-way ANOVA on log-transformed data). p-values depicted in the graphs represent the three-group comparison over time calculated using two-way ANOVA (interaction term) on log-transformed data. RIPC: remote ischaemic preconditioning.

Circulating concentrations of the anti-inflammatory cytokines IL-10 ($p<0.0001$; Figure 3) and IL-1RA ($p<0.0001$; Supplemental Figure 1) were also increased in all groups. The kinetics of all measured cytokines were similar between the three experimental groups. LPS administration typically resulted in a biphasic pattern in leukocyte counts: initial leukocytopenia followed by leukocytosis ($p<0.0001$ within all groups, Supplemental Table 1). A transient lympho- and monocytopenia was observed ($p<0.0001$ within all groups, Supplemental Table 1). RIPC did not affect LPS-induced changes in numbers of total leukocytes or their differential counts.

Cytokine production of ex vivo stimulated whole blood

Ex vivo stimulation of whole blood with LPS induced robust production of TNF-α, IL-6, and IL-10 (absolute values provided in legend to Figure 4).

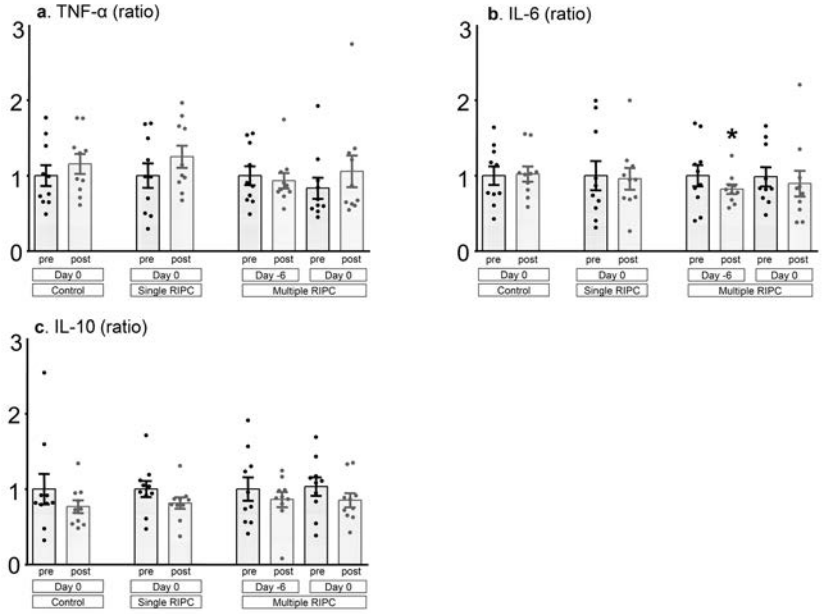

Figure 4: Cytokine production of *ex vivo* stimulated whole blood pre- and post-RIPC. a. Tumor necrosis factor (TNF)-α. **b.** Interleukin (IL)-6. **c**. IL-10. For the control group, RIPC was not applied but blood samples were obtained at the same timepoints. Data are presented as mean and SEM ratio (compared with the respective pre-condition). Absolute values of the pre-condition measurements in pg ml^{-1}: TNF-α: 1256 (172) (Control), 1478 (239) (single RIPC), 1823 (227) (multiple RIPC); IL-6: 12510 (1547) (Control), 13182 (2546) (single RIPC), 15676 (2194) (multiple RIPC); IL-10: 273 (55) (Control), 290 (31) (single RIPC), 248 (38) (multiple RIPC). * indicates $p<0.05$ compared with pre-RIPC (calculated using Student T-tests). RIPC: remote ischaemic preconditioning.

On the endotoxaemia experiment day (day 0), *ex vivo* cytokine production was similar before RIPC (T=-1 hours) compared with immediately after RIPC (T = 0, just before LPS administration) in both RIPC groups, and at the corresponding timepoints in the control group (Figure 4). A decrease in IL-6 production was observed in the multiple RIPC group after the first bout of RIPC on day -6 (Figure 4). *Ex vivo* cytokine production before and after RIPC in the multiple RIPC group was similar between days -6 and 0 (all p-values >0.10).

Renal tubular stress as assessed by urinary TIMP2*IGFBP7 levels

Concentrations of [TIMP2]*[IGFBP7] were significantly higher in urine collected immediately after RIPC (T=0, just before LPS adminstration) than in urine collected before RIPC (T=-2 hours; p=0.008 and p=0.03 for the single and multiple RIPC groups, respectively, Figure 5A).

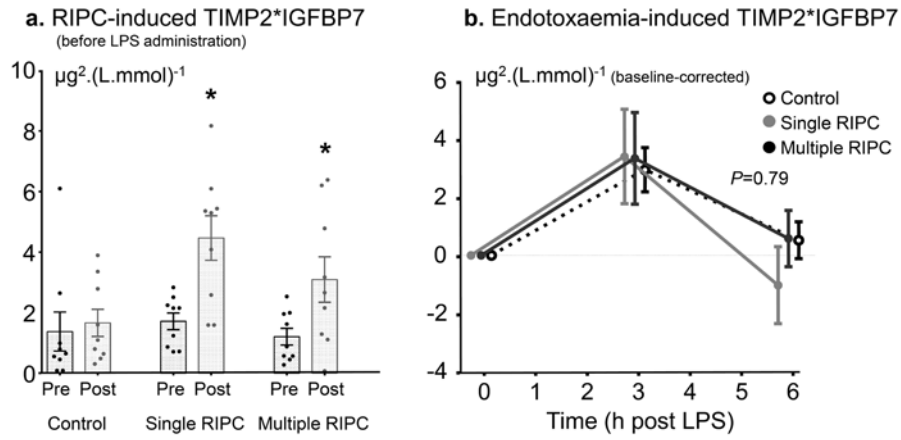

Figure 5: Urinary TIMP2*IGFBP7 levels. a. Urinary concentrations of TIMP2*IGFBP7 before and after RIPC (both before LPS administration). For the control group, RIPC was not applied but urine samples were obtained at the same timepoints. Data are presented as mean and SEM. * indicates p<0.05 compared with pre-RIPC (calculated using Student T-tests). **b.** Urinary levels of TIMP2*IGFBP7 during experimental endotoxaemia. Baseline-corrected data are presented as mean and SEM (baseline: T=0 [time of LPS administration]). Within-group changes over time were significant in all groups (p=0.003, p=0.045, and p=0.012 in the control, single RIPC, and multiple RIPC groups, respectively, calculated using one-way ANOVA). The p-value depicted in the graph represents the three-group comparison over time calculated using two-way ANOVA (interaction term). RIPC: remote ischaemic preconditioning.

In the control group, no change was observed in urine collected at the same timepoints (p=0.48, Figure 5A). The magnitude of the increase in urinary [TIMP2]*[IGFBP7] was similar between the single and multiple RIPC groups (increase of 2.75 (0.78) vs. 1.89 (0.69) ng.mL^2, respectively, p=0.42). LPS administration increased urinary [TIMP2]*[IGFBP7] in all groups (Figure 5B). The LPS-induced increase in TIMP2*IGFBP7 was not altered by RIPC (Figure 5B).

Discussion

Our study demonstrates that RIPC does not modulate increases in plasma cytokine levels induced by LPS administration in healthy male volunteers. In addition, both RIPC and LPS increased urinary [TIMP2]*[IGFBP7], but the additional LPS-induced increase in [TIMP2]*[IGFBP7] was not affected by RIPC. These results indicate that the putative protective effects of RIPC may not be mediated by modulation of the systemic inflammatory response, as reflected by plasma cytokine levels. Furthermore, RIPC does not attenuate biomarkers indicative of tubular stress after LPS administration.

We did not observe any RIPC-induced effects on plasma cytokine levels, although *ex vivo* IL-6 production was modestly reduced in the multiple RIPC group after the first bout of RIPC. The latter finding may represent a type I error, as no effect was observed in the single RIPC group and production of other cytokines was similar across experimental groups. Our findings, obtained in a homogenous group of healthy young adults in a highly standardized and reproducible model of systemic inflammation, appear to contrast with previous studies reporting immunomodulatory effects of RIPC in patients. For instance, in 216 patients after lung resection RIPC attenuated circulating IL-6 and TNF-α concentrations, [7] whereas increased IL-10 levels 24 hours after RIPC were reported in 30 infants receiving RIPC before repair of simple congenital heart defects. [25] In contrast, RIPC was not associated with differences in levels of the pro-inflammatory cytokines IL-6 and TNF-α in patients after major cardiac surgery. [26, 27] The heterogeneity of patients in these trials is probably an important factor accounting for these discrepant observations. None of these studies were primarily designed to investigate the effects of RIPC on systemic inflammation, as they were performed in patients undergoing various types of surgery. The immune response in these complex procedures is a result of various insults, of which ischaemia-reperfusion injury is just one. The experimental human endotoxaemia model by itself does not induce ischaemia-reperfusion injury. Therefore, the observed mitigation of the inflammatory response by RIPC in surgical patients may result from protection from ischaemia-reperfusion injury, rather than being the consequence of a direct immunomodulatory effect.

Both RIPC and endotoxaemia increased urinary [TIMP2]*[IGFBP7] in our study. To the best of our knowledge, we are the first to reveal that pathogen-associated molecular patterns (PAMPs) like LPS induce TIMP2 and IGFBP7 in humans. Urinary [TIMP2]*[IGFBP7] is a marker of cell cycle arrest in the kidney reflecting tubular cell stress, but not the occurrence of actual cell damage. [28] As such, increased levels of this marker combination are a precursor for the occurrence of actual AKI, which is nevertheless still reversible at that moment (i.e. cell cycle arrest must be prolonged for AKI to develop). In accordance, human endotoxaemia (at least using 2 ng/kg bolus administration as used in this study) does not cause AKI, as diuresis, the levels of creatinine, urea clearance and the fraction of sodium excreted are not affected. [29] The increase of [TIMP2]*[IGFBP7] following LPS administration thus likely results from direct interaction of LPS with tubular epithelial cells [30]. The RIPC-induced increase of [TIMP2]*[IGFBP7] is probably due to the induction of tubular stress by mediators released during cycles of ischaemia-reperfusion.

The clinical effects of RIPC applied before surgery have been evaluated in several large intervention trials, with some reporting beneficial effects, [6, 7, 10] whereas other, more recently performed large studies, did not. [8, 9] The reasons for the observed discrepancies remain to be fully elucidated, but may involve the use of different anaesthetic regimes, differences in patient selection, and misclassification of the a priori risk for developing organ failure such as AKI. [31-33] Similar to the aforementioned controversial effects on inflammation, these conflicting data strengthen the argument that more basic research is required into understanding mechanisms underlying RIPC.

Our study has several strengths but also limitations. We used a similar RIPC protocol applied to the forearm model used in clinical trials that showed beneficial effects of RIPC, including attenuation of the inflammatory response. [6, 7, 10] We directly compared two different RIPC protocols to investigate both the early (the `first window of protection`) and late effects of RIPC (the `second window of protection`). RIPC-induced increases in urinary [TIMP2]*[IGFBP7] suggest that the RIPC intervention directly caused detectable effects on the kidneys. Although 10 participants per group appears to be low, our established endotoxaemia model has consistently revealed significant between-group differences in several interventional studies. [34-39] Examining the systemic inflammatory response of younger male adults may be a limitation, since this is unlikely to be comparable to the inflammatory response in older patients with comorbidities who may possibly benefit from RIPC. Nevertheless, effects of RIPC on endothelial function were previously demonstrated in healthy young volunteers. [40] We cannot exclude that the endotoxaemia model is too mild or transient to detect an (possibly delayed) effect of RIPC on systemic inflammation and organ injury. Although we focused on *in vivo* and *ex vivo* cytokine production and markers of tubular stress, we cannot rule out effects of RIPC on other innate immune functions including leukocyte phagocytosis capacity, respiratory burst and formation of neutrophil extracellular traps. Finally, the largest body of evidence on RIPC's anti-inflammatory and tissue-protective effects has been obtained in animals or patients who sustain ischaemia-reperfusion injury. It might be argued that the immune response elicited by LPS differs from that induced by ischaemia-reperfusion injury. Nevertheless, remote ischaemic conditioning attenuated the LPS-induced release of pro-inflammatory TNF-α and IL-6, and enhanced anti-inflammatory IL-10 levels in mice. [13, 14]

Conclusions

We demonstrate that RIPC does not affect the *in vivo* systemic inflammatory response induced by administration of LPS in humans. These results suggest that the putative beneficial effects of RIPC may not be mediated through direct systemic anti-inflammatory effects. The endotoxin-induced increase in urinary excretion of a tubular stress biomarker was not attenuated by RIPC. The absence of immunomodulatory effects of RIPC in this experimental medicine study demonstrates the need for further investigation into the mechanisms of RIPC and its' potential role in reducing perioperative AKI.

Acknowledgements

The authors would like to thank the research nurses of the Department of Intensive Care for their help with the endotoxaemia experiments, Jelle Gerretsen for cytokine determinations, and Astute Medical Inc. (San Diego, CA, USA) for providing assay kits for TIMP2*IGFBP7.

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Supplemental Methods

Plasma cytokine measurements

Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was immediately centrifuged at 2000 g for 10 minutes at 4 °C after which plasma was stored at –80 °C until analysis. Concentrations of TNFα, IL-6, IL-8, and IL-10 were analyzed batch-wise using a Luminex assay according to the manufacturer's instructions (Bio-Plex, Bio-Rad, Hercules, CA, USA).

Ex vivo whole blood LPS stimulation

Whole blood was challenged with LPS in pre-filled tubes as described previously. [1] Briefly, 0.5 mL of blood was added to tubes pre-filled with 2 mL culture medium or 2 mL culture medium supplemented with 12.5 ng.mL^{-1} LPS (end concentration of LPS 10 ng.mL^{-1}). Cultures were incubated at 37 °C for 24 hours, centrifuged, and supernatants were stored at –80 °C until analysis. Concentrations of TNFα, IL-6, and IL-10 were determined using enzyme-linked immunosorbent assays according to the manufacturer's instructions (Duoset, R&D systems, Minneapolis, MN, USA).

Urinary TIMP2*IGFBP7 levels

Timepoints at which urine was collected are depicted in Figure 1 (the urine sample at T=-2 represents morning urine). Urine was aliquoted and stored at –80 °C until analysis. Urinary levels of TIMP-2 and IGFBP7 were measured with the NephroCheck™ Test (Astute Medical, San Diego, CA, USA). [2] Values of TIMP-2 and IGFBP7 were corrected for urinary creatinine content (enzymatic determination via random acces analyzer Cobas C8000, Roche Diagnostics, Almere, The Netherlands).

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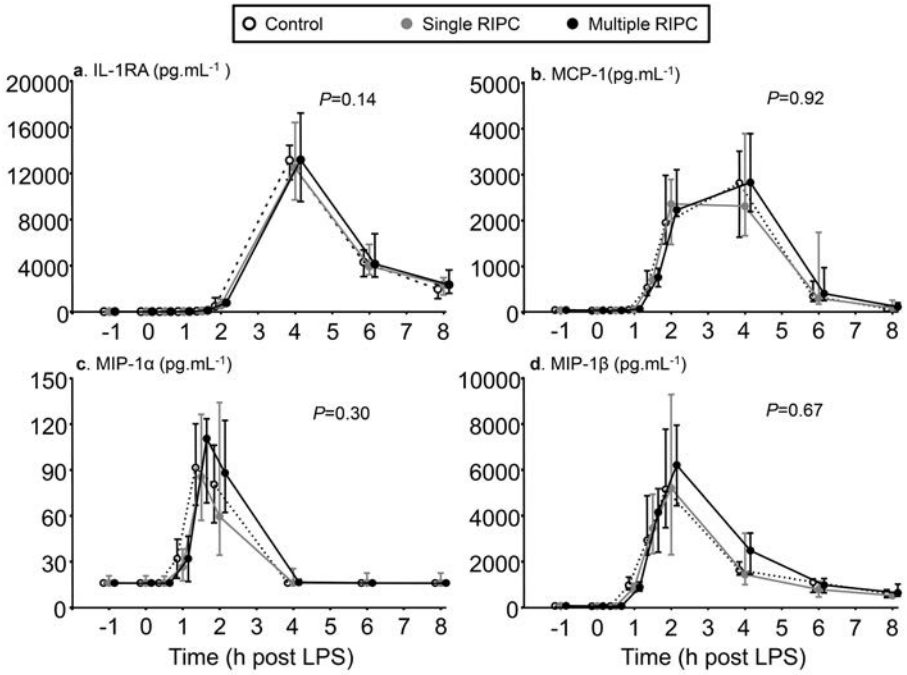
Supplemental Figures and Tables



Supplemental Figure 1: Plasma concentrations of inflammatory cytokines and chemokines during experimental endotoxaemia.

A. Interleukin (IL) 1-receptor antagonist (RA). **B.** Monocyte Chemoattractant Protein-1 (MCP-1) **C.** Macrophage Inflammatory Protein (MIP)-1α. **D**. MIP-1β. Data are presented as median and IQR. Within-group changes over time were significant for all cytokines within all groups ($p < 0.0001$, calculated using one-way ANOVA on log-transformed data). p-values depicted in the graphs represent the three-group comparison over time calculated using two-way ANOVA (interaction term) on log-transformed data. RIPC: remote ischaemic preconditioning.

Supplemental Table 1: Total leukocyte and differential counts

Leukocyte subtype	Group	T=-1	T=0	T=1	T=2	T=4	T=6	T=8	p value
Leukocytes *10^{9}.L^{-1}	Control	5.1 (0.4)	5.4 (0.7)	3.8 (0.7)	6.3 (0.5)	9.4 (0.6)	11.4 (0.6)	10.7 (0.50	0.98
	Single RIPC	4.9 (0.3)	5.0 (0.3)	4.1 (0.8)	7.0 (0.6)	10.0 (0.6)	11.4 (0.5)	10.8 (0.6)	
	Multiple RIPC	5.2 (0.4)	5.4 (0.5)	3.7 (0.5)	6.8 (0.6)	9.3 (0.7)	11.2 (0.6)	10.8 (0.6)	
Granulocytes (% of leukocytes)	Control	56 (1.6)	64 (2.5)	67 (4.3)	85 (2.0)	94 (0.4)	91 (0.8)	87 (1.0)	0.99
	Single RIPC	54 (2.6)	61 (3.4)	67 (4.4)	85 (2.0)	93 (0.7)	90 (1.1)	86 (1.6)	
	Multiple RIPC	58 (3.6)	64 (3.5)	67 (4.2)	87 (1.0)	94 (0.8)	91 (1.3)	87 (1.7)	
Lymphocytes (% of leukocytes)	Control	30 (1.8)	24 (2.0)	29 (3.9)	12 (1.9)	4 (0.2)	4 (0.4)	7 (0.7)	0.97
	Single RIPC	33 (2.0)	29 (2.6)	34 (5.7)	13 (1.5)	4 (0.3)	5 (0.5)	8 (1.0)	
	Multiple RIPC	28 (2.7)	25 (2.9)	28 (4.0)	11 (0.8)	4 (0.5)	4 (0.7)	7 (1.1)	
Monocytes (% of leukocytes)	Control	10 (0.6)	8 (0.4)	0.6 (0.1)	0.4 (0.1)	2 (0.3)	4 (0.4)	5 (0.4)	0.94
	Single RIPC	10 (0.6)	8 (0.7)	0.6 (0.1)	0.4 (0.1)	2 (0.5)	5 (0.7)	6 (0.7)	
	Multiple RIPC	9 (0.7)	8 (0.6)	1 (0.2)	0.3 (0.1)	2 (0.6)	4 (0.6)	4 (0.6)	

Data are presented as mean (SEM). Within-group changes over time were significant for all parameters within all groups ($p < 0.0001$, calculated using one-way ANOVA). p-value indicates between-group differences over time calculated by repeated measures two-way ANOVA (interaction term).

Chapter 5

Involvement of lactate and pyruvate in the anti-inflammatory effects exerted by voluntary activation of the sympathetic nervous system

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Abstract

We recently demonstrated that the sympathetic nervous system can be voluntarily activated following a training program consisting of cold exposure, breathing exercises, and meditation. This resulted in profound attenuation of the systemic inflammatory response elicited by lipopolysaccharide (LPS) administration. Herein, we assessed whether this training program affects the plasma metabolome and if these changes are linked to the immunomodulatory effects observed. A total of 224 metabolites were identified in plasma obtained from 24 healthy male volunteers at six timepoints, of which 98 were significantly altered following LPS administration. Effects of the training program were most prominent shortly after initiation of the acquired breathing exercises but prior to LPS administration, and point towards increased activation of the Cori cycle. Elevated concentrations of lactate and pyruvate in trained individuals correlated with enhanced levels of anti-inflammatory IL-10. In vitro validation experiments revealed that co-incubation with lactate and pyruvate enhances IL-10 production and attenuates the release of pro-inflammatory IL-1β and IL-6 by LPS-stimulated leukocytes. Our results demonstrate that practicing the breathing exercises acquired during the training program results in increased activity of the Cori cycle. Furthermore, this work uncovers an important role of lactate and pyruvate in the anti-inflammatory phenotype observed in trained subjects.

Introduction

Recent work from our group revealed that the sympathetic nervous system can be voluntarily activated through a training program consisting of exposure to cold, breathing exercises, and meditation [1]. Compared to an untrained control group who did not practice any exercise, a strong increase in endogenous epinephrine levels was observed in trained healthy volunteers shortly after initiation of the learned breathing exercises. This resulted in augmentation of plasma concentrations of IL-10, a pivotal anti-inflammatory cytokine, and suppression of the pro-inflammatory response during experimental human endotoxemia, a standardized and reproducible model of systemic inflammation elicited by lipopolysaccharide (LPS) administration [1]. Activation of the sympathetic nervous system is also known to profoundly impact cellular metabolism [2]. Furthermore, interaction between immune responses and host metabolism, also termed "immunometabolism", has gained renewed interest, as it was recently discovered that metabolic reprogramming of innate immune cells plays a critical role in mounting effective immune responses and in generation of innate immune memory [3,4].

Metabolomic profiling, for instance using reversed phase chromatography coupled to electrospray mass spectrometry (ESI–MS), enable the detection of highly polar compounds in blood [5]. These techniques allow for simultaneous investigation of a large number of metabolites, thereby providing an indication of the "metabolic status" of an individual [6]. Using such an established platform [7], we performed metabolomic profiling of plasma samples obtained in the abovedescribed study [1]. Our primary aim was to determine changes in the plasma metabolome of trained subjects [1], and to assess if these changes could play a role in the immunomodulatory effects observed.

Materials and Methods

Subjects and experimental design

Metabolomic profiling was performed in plasma samples obtained in a previously published parallel randomized controlled study registered at ClinicalTrials.gov as NCT01835457 [1]. The study protocol is described in detail elsewhere [1]. Briefly, after approval from the local ethics committee of the Radboud university medical centre (CMO 2012/455), 24 healthy nonsmoking male volunteers with a median age of 22 years (range 19-27) provided written informed consent and were included in the study. All study procedures were in accordance with the Declaration of Helsinki, including current revisions, and Good Clinical Practice guidelines. Subjects were screened before the study and had a normal physical examination, electrocardiography, and routine laboratory values. Exclusion criteria were: febrile illness during the 2 weeks before the endotoxemia experiment, taking any prescription medication, history of spontaneous vagal collapse, practicing or experience with any kind of meditation, or participation in a previous trial where LPS was administered. The subjects were randomly allocated to the trained group (n=12) or the control group (n=12) by the opening of sealed envelopes prepared by unblinded staff not involved in the study. The trained group underwent a 10-day training program provided by Dutch individual Wim Hof, which consisted of three main elements: meditation, exposure to cold, and breathing exercises (see [1] for a detailed description). After completion of the training, subjects of both groups (n=24) underwent experimental human endotoxemia at our intensive care research unit, consisting of administration of an intravenous bolus of 2 ng/kg of US Standard Reference *Escherichia coli* endotoxin (*E. coli* O:113 [LPS], Clinical Center Reference Endotoxin; National Institutes of Health, Bethesda, MD, USA). As part of our standard endotoxemia protocol [31,32], the subjects received 1.5 L 0.9% NaCl during one hour starting one hour before endotoxin infusion (pre-hydration) followed by 150 ml/h until 6 hours after endotoxin infusion and 75 ml/h until the end of the experiment. The experimental human endotoxemia protocol is provided in detail elsewhere [1,32]. The control group did not undergo any training procedures and also underwent experimental human endotoxemia. Subjects in the trained group started practicing the breathing exercises acquired during the training program 30 minutes before LPS administration until two-and-a-half hours afterwards. The control group did not practice any exercise throughout the endotoxemia experiment. As described in detail elsewhere [1], the breathing exercises consisted of two exercises. In brief, the first exercise comprised cycles of vigorous hyperventilation (approximately 30 breaths) followed by breath holding for several minutes at the discretion of the subject. The second exercise was similar,

but at the end of the hyperventilation period, breath was only held for 10 seconds during all body muscles were tightened.

Sample preparation for LC-MS analysis

Ethylenediaminetetraacetic acid (EDTA) anti-coagulated blood was obtained one hour before LPS administration (baseline), and at T=0, T=1, T=2, T=4 and T=8 h. Blood was immediately centrifuged at 2000 g for 10 minutes at 4 °C after which plasma was stored at –80 °C until analysis. Sample extraction was performed as described previously [7], with some modifications. Briefly, 50 µL of plasma was mixed with 450 µL of 90% (v/v) methanol containing internal standards and incubated for 15 min at 37°C with 1150 rpm. Precipitated proteins were separated from the extract by centrifugation for 12 min at 15000 rpm, after which supernatants were stored at -80 °C until further analysis.

LC-MS analysis

Modified reversed-phase chromatography in combination with high resolution mass spectrometry (HRMS) was employed in this study. Samples were analyzed on an Agilent 1290 UPLC system (Agilent) with a Discovery HS F5-3 column (15 cm x 2.1 mm, 3 µm, Supelco, Sigma Aldrich) coupled to a high-resolution 6540 QTOF/MS Detector (Agilent) operated in both positive and negative ESI mode in a detection range of 50 to1700 *m/z* at 2 GHz in extended dynamic range. The LC solvent consisted of 95% 10 mM ammonium formate with 0.1% formic acid and 5% acetonitrile (A), and 95% acetonitrile with 5% 10 mM ammonium formate with 0.1% formic acid (B). A multi-step gradient was used, with 5% B from 0-0.1min to 35% B at 1.5 min, to 95% B at 2.05 min which was kept constant until 3.2 min, to 5% B at 3.21 min and washing until 4.3 min with 5% B. The flow rate was kept constant at 700 µL/min from 0 min to 2.2 min, and increased up to 900 µL/min from 2.2 min to 2.5 min, after which flow rate was kept constant until 3.2 min. The flow rate was decreased from 900 µL/min to 800 µL/min from 3.2 min to 3.1 min and kept constant until 3.7 min, when flow rate was changed to 700 µL/min. The run time was 4.3 min, 1 µl of sample was injected and the column heated to 40 °C. The DualAJS ESI source was set to the following parameters: Gas temperature 200 °C, drying gas 8 L/min, nebulizer 35 psig, sheath gas temp: 350 °C, sheath gas flow 11 L/min, VCAp 3500 V and nozzle voltage of 0 V. Online calibration of the instrument was performed throughout the data acquisition using Agilent ES-TOF Reference Mass Solution Kit.

Cytokine and lactate determinations

Plasma concentrations of the cytokines TNFα, IL-6, IL-8, and IL-10 at various timepoints during the endotoxemia experiment were determined by Luminex

assay (Milliplex, Millipore) as described previously [1]. The area under the cytokine plasma concentration-time curves (AUC) was used as an integral measure of the subjects' *in vivo* cytokine responses. Furthermore, we validated the LC-MS analysis for lactate by comparing LC-MS data with lactate concentrations measured by a point-of-care blood analyzer (i-STAT, Abbot) as described previously [1].

PBMC stimulation experiments

After approval from the local ethics committee of the Radboud university medical center (CMO 2010/10), EDTA anticoagulated blood was obtained from 6 healthy donors. Isolation of PBMCs was performed by differential centrifugation over Ficoll-Paque PLUS (GE Healthcare Biosciences) in SepMate tubes (STEMCELL technologies). PBMCs were washed thrice with PBS, counted, and 5 x 10^5 PBMCs/well were seeded 96-well round-bottom plates in RPMI (Dutch Modification, Invitrogen) culture medium supplemented with 50 µg/mL gentamycin (Thermo Fisher Scientific) and 2 mM Glutamax (Invitrogen). Cells were incubated with RPMI (control), or 1, 3, 10, or 20 mM of sodium lactate (provided by the Department of Pharmacy, Radboud university medical center, Nijmegen, The Netherlands), pyruvate (Invitrogen), or a combination of sodium lactate and pyruvate for 1 hour at 37 °C and 5% CO_2. Subsequently, RPMI or 10 ng/mL *E. coli*-derived LPS (serotype O55:B5, Sigma Aldrich) was added and PBMCs were incubated for 48h at 37 °C and 5% CO_2. Concentrations of IL-10, IL-1β, IL-6, and TNFα in the cell culture supernatants were measured by ELISA (R&D systems).

Raw data processing and statistical analyses

Chromatograms were generated by the LC-MS instrument in .d format. Raw data were converted into mzXML and chromatogram peaks were extracted using XCMS v1.42.0 [33]**,** which was optimized using the IPO R package [34] with the following settings: peakwidth=c(10, 70), ppm= 20, snthresh=10, mzdiff=0.0034, prefilter=c(3, 100), noise=100, gapInit=0.8448, gapExtend=2.0544, bw=5, mzwid=0.015, minfrac=0.5, max=50. All further analyses were performed in R programming language [35], Metaboanalyst 4.0 [9], and GraphPad Prism version 5.0 (GraphPad Software). IDEOM software (http://mzmatch.sourceforge.net/ideom.php) [36] was used to eliminate noise and for putative peak annotation by exact mass within ±10 ppm against the Metabolomic Discoveries in house metabolite library [37] in negative and positive ESI mode, respectively. Retention time prediction was applied to aid metabolite annotation. 972 peaks were detected. Not annotated metabolites and potential peptides were removed (n=748), leaving 224 metabolites in the dataset. Furthermore, 6 outlier samples were identified using ROBPCA by defining the sample distances within the orthogonal to the projection

plane [38]. This left a total of 138 samples to be analyzed. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed to visualize the metabolic alterations between samples after log-transformations followed by mean-centering and dividing by the standard deviation of each variable. OPLS-DA models were validated by permutation tests (1000 permutations) [39]. Differential analyses were performed using paired (within-group comparisons) and unpaired (between-group comparisons) t-tests with multiple-testing correction using Benjamini-Hochberg FDR [40]. Analogous to previous work [15,41], a FDR-adjusted p-value of 0.1 was set as threshold for statistical significance in the differential analyses. Targeted analyses (permutation tests, Spearman correlation between metabolites and cytokines, and paired t-tests for the PBMC stimulation data) were performed without applying FDR correction, and the threshold for significance was set at $p<0.05$ for these data. We restricted correlation analyses to metabolite levels at early timepoints (T=0 and T=1), because these can still relevantly affect the immunological response, which is orchestrated in the first hour and peaks 1.5-2 hours after LPS administration [1].

The metabolomics dataset is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org, where it has been assigned Project ID PR00083. The data can be accessed directly via it's Project DOI: 10.21228/M8C671. Metabolomics Workbench is supported by NIH grant U2C-DK119886.

Results

Characteristics of the study population are listed in Table S1 in the Supplementary Material and reveal no differences between the control group and the trained group.

Effects of LPS administration on plasma metabolites

We first examined LPS-induced changes on the plasma metabolome. To this end, we restricted the analyses to the control group. Expectedly, LPS administration resulted in typical flu-like symptoms, fever, hemodynamic changes and increases in plasma concentration of pro and anti-inflammatory cytokines (results reported in detail elsewhere [1], baseline, peak, and area under curve (AUC) plasma cytokine levels are provided in Table S2 in the Supplementary Material). We compared levels of plasma metabolites 0, 1, 2, 4, and 8 hours after LPS administration to those at baseline (one hour before LPS administration). PCA plots are depicted in Figure 1a, and show the clearest separation at 4 hours post-LPS.

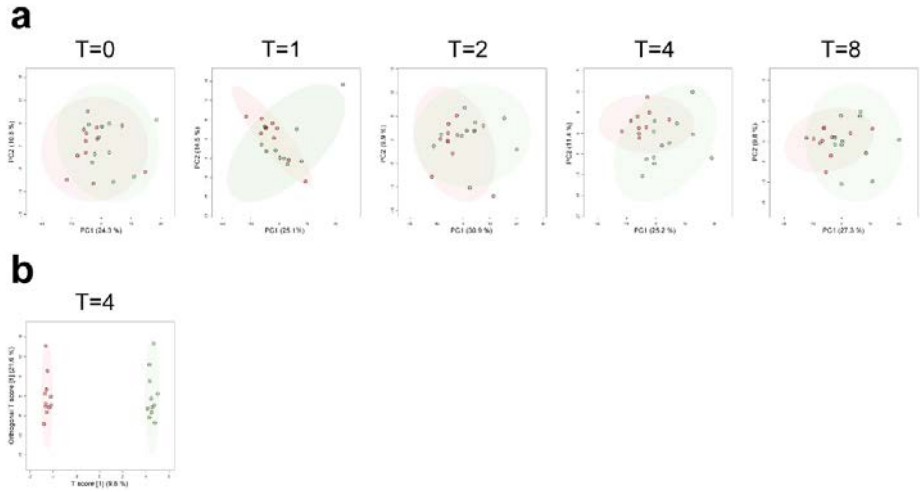

Figure 1. Effects of endotoxemia on the plasma metabolome. (a) Principal component analysis (PCA) of the plasma metabolome at T=0 (just prior to LPS administration), 1, 2, 4, and 8 hours following LPS administration in the control group. The red dots indicate baseline (one hour before LPS administration) and the green dots the timepoints indicated above the graphs. The shaded areas indicate the 95% confidence intervals; **(b)** Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the plasma metabolome at 4 hours after LPS administration in the control group. The red dots indicate baseline (one hour before LPS administration) and the green dots 4 hours post-LPS administration. The shaded areas indicate the 95% confidence intervals.

Subsequently, we constructed OPLS-DA models. Permutation tests revealed that only the model constructed for the comparison of T=4 hours vs. baseline showed significant separation (1+3 components, R^2X_{cum}: 0.448, R^2Y_{cum}: 0.997, Q^2_{cum}: 0.404; permutation tests: R^2Y: $p<0.001$, Q^2: $p=0.03$, score plot depicted in Figure 1b). Differential analyses revealed 98 significantly altered metabolites out of a total of 224 identified metabolites (FDR-adjusted p-value of <0.1) across all timepoints. The top 25 significantly altered metabolites are depicted in Figure 2 (all decreased compared with baseline); a complete overview of all metabolites on all timepoints is provided in Tables S3 and S4 in the Supplementary Material.

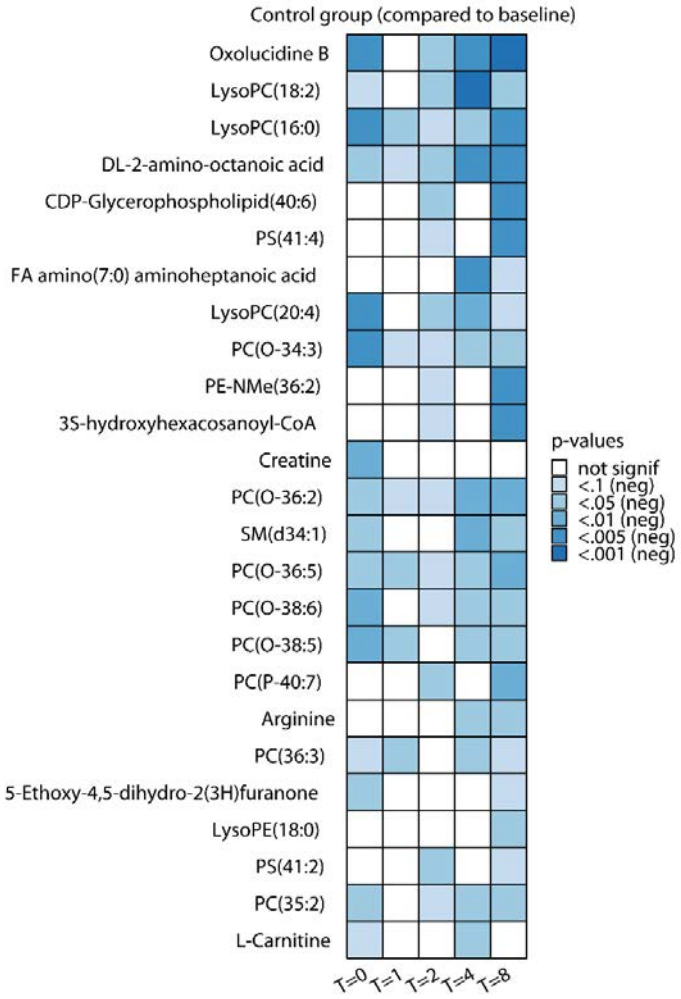

Figure 2. Top differentially regulated metabolites during endotoxemia. Top 25 most significantly (according to p-value) regulated metabolites at T=0 (just before LPS administration), 1, 2, 4, and 8 hours post-LPS administration compared with baseline (one hour before LPS administration) in the control group. Blue color indicates a decrease compared with baseline (this top 25 only comprises decreased metabolites). p-values were calculated using paired t-tests with multiple-testing correction (Benjamini-Hochberg FDR).

The relatively large number of decreased metabolites at T=0 is likely a result of dilution, as all subjects were prehydrated with 1.5 L of saline/glucose solution between baseline and T=0 as part of our standard experimental endotoxemia protocol [8]. Because PCA and OPLS-DA models showed the most pronounced separation at T=4 hours, we focused on that timepoint, and all 44 significantly altered metabolites (10 increased, 34 decreased) at T=4 hours are listed in Table 1.

Table 1: Significantly altered metabolites 4 hours after LPS administration in the control group.[1]

Metabolite	Class	Fold change vs. baseline [1]	FDR adjusted p-value [2]
Increased			
5,8-Dihydro-6-(4-methyl-3-pentenyl)-1,2,3,4-tetrathiocin	Prenol lipids	+1.25	0.044
6-Phosphonoglucono-D-lactone	Monosaccharides	+3.31	0.051
Deoxycytidine	Nucleosides, nucleotides and derivatives	+24.77	0.061
Artonin K	Flavonoids	+1.43	0.065
Hexane-6-keto-1,3,4,6-tetracarboxylate	Unknown	+3.91	0.065
Cis-(homo)2aconitate	Metabolism of cofactors and vitamins	+1.25	0.071
Deoxyuridine triphosphate	Nucleosides, nucleotides and derivatives	+4.46	0.076
Imidazoleacetic acid riboside	Nucleosides, nucleotides and derivatives	+1.31	0.076
Lactic acid	Hydroxy acids and derivatives	+2.03	0.091
Gnidicin	Unknown	+6.87	0.096
Decreased			
LysoPC(18:2)	Glycerophospholipids	-2.22	0.0008
DL-2-amino-octanoic acid	Amino acids and derivatives	-1.51	0.002
Oxolucidine B	Unknown	-2.27	0.005
FA amino(7:0) aminoheptanoic acid	Fatty acyls	-1.59	0.005
LysoPC(20:4)	Glycerophospholipids	-2.85	0.007
PC(O-36:2)	Glycerophospholipids	-1.26	0.007
SM(d34:1)	Sphingolipids	-1.21	0.009
Arginine	Amino acids and derivatives	-1.40	0.013
PC(36:3)	Glycerophospholipids	-1.29	0.015
PC(O-36:5)	Glycerophospholipids	-1.24	0.019
PC(O-34:3)	Glycerophospholipids	-1.28	0.021
L-Carnitine	Alkylamines	-1.32	0.022
Succinic acid semialdehyde	Fatty acids and conjugates	-1.47	0.022
PC(O-38:5)	Glycerophospholipids	-1.20	0.023
PS(21:0)	Glycerophospholipids	-3.43	0.024
L-Acetylcarnitine	Fatty acid esters	-1.67	0.026
PC(O-38:6)	Glycerophospholipids	-1.21	0.027
Glutamine	Amino acids and derivatives	-1.45	0.028

Table 1: Continued

Metabolite	Class	Fold change vs. baseline [1]	FDR adjusted p-value [2]
PC(36:5)	Glycerophospholipids	-1.65	0.035
PC(36:4)	Glycerophospholipids	-1.20	0.035
LysoPC(16:0)	Glycerophospholipids	-2.20	0.038
PC(P-40:6)	Glycerophospholipids	-1.19	0.045
PC(35:2)	Organic phosphoric acids and derivatives	-1.25	0.045
Narciclasine	Unknown	-1.86	0.045
PC(40:6)	Glycerophospholipids	-1.69	0.045
Lenticin	Unknown	-1.35	0.065
PC(36:2)	Glycerophospholipids	-1.35	0.066
PC(34:3)	Glycerophospholipids	-1.23	0.071
PC(38:4)	Glycerophospholipids	-1.20	0.071
PE(39:1)	Glycerophospholipids	-1.33	0.071
PC(O-34:2)	Glycerophospholipids	-1.30	0.071
SM(d34:2)	Sphingolipids	-1.20	0.078
PC(38:5)	Glycerophospholipids	-1.17	0.087
TG(41:0)	Glycerolipids	-1.20	0.091

Baseline represents one hour before LPS administration.[2] P-values were calculated using paired t-tests with multiple-testing correction (Benjamini-Hochberg FDR).

Three of the increased metabolites belonged to the class of nucleosides, nucleotides and derivatives. The majority of decreased metabolites were comprised of glycerophospholipids. Metabolite Set Enrichment Analysis (MSEA) [9] revealed that the most significantly affected pathways included glutamate metabolism, oxidation of various fatty acids, pyrimidine metabolism, the urea cycle, and the Warburg effect (Figure S1 in the Supplementary Material).

Differences in plasma metabolites in trained and untrained individuals

We performed PCA and constructed OPLS-DA models for the control vs. trained group at each timepoint. PCA plots did not show clear separation (Figure 3a).

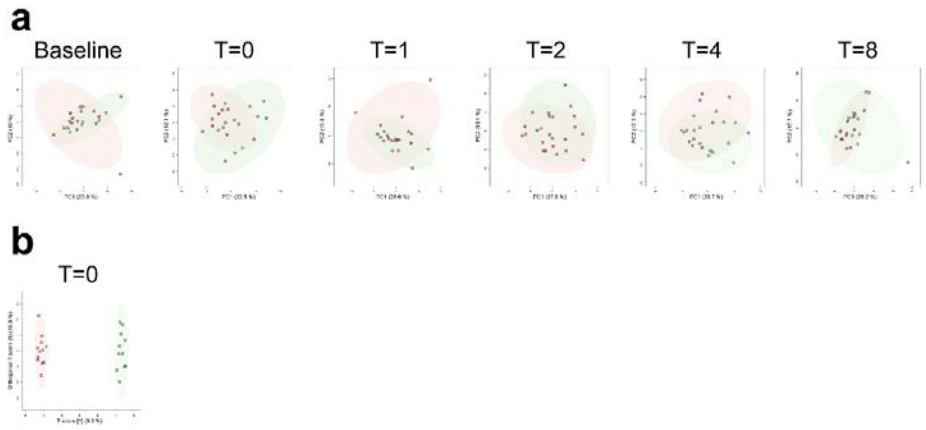

Figure 3. The plasma metabolome in trained and untrained subjects.

Principal component analysis (PCA) of the plasma metabolome at baseline (one hour before LPS administration, T=0 (just before LPS administration), and 1, 2, 4, and 8 hours post-LPS administration in the control (red dots) and trained (green dots) group. The shaded areas indicate the 95% confidence intervals; **(b)** Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the plasma metabolome at T=0 in the control (red dots) and trained (green dots) group. The shaded areas indicate the 95% confidence intervals.

Permutation tests revealed that the model constructed for the comparison between both groups at T=0 hours (when the trained individuals had been practicing the breathing exercises for 30 minutes but LPS had not yet been administered) showed significant separation (1+3 components, R^2X_{cum}: 0.439, R^2Y_{cum}: 0.995, Q^2_{cum}: 0.649; permutation tests: R^2Y: $p = 0.03$, Q^2: $p = 0.001$, score plot depicted in Figure 3b), whereas models constructed at other timepoints did not. Differential analyses revealed a total of 19 significantly altered metabolites between trained and untrained groups across all timepoints (Figure 4).

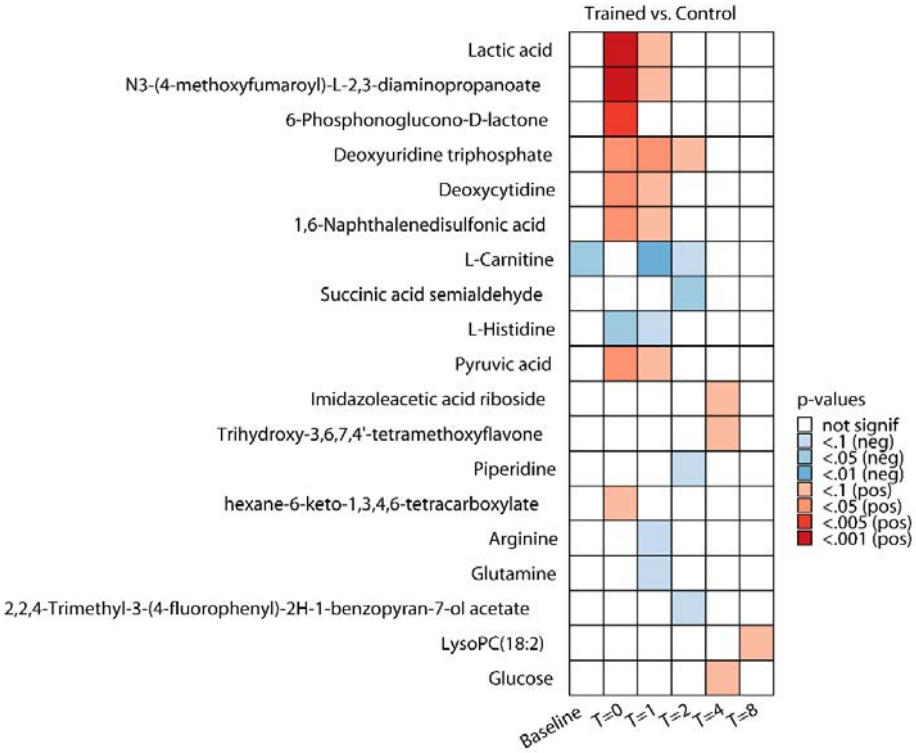

Figure 4. Differentially regulated metabolites in the trained group. Significantly regulated metabolites at baseline (one hour before LPS administration), T=0 (immediately prior to LPS administration), and 1, 2, 4, and 8 hours following LPS administration in the training group compared with the control group. Blue color indicates a decrease compared with the control group; red color indicates an increase compared with the control group. p-values were calculated using unpaired t-tests with multiple-testing correction (Benjamini-Hochberg FDR).

At baseline (after the trained subjects had completed the training program but before any endotoxemia-related procedures or breathing exercises were performed), L-carnitine was lower in the trained group. Nevertheless, the vast majority of significant between-group changes were observed at timepoints between T=0 and T=2 hours, during which the trained group practiced the breathing exercises acquired during their training program. A list of all metabolites across all timepoints in both groups is provided in Tables S3 and S4 in the Supplementary Material. Based on the OPLS-DA model that showed significant separation between both groups at T=0, we focused on that particular timepoint, and all 9 significantly altered metabolites (8 up, 1 down) at T=0 are listed in Table 2.

Table 2: Significantly altered metabolites between the trained and control group at T=0 (30 minutes after initiation of the breathing exercises and prior to LPS administration).[1]

Metabolite	Class	Fold change (trained/control)	FDR adjusted p-value [1]
Increased			
N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoate	Unknown	+3.15	0.0007
Lactic acid	Carbohydrate metabolism	+3.00	0.0007
6-Phosphonoglucono-D-lactone	Lactones	+3.19	0.005
Deoxyuridine triphosphate	Nucleosides, nucleotides and derivatives	+4.91	0.006
Deoxycytidine	Nucleosides, nucleotides and derivatives	+9.15	0.007
1,6-Naphthalenedisulfonic acid	Unknown	+2.19	0.007
Pyruvic acid	Alcohols and polyols	+2.07	0.035
Hexane-6-keto-1,3,4,6-tetracarboxylate	Unknown	+3.52	0.083
Decreased			
L-Histidine	Amino acids and derivatives	-2.72	0.026

p-values were calculated using unpaired t-tests with multiple-testing correction (Benjamini-Hochberg FDR).

Lactic acid (lactate) was one of the most significantly increased metabolites in the trained group. We previously measured this metabolite at the bedside using a point-of-care blood analyzer (i-STAT) [1], which provided us with an opportunity to validate the LC-MS analysis for this metabolite. Lactate concentration measurements were virtually identical between the two methods in both groups (Figure S2A-B in the Supplementary Material). Furthermore, there was excellent correlation between both methods, with only a single outlier sample observed (r values of 0.90 and 0.97 with and without the outlier sample in the analysis, respectively, Figure S2C in the Supplementary Material). Two other metabolites that were increased (deoxyuridine triphosphate and deoxycytidine) belong to the nucleosides, nucleotides and derivates class, while 6-phosphonoglucono-D-lactone is an intermediate in the pentose phosphate pathway, and pyruvic acid (pyruvate) is a well-known metabolite of the alcohols and polyols class. The only decreased metabolite was L-histidine, an amino acid used in the biosynthesis of proteins. MSEA enrichment analysis revealed that the most significantly affected pathways included ammonia recycling, pyruvate metabolism, the Warburg effect, pyrimidine metabolism, gluconeogenesis, glutamate metabolism, and amino sugar metabolism (Figure 5).

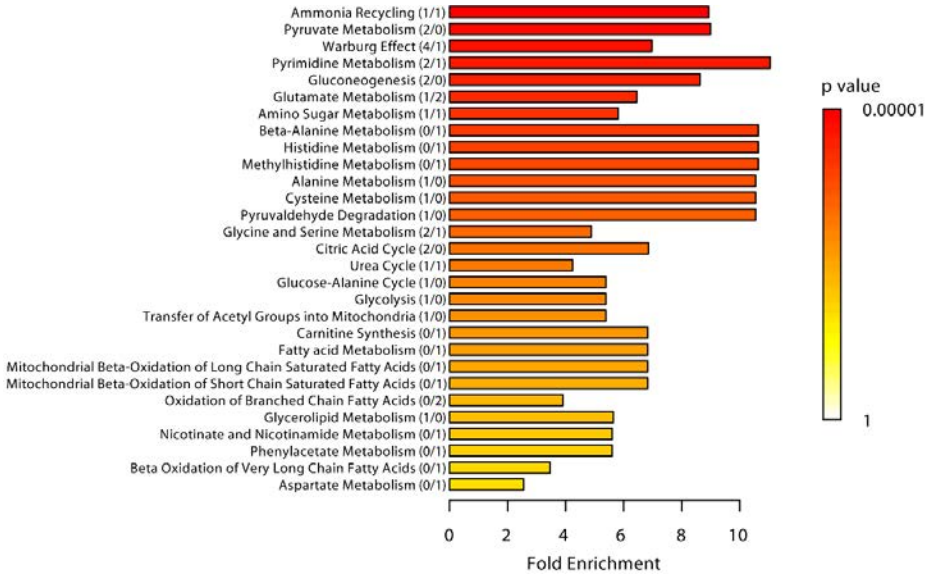

Figure 5. Enriched pathways in the trained group. Significantly enriched pathways at T=0 in the trained group compared with the control group. Threshold for significance was set at a Benjamini-Hochberg FDR-adjusted p-value of less than 0.1. Number of significantly increased/decreased metabolites within each pathway are indicated in parentheses.

Relationship between differentially regulated metabolites and inflammatory response mediators

We evaluated whether in trained individuals practicing the breathing exercises, plasma concentrations of the significantly altered metabolites listed in Table 2 at early timepoints correlated with *in vivo* cytokine responses, which were profoundly modulated in these subjects (i.e. strongly increased plasma levels of the anti-inflammatory cytokine IL-10 and attenuated concentrations of pro-inflammatory cytokines TNFα, IL-6, and IL-8, see Table S2 in the Supplementary Material and [1]). At T=0, only one significant correlation was found, between L-histidine and IL-8 (r=0.62, p=0.048). At T=1, significant correlations between several metabolites and plasma levels of IL-10, which were enhanced by threefold in trained subjects compared with the control group (Table S2 in the Supplementary Material and [1]), were found. These include 1,6-naphthalenedisulfonic acid (r=0.76, p=0.02), deoxyuridine triphosphate (r=0.78, p=0.03), and deoxycytidine (r=0.74, p=0.03). Interestingly, also lactate and pyruvate levels at T=1 correlated with the AUC IL-10 response (r=0.60, p=0.04 and r=0.66, p=0.03, respectively, Figure 6a-b).

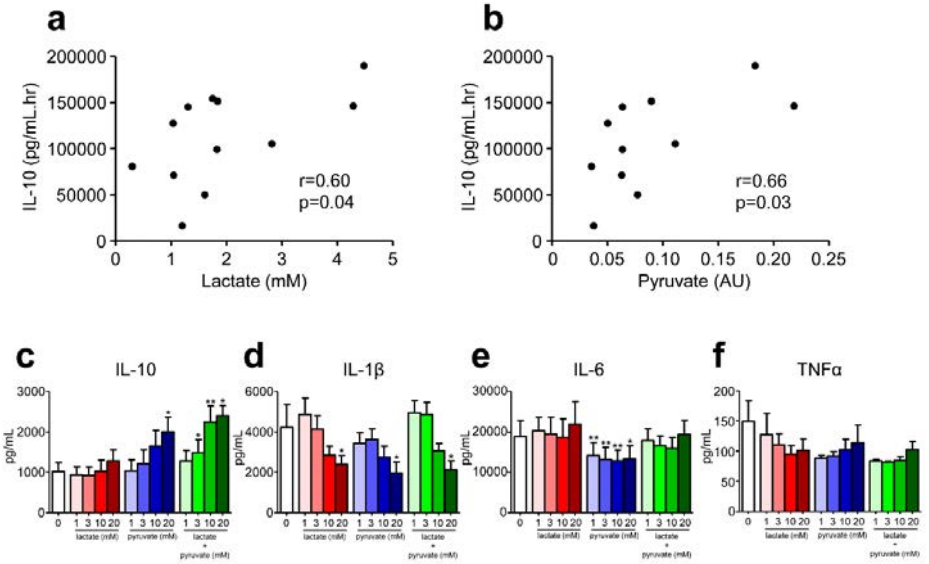

Figure 6. Role of lactate and pyruvate in IL-10 induction. (a-b) Relationship between plasma lactate (a) and pyruvate (b) levels at T=1 and the plasma IL-10 response (expressed as area under the time-concentration curve [AUC]). **(c-f)** IL-10, IL-1β, IL-6 and TNFα production by LPS-stimulated PBMCs incubated with medium alone (white bar) or different concentrations of lactate (red bars), pyruvate (blue bars) or a combination of the two (green bars). Spearman correlation was used to calculate r and p-values in panels a-b. Data in panels c-f are depicted as mean ± SEM of 6 (panels C-E) or 3 (panel F) donors.* p<0.05, ** p<0.01 vs. medium control (paired t-tests).

Furthermore, these two metabolites were highly intercorrelated (r=0.94, p<0.0001), but were not related to the profoundly increased epinephrine levels observed in trained subjects practicing the breathing exercises at any of the measured timepoints (p-values >0.15), which were previously shown to be an important driver of enhanced IL-10 production [1]. Because both lactate and pyruvate have been implicated to exert anti-inflammatory effects [10,11], we set out to validate the observed associations using *in vitro* experiments with PBMCs obtained from healthy individuals. High concentrations of pyruvate, but not lactate, enhanced LPS-induced IL-10 production, and the combination of both metabolites resulted in an even more pronounced and statistically robust increase (Figure 6c). Furthermore, pyruvate attenuated production of the pro-inflammatory cytokines IL-1β and IL-6 (Figure 6d-e), whereas high concentrations of lactate as well as the combination of both metabolites mitigated IL-1β production (Figure 6d). LPS-induced TNFα production was rather low; it was detectable in cell culture supernatants of only three out of the six healthy donors (data of these subjects are shown in Figure 6F). This is likely due to the fact that the 48-hour incubation time is too prolonged to

reliably measure TNFα responses, as levels in supernatants were previously shown to decline as early as 6 hours following LPS stimulation [12]. Nevertheless, lactate, pyruvate, and the two metabolites combined also tended to attenuate LPS-induced TNFα production, although significance was not reached, probably due to the low remaining sample size.

Discussion

In the present work, we demonstrate that the systemic inflammatory response induced by LPS in healthy volunteers significantly alters the plasma metabolome, with the most profound changes taking place 4 hours following LPS administration. Endotoxemia mainly led to an increase of several nucleosides, nucleotides and derivatives, and a decrease in many glycerophospholipids. Before LPS administration, trained subjects practicing the learned breathing exercises exhibited higher levels of lactate and pyruvate compared with a control group who did not practice any exercise, and concentrations of these metabolites correlated with the profoundly enhanced levels of the anti-inflammatory cytokine IL-10 observed in trained individuals following LPS administration. We subsequently validated these findings *in vitro*, by showing that co-incubation with lactate and pyruvate enhances LPS-induced IL-10 release and attenuates pro-inflammatory cytokine production by primary human leukocytes.

Two of the most profoundly enhanced metabolites after the LPS challenge were deoxyuridine triphosphate and especially deoxycytidine, both involved in the pyrimidine metabolism pathway which was enriched following LPS administration. Increased plasma deoxycytidine levels result from DNA degradation [13]. We have previously shown that plasma levels of both nuclear and mitochondrial DNA show a transient increase during endotoxemia, reaching their maximum levels 3 hours after LPS administration, after which they gradually return to baseline in the following hours [14]. Hence, it might be speculated that the breakdown of plasma DNA is one of the main drivers of the increased deoxycytidine concentrations observed 4 hours after LPS administration. Many metabolites belonging to the class of glycerophospholipids were distinctly decreased following administration of LPS. Related to this, various pathways related to lipid metabolism were enriched, which is in accordance with previous findings obtained in healthy volunteers undergoing experimental endotoxemia [15]. Glycerophospholipids are the main components of cell membranes and function as precursors to signaling molecules involved in many cellular and physiological processes [16]. The majority of the decreased metabolites

belonging to this class consisted of phosphatidylcholines. Previous work from our group has shown that plasma levels of secretory phospholipase A2 ($sPLA_2$), the principal catalysts of glycerophospholipid hydrolysis, greatly increase during endotoxemia [17], which may be an explanation for the reduced plasma glycerophospholipid concentrations. The Warburg effect is another pathway of interest that was enriched during systemic inflammation, with four significantly increased (6-phosphonoglucono-D-lactone, glucose, lactate, pyruvate) and one significantly decreased (L-glutamine) metabolite. This process entails the shift from oxidative phosphorylation (OXPHOS) as the primary energy source towards aerobic glycolysis and was recently shown to play a critical role in mounting (LPS-induced) immune responses, by, among other things, facilitating rapid production of inflammatory cytokines [18-20].

Following the training program, trained subjects exhibited lower plasma levels of L-carnitine compared with untrained controls. This relative depletion of carnitine from plasma may indicate an overall increment in Acyl-shuttling mechanism in mitochondria by CPT1/CPT2 and might be related with lipid beta-oxidation intensification [21]. The most pronounced changes in the plasma metabolome of trained subjects compared with controls were however observed during the period that the breathing exercises were carried out. This was not unexpected given the major changes in cardiorespiratory parameters, markers of autonomic nervous system activity, and inflammatory molecules and symptoms observed during this period, as reported elsewhere [1]. At T=0, when the trained individuals had been practicing the breathing exercises for 30 minutes but just prior to LPS administration, the Warburg effect was one of the top enriched pathways in trained individuals compared with controls. Plasma levels of four metabolites (lactate, 6-phosphonoglucono-D-lactone, pyruvate, and isocitric acid) of this pathway were increased in the trained group, whereas concentrations of L-glutamine were lower. This finding may be counterintuitive, as the Warburg effect is mainly associated with pro-inflammatory effects, whereas trained subjects exhibited a distinct anti-inflammatory phenotype [1]. The fact that LPS, a strong inducer of the Warburg effect [20], had not yet been administered at this early timepoint renders this finding biologically implausible as well. Finally, as alluded to before, the Warburg effect encompasses a shift from oxidative phosphorylation (and thus usage of the citric acid cycle) towards glycolysis, but the citric acid cycle pathway was also enhanced in the trained group. Therefore, we hypothesize that the observed effect is not due to a true Warburg effect (*aerobic* glycolysis), but rather driven by the profoundly increased lactate levels in this group as a result of classical *anaerobic* glycolysis in the muscles. The latter may have been caused by the combination of

vigorous breathing (including repeated muscle tightening), intermittent hypoxia, and epinephrine-induced vasoconstriction in this group [1]. The breathing exercises could certainly be regarded as exercise, given that, similar to exercise, they resulted in profuse sweating and exhaustion. The fact that lactate and pyruvate levels were highly intercorrelated provides a strong indication of increased gluconeogenesis (i.e. the generation of glucose from non-carbohydrate substrates such as lactate, with pyruvate as the intermediate metabolite, which mainly takes place in the liver). Gluconeogenesis was also one of the top enhanced pathways in the trained group during practicing of the breathing exercises. Because epinephrine is a well-known strong inducer of gluconeogenesis [22], the profoundly increased plasma epinephrine levels observed shortly after initiation of the breathing exercises [1] likely play a pivotal role in this effect. Combining the findings of enhanced anaerobic glycolysis with increased gluconeogenesis, our data provide evidence of increased activation of the Cori cycle in trained subjects during practicing of the breathing exercises, in which lactate is produced by muscle cells and subsequently converted back, via pyruvate, into glucose in the liver [23]. However, it needs to be acknowledged that we can only infer activation of the Cori cycle, as we did not perform isotype labeling experiments.

Correlation analysis between differentially regulated metabolites and inflammatory cytokine levels in trained subjects revealed that both lactate and pyruvate were related to plasma concentrations of the key anti-inflammatory cytokine IL-10. Of note, production of this cytokine was profoundly accelerated and enhanced in trained subjects practicing the breathing exercises, and its plasma levels were strongly correlated to the subsequent attenuation of pro-inflammatory cytokine responses [1]. Our PBMC stimulation experiments demonstrate that pyruvate alone, but especially the combination of both pyruvate and lactate exert a robust enhancing effect on LPS-induced IL-10 production. These results may substantiate the correlations found during endotoxemia *in vivo*. Furthermore, both lactate and pyruvate individually, as well as the combination of both metabolites attenuate IL-1β production, and lactate mitigated release of IL-6, another pro-inflammatory cytokine. Both lactate and pyruvate have been demonstrated previously to exert anti-inflammatory effects. For instance, very recent work demonstrates that addition of lactate to LPS-stimulated primary human monocytes causes a distinct metabolic shift by decreasing aerobic glycolysis and increasing oxidative phosphorylation, a metabolic state characteristic for anti-inflammatory responses [24]. In the same study, lactate was shown to decrease LPS-induced production of pro-inflammatory cytokines by human PBMCs [24]. The authors proposed that immunomodulatory effects of lactate may serve as a feedback signal to limit excessive inflammatory

responses of highly glycolytic pro-inflammatory immune cells [24]. Pyruvate has been shown to decrease mRNA expression and protein levels of pro-inflammatory cytokines TNFα and IL-6 in LPS-stimulated canine PBMCs, whereas IL-10 expression and production was increased [25]. Furthermore, administration of pyruvate significantly lowered IL-6 and enhanced IL-10 plasma concentrations in LPS-treated rats, leading to prolonged survival, and incubation with ethyl pyruvate blocked activation of NF-κB, a critical pro-inflammatory transcription factor, in LPS-stimulated murine macrophages [26]. These data strengthen the notion that also in the human *in vivo* situation, the observed increases in lactate and pyruvate at least partly account for the immunomodulatory effects observed in trained subjects.

Several limitations and aspects of the present work deserve attention. First, only male volunteers were included. In earlier work, we observed that the endotoxin-induced pro-inflammatory immune response is more pronounced in females than in males [27]. Furthermore, menstrual cycle-induced variation in hormone levels can also impact immune parameters [28,29], thereby further increasing variation. Therefore, inclusion of both sexes yields more interindividual variation and would necessitate larger group sizes. Because endotoxemia studies are very labor-intensive and costly, the choice was made to only include males, which nevertheless limits this study's generalisability. A second limitation, which applies to all plasma metabolomics studies, pertains to the uncertainty of the source of the measured metabolites. Virtually all identified metabolites can be produced by a wide variety of cells in many organs. Especially in immunological studies like this, combining plasma metabolomics with the determination of intracellular metabolites in immune cells (e.g. leukocytes) would represent a more powerful approach. Unfortunately, no samples were stored for this purpose.

Third, the current study does not allow to deduce which (combination) of the three elements of the training program is responsible for the observed effects, and several of our ongoing studies are aimed at elucidating this question. Nevertheless, as alluded to in a previous section, the breathing exercises probably play a pivotal role. Fourth, because metabolomic profiling was performed on samples stored for three years at -80 °C, sample degradation could be an issue. Nevertheless, the high correlation between lactate measured during the experiments using a point-of-care analyzer and by LC-MS indicates that, at least for this metabolite, no significant degradation occurred during storage. Furthermore, because storage time was virtually identical across samples (all were collected within 1 month), the extent of degradation of certain metabolites would have been similar across all samples.

Provided that metabolites did not completely degrade, this would therefore have little impact on the results. Fifth, as the PBMCs stimulation experiments with lactate and pyruvate contained a mixture of different cell types (predominantly monocytes and lymphocytes), we cannot be certain about the cellular origin of the effects observed. However, it is well established that monocytes are the main cytokine producers in short-term whole blood LPS stimulation assays (which next to PBMCs also contain granulocytes) [30]. Furthermore, as discussed earlier on, anti-inflammatory effects of lactate and pyruvate have been reported in primary human monocytes [24] and murine macrophages [26], the latter of which show many similarities to monocytes. Therefore, it is likely that the effects of pyruvate and lactate we observed can predominantly be ascribed to monocytes. Finally and importantly, all training procedures described in this study were conducted in the presence of medical personnel. Because of profound physiological effects of the breathing exercises (e.g. acid-base shifts, intermittent hypoxia) and exposure to cold [1], potential health risks while practicing these elements of the training program should be considered.

In conclusion, the present study extends our previous findings regarding the effect of a training intervention consisting of cold exposure, breathing exercises, and meditation on the LPS-induced immune response in healthy volunteers. Practicing the breathing exercises acquired during the training program results in enhanced activity of the Cori cycle, and next to the previously established relationship between epinephrine and IL-10 induction [1], the current data indicate a role of lactate and pyruvate in the enhanced production of this key anti-inflammatory mediator and in the overall anti-inflammatory phenotype observed in trained subjects.

Acknowledgments

We greatly appreciate the help of Manuela Hische and Michael Heiser for their support with the analysis, Judith Lenk and Monique Thiele for the technical assistance.

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Supplementary Material

Table S1: Subject characteristics.

Parameter	Control group (n = 12)	Trained group (n = 12)	p-value
Age, yrs	22 (19-27)	24 (19-27)	0.43
Height, cm	185 (179-189)	181 (172-190)	0.30
Weight, kg	78 (65-91)	75 (58-92)	0.25
BMI, kg/m^2	23 (20-27)	23 (19-26)	0.98
HR, beats/min	61 (40-75)	60 (41-80)	0.88
MAP, mmHg	94 (78-105)	92 (82-113)	0.89

Parameters were measured during the screening visit (so before start of the training in the trained group). BMI: body mass index; HR: heart rate; MAP: mean arterial blood pressure. Data are presented as median (range). p-values were calculated using Mann-Whitney U-tests.

Table S2: Baseline, peak, and area under curve (AUC) plasma cytokine responses in control and trained groups.

Parameter	Baseline (pg/mL)		Peak (pg/mL)		AUC (x103 pg/mL.h)	
	Control	Trained	Control	Trained	Control	Trained
TNFα	6 (4-8)	5 (3-8)	458 (281-753)	213 (180-553)#	60 (45-94)	28 (21-68)*
IL-6	3 (3-3)	3 (3-3)	520 (319-703)	300 (169-386)*	68 (40-84)	29 (18-49)*
IL-8	6 (4-10)	4 (3-7)	590 (422-677)	368 (266-540)*	95 (58-109)	47 (36-58)*
IL-10	3 (3-5)	3 (3-6)	268 (220-648)	962 (498-1208)*	40 (38-81)	116 (74-190)*

TNF: tumor necrosis factor; IL: interleukin. Data are presented as median (interquartile range) of 12 subjects per group. # p = 0.05 - 0.10 vs. control, * p < 0.05 vs. control (Mann-Whitney U-tests). These data were published previously [1].

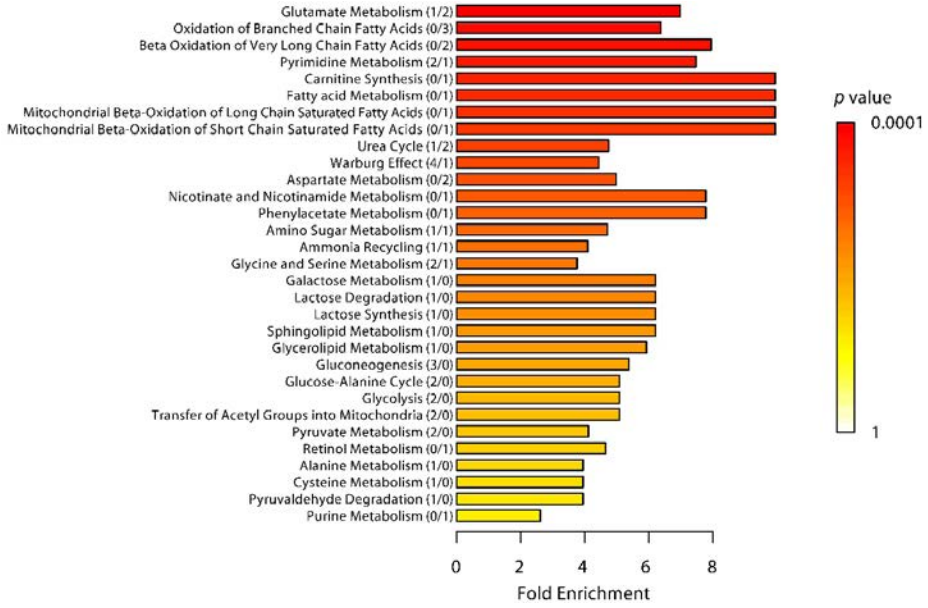

Figure S1. Enriched pathways after LPS administration. Significantly enriched pathways 4 hours after LPS administration compared with baseline (one hour before LPS administration). Threshold for significance was set at a Benjamini-Hochberg false discovery rate (FDR)-adjusted *p*-value of less than 0.1. Number of significantly increased/decreased metabolites within each pathway are indicated in parentheses.

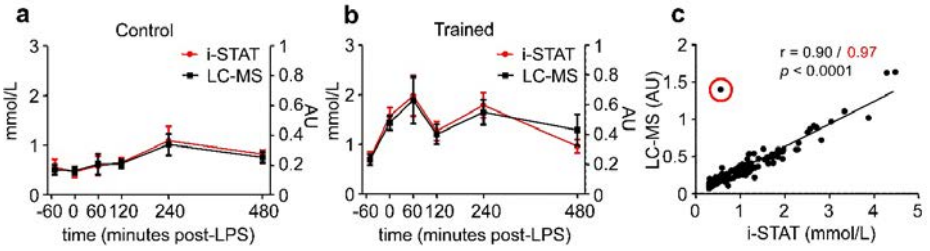

Figure S2. Comparison of plasma lactate concentrations measured by LC-MS and a point-of-care analyzer. (a) Plasma lactate concentrations over time in the control group, measured by a point-of-care blood analyzer (i-STAT) or by liquid chromatography – mass spectrometry (LC-MS); **(b)** Plasma lactate concentrations over time in the trained group, measured by i-STAT or LC-MS; **(c)** Correlation between plasma lactate concentrations measured by i-STAT or LC-MS. r and *p*-values were calculated using Pearson correlation. The r value in red was calculated after exclusion of the outlier indicated by the red circle. The i-STAT data presented in this figure were published previously [1]. Data in panels A-B are depicted as mean ± standard error of the mean (SEM) of 12 subjects per group.

Chapter 6

Modulation of pain sensitivity by a hyperventilatory breathing exercise and cold exposure training

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Abstract

Background: Evidence indicates that healthy individuals who follow a training program comprised of hyperventilatory breathing exercises and cold exposure can voluntarily activate their sympathetic nervous system and attenuate their systemic inflammatory response during experimental endotoxemia (intravenous administration of bacterial endotoxin). Furthermore, trained participants reported less endotoxemia-induced flu-like symptoms. However, it remained to be determined whether the effects on symptoms are due to the mitigated inflammatory response or involve direct analgesic effects of (elements of) the training program.

Methods: In the present study, we used Nijmegen-Aalborg Screening Quantitative sensory testing (NASQ) to objectively map pain sensitivity using non-invasive stimuli to address this question. First, NASQ parameters were evaluated in 20 healthy volunteers before, during, and after the conduct of the hyperventilatory breathing exercise. Second, NASQ measurements were performed before and after 48 healthy volunteers followed different modalities of the training program: breathing exercise training, cold exposure training, the combination of both, or no training. Lastly, NASQ measurements were performed in these 48 subjects during experimental endotoxemia.

Results: Electrical pain detection thresholds increased during the breathing exercise ($p=0.001$) as well as four hours afterwards ($p=0.03$). Furthermore, cold exposure training resulted in lower VAS scores during hand immersion in ice water ($p<0.001$). Systemic inflammation induced by administration of endotoxin nullified the decreased pain perception during the ice water test in subjects trained in cold exposure.

Conclusions: A hyperventilatory breathing exercise decreases pain perception induced by an electrical stimulus. Furthermore, cold exposure training may decrease pain perception induced by hand immersion in ice water.

Introduction

Healthy volunteers who followed a training program comprised of two different breathing exercises, cold exposure training, and meditation are able to activate their sympathetic nervous system voluntarily, reflected by high epinephrine levels [1]. This results in attenuation of the inflammatory response during experimental endotoxemia, a standardized *in vivo* model of systemic inflammation induced by intravenous administration of bacterial endotoxin [1]. A recent detailed analysis of the different components in this training program revealed that the combination of cold exposure with one of the breathing exercises is responsible for these effects [2]. This training program, or elements of it, could translate into a novel treatment modality for patients with inflammatory conditions.

Strikingly, next to lower levels of pro-inflammatory cytokines, the subjective self-reported flu-like symptoms during endotoxemia were attenuated in trained volunteers [1, 2]. This is a significant finding, as subjective and patient-reported outcome measures (PROM) are important end-points in clinical studies into inflammatory diseases such as rheumatoid arthritis [3] and inflammatory bowel disease (IBD) [4]. However, it is currently unclear whether the effects on symptoms observed in our previous studies [1, 2] are either a consequence of the mitigated inflammatory response, or involve direct analgesic effects caused by the training program. Moreover, objectifying signs and symptoms of people (either patients or healthy volunteers) that experience pain and discomfort has proved to be as difficult as it is relevant [5]. Complaints of pain are inherently subjective, thus standardizing the manner in which the perception of pain is objectified is of pivotal importance [6].

Different efforts to protocolize and standardize pain measurements are reported under the general term `Quantitative Sensory Testing` (QST). The Nijmegen-Aalborg Screening QST (NASQ) was developed to map pain sensitivity at multiple body locations by non-invasive stimuli [7, 8]. In recent years, this NASQ battery has been optimized and calibrated for use in clinical practice as well as in research, for instance in patients with head and neck pain [9]; breast cancer [10], neuropathic pain [11], and in healthy volunteers [12].

In the present study, we investigated whether different elements of the aforementioned training program alter pain perception assessed by NASQ measurements. We hypothesized that elements of the training program decrease pain perception.

Methods

This manuscript describes NASQ measurements performed during two studies recently reported on [2]. These studies primarily focused on the contribution of the different components of the aforementioned training program on circulating epinephrine levels and inflammatory parameters [2]. In the first study (breathing exercises study), NASQ measurements were performed before, during, and after the conduct of one of the breathing exercises described further below (and in [2]). In the second study (experimental human endotoxemia study), NASQ parameters were evaluated before and after participants followed a training program involving both a breathing exercise and exposure to cold in four different modalities: participants were randomized to be trained in the breathing exercise, only in cold exposure, the combination of both, or to a control group that did not receive any training (see below and [2]). Furthermore, in the context of this latter study, NASQ parameters were evaluated during experimental endotoxemia, in which all participants of the four training groups took part (see below and [2]). Previous data from our group revealed that the systemic inflammatory response induced in this model results in decreased pain thresholds [13], and we explored whether the different training modalities influenced this effect.

Ethics approval

All procedures were approved by the local ethics committee of the Radboud university medical center (CMO Arnhem-Nijmegen, reference and trial registration 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 participants 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 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 start of the training program, surgery or trauma with significant blood loss or blood donation, hospital admission or surgery with general anesthesia, participation in another study within three months prior to the experimental day, or clinically significant acute illness and/or infections within four weeks before the start of the training program.

Breathing exercises study

After ethics approval (reference number: 2014-1374/NL51237.091.14), 40 males provided written informed consent to participate in a prospective randomized study

registered at https://clinicaltrials.gov/ (NCT02417155). The study was carried out in the research department of the Radboud university medical center from December of 2014 to February of 2015. An extensive description of the methods is described elsewhere [2]. A schematic overview of the study procedures is depicted in Figure 1.

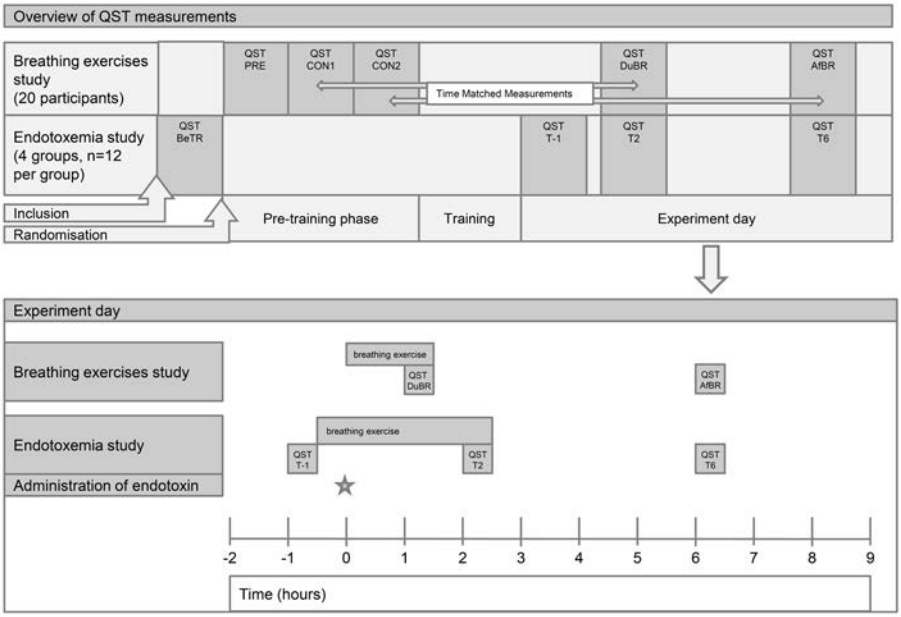

Figure 1. Overview of study procedures.

PRE – NASQ measurements immediately after inclusion, before training procedures.

CON1 – The first NASQ measurement on the control day, time-matched (around 10am) with the first NASQ measurement on the experiment day (DuBR).

CON2 – The second NASQ measurement on the control day, time-matched (around 3pm) with the second NASQ measurement on the experiment day (AfBR)

DuBR – The first NASQ measurement on the experiment day, time-matched with CON1, taken during conduct of the breathing exercise.

AfBR – The second NASQ measurement on the experiment day, time-matched with CON2, taken 4 hours after cessation of the breathing exercise.

BeTR – The baseline NASQ measurement in the endotoxemia study, taken immediately after inclusion and before randomization to the training groups.

T-1 – The first NASQ measurement on the endotoxemia experiment day, taken 1 hour before administration of LPS.

T2 – The second NASQ measurement on the endotoxemia experiment day, taken 2 hours after administration of LPS, during conduct of the breathing exercise in the groups trained in this exercise (BRT and CBR groups).

T6 – The third NASQ measurement on the endotoxemia experiment day, taken 6 hours after administration of LPS, 3.5 hours after cessation of the breathing exercise in the groups trained in this exercise (BRT and CBR groups).

During the informed consent procedure, all participants were verbally familiarized with the different training procedures that were part of the current study and subsequently gave consent to participate in any of the four groups ahead of randomization. Participants were randomized to four different groups (n=10 per group) by an independent research nurse using the sealed envelope method: extensive training by the creator of the intervention, extensive training by an independent trainer, short training by the creator of the intervention, and short training by an independent trainer. All participants were trained in the week before the experiment day. NASQ assessments described in the current manuscript were performed in a subset of 20 volunteers who were trained in a breathing exercise which consisted of hyperventilation for an average of 30 breaths using deep and powerful breaths ("hyperventilation phase") followed by exhalation breath holding for approximately two minutes ("retention phase"). The duration of breath retention was entirely at the discretion of the participant. Breath retention was followed by a deep inhalation breath, that was held for 10 seconds. Subsequently a new cycle of hyper/hypoventilation began.

A total of five NASQ assessments were performed. The first NASQ assessment was performed after inclusion and randomization but before the start of the training program ($NASQ_{PRE}$, Figure 1). Furthermore, on a separate day, also after randomization but before start of the training program, two NASQ assessments were performed which were time-matched to those performed on the experiment day (Figure 1). The first NASQ assessment on the control day ($NASQ_{CON1}$) was time-matched with the first NASQ assessment on the experiment day ($NASQ_{DuBR}$, at approximately 10 am), which was performed during execution of the breathing exercise. The second NASQ assessment on the control day ($NASQ_{CON2}$) was time-matched with the second NASQ assessment on the experiment day ($NASQ_{AfBR}$, at approximately 3 pm), which was performed 4 hours after cessation of the breathing exercise. On the experiment day, participants practiced the breathing technique for 1.5 hours and NASQ assessment was started one hour following start of the breathing exercise and lasted 30 minutes. Due to technical issues, the NASQ assessment could not be analyzed in one participant of the breathing exercises studies. Data of all groups were combined, resulting in a total of 19 participants for the breathing exercises study. We analyzed these data together, as the increase in epinephrine levels in the breathing exercises study was similar in all four groups [2]. Also, no between-group differences were observed in any of the NASQ parameters (data not shown).

Experimental human endotoxemia study

After ethics approval (reference number 2016-2312/NL56686.091.16), 48 males provided written informed consent to participate in this prospective randomized controlled study registered at https://clinicaltrials.gov/ (NCT03240497). The study was carried out in the research department of the Radboud university medical center from April to June of 2016. An extensive description of the methods is described elsewhere [2]. A schematic overview of the study is depicted in Figure 1. During the informed consent procedure, all participants were verbally familiarized with the different training procedures that were part of the current study and subsequently gave consent to participate in any of the four groups ahead of randomization. We employed a 2 by 2 design, in which 48 participants were randomized by an independent research nurse using the sealed envelope method to 4 different groups (n=12 per group): cold exposure (CEX), breathing exercise (BRT), cold exposure and the breathing exercise (CBR), and a control group (CG). Participants of all groups except the control group were trained in the week leading up to the endotoxemia experiment day (further detailed in section `endotoxemia procedures` below). Briefly, the participants in the CEX group followed an intensive 4-day cold exposure training program, 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 (see video material accompanying our previous publication [2]). Furthermore, participants were instructed to end their daily shower with a period of 60 seconds of cold water until the endotoxemia experiment day. Participants in the BRT group were trained in the breathing exercise as described in the previous subsection, but without the prolonged breath retention phase. Instead, participants held their breath for only 10 seconds, during which all body muscles were tightened, after which a new cycle of hyperventilation was initiated. We used this exercise as we showed that it is equally effective in increasing plasma epinephrine levels (the main driver of the anti-inflammatory effects) as the exercise with prolonged retention [2], is easier to learn, and potentially safer. Participants randomized to the CBR group followed both cold and breathing exercise training procedures and participants in the control group did not receive any training.

In total, four NASQ assessments were performed during this study. One NASQ assessment after inclusion but before randomization and before training (BeTR). On the endotoxemia experiment day, the first NASQ assessment was performed one hour before endotoxin administration (T-1). The second NASQ assessment was timed two hours after endotoxin administration (T2), this was during execution of the breathing exercise. The third NASQ assessment was timed six hours after administration of endotoxin (T6), 3.5 hours after cessation of the breathing exercise.

All participants, regardless of the randomization, underwent experimental endotoxemia at the research unit of the Intensive Care department of the Radboud university medical center according to our standard protocol [14] also used in our previous studies into this intervention [1, 2]. Participants refrained from caffeine and alcohol 24 hours before the experiment, and refrained from any intake of food and drinks 10 hours before the experiment. Fasting was maintained until 4.5 hours after administration of endotoxin. 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 anesthesia using a 20-gauge arterial catheter for continuous arterial monitoring of vital signs. Purified endotoxin (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 body weight.

Nijmegen-Aalborg Screening Quantitative Sensory Testing measurements

The measurements of pressure pain threshold (PPT), electrical pain detection threshold (EPDT), electrical pain tolerance threshold (EPTT) and conditioned pain modulation (CPM) test are extensively described [7, 8] and visualized [11]. All measurements were conducted in a stimulus-poor room in our university hospital with a constant temperature (20.5-22°C) and humidity (with a set-point of 6 g/Kg that results in a relative humidity of 45-55%). Measurements were performed by two researchers (HT and JvG), both extensively trained in NASQ measurements. The musculus deltoideus was marked as the training site to help the participants to get used to the assessments via pressure algometry as well as electrical threshold assessment. The following areas were marked bilaterally as test sites: the musculus rectus femoris (15 cm above the patellar ridge), the musculus trapezius (pars medialis, level Th3), the thenar muscle, the musculus abductor hallucis. A description of the specific test sites used for each measurement are provided below.

PPT

Pressure pain threshold (PPT) was measured on both the left and the right side of the body at the m. deltoideus, m. rectus femoris, thenar and m. abductor hallucis. The measurements were first performed on the training site (m.deltoideus), and secondly on the study sites (directly on the specific muscle). Pressure was manually delivered with the pressure algometer (Wagner instruments, Force TEN™ Digital Force Gage FDX 50, Greenwich, CT, USA) with a 1.0 cm^2 probe under a 90° angle.

A ramping rate of ~5 Newton(N)/s was used by manually adjusting the applied pressure based on visual feedback using the display of the pressure algometer. Pressure was started at 0 N and applied up to a maximum of 250 N for safety purposes. The participants were instructed to say "stop" when they felt a burning, painful or stitching sensation alongside the feeling of pressure. The participants were asked to rate the associated pain on a VAS scale using a 10 cm line printed on A4 paper. Test pressure values and VAS scores were noted on a sheet while making sure the participants could not read the values of the measurements during the execution of the tests. The PPT measured with a pressure algometer showed a good test–retest (r=0.88) and interobserver reliability (r=0.84) [15]. In healthy individuals, the ICC values showed excellent reliability (ICC=0.74) on the thenar [16]. In another study including healthy volunteers, intra-rater reliability was shown to be excellent (ICC > 0.9) [17]. In a recently conducted systematic review test-retest ICC for the VAS scale was 0.77-0.90 [18].

EPDT

Electrical pain detection threshold (EPDT) was measured on the m. rectus femoris and m. trapezius test sites. A QST stimulator (QST-III; JNI Biomedical ApS, Klarup Denmark) was used to obtain the electrical pain detection threshold. The QST stimulator delivers tetanic stimulation at 100 Hz with 0.2 ms square waves. The ramping rate was set to 1 mA/s. The initial current was set to 0 mA, the maximum current was automatically set to 50 mA for safety purposes. The participants were instructed to press the power button to start the flow of current and to release the button at the moment the sensation started to be painful and annoying. At each site, three measurements were taken, allowing at least 15 seconds in between measurements to avoid windup effects. Electrical values and VAS scores were noted on a sheet while making sure the participants could not read the values of the measurements during the execution of the tests. The mean value for each EPDT test location was calculated. Data on the reliability of EPDT testing in healthy volunteers are, to our knowledge, not available. In patients with painful chronic pancreatitis, the test-reliability was poor in pancreatic viscerotomes (ICC 0.15-0.43) [19].

EPTT

Electrical pain tolerance threshold (EPTT) was measured on the m. rectus femoris of the non-dominant leg. The same QST stimulator was used as described for the EPDT measurement above. The participants were instructed to press the power button to start the flow of current and release the button at the moment the feeling was the maximum tolerable pain. Again, electrical values and VAS scores were noted on a sheet while making sure the participants could not read the values of the

measurements during the execution of the tests. In patients with painful chronic pancreatitis the ICC was shown to be fair: 0.48-0.49 [19].

CPM (`Ice water test`)

Conditioned pain modulation (CPM) was measured using a bucket of water with melting ice with a target temperature range of one to four degrees Celsius. Throughout the experiment, a temperature probe was used to check if the water was at the intended target temperature. Ice was added if necessary. The PPT and the EPTT measurements described above were used as a preconditioning test stimulus. The participants were asked to immerse one hand into the ice water in the bucket until the wrist and with the fingers spread without touching the wall or bottom of the bucket. The participants were told to remove their hand from the water after three minutes of immersion or sooner if the pain became intolerable. During the immersion, the participants were asked to rate the pain on a 0 to 100 scale on a 10 cm line printed on A4 paper every 10 seconds, in which 0 represents no pain and 100 unbearable pain. Finally, the PPT and EPTT measurement were performed again directly after taking the hand out of the ice water bucket to determine test stimulus post-conditioning. CPM was calculated by using the difference of the pre- and postconditioning measurements as a proportion of the preconditioning measurement expressed as a percentage (post-conditioning minus preconditioning / preconditioning)*100%. Using the combination of the PPT handheld algometer and ice water stimuli to assess the CPM, is one of the most reliable methods described, with a modest test–retest reliability (ICC = 0.49; coefficient of variation = 63.6%) [20].

Statistical analysis

As this manuscript describes secondary endpoints of two studies that primarily focused on the contribution of the different components of the aforementioned training program on circulating epinephrine levels and inflammatory parameters [2], the study was not formally powered for the NASQ endpoints.

For PPT and EPDT data, the median value of pain pressure threshold in Newton or electrical stimulus threshold in milliampere (mA) was calculated from the values obtained at each measurement site for each participant. During all ice water tests in both studies, the majority of participants reached the maximum time of 180 seconds immersion in ice water, rendering comparisons between average times of little value. As such, the proportions of participants reaching the maximum time were compared. For ice water VAS, the highest levels of pain reported by each individual participant during the ice water test was used for analysis. Group data are presented as median [interquartile range], means ± standard error of the mean

(SEM) or number (%). Differences were analyzed using Fisher exact tests, paired t-tests, repeated measures one-way analysis of variance (ANOVA) followed by Tukey post-hoc tests, or Kruskal-Wallis tests. Pearson correlation was used. Bonferroni correction was applied to adjust for multiple testing. Analyses were performed using Graphpad Prism V5.03 (Graphpad Software, San Diego, CA, USA) and SPSS V25.0.0.1 (IBM Corp, Armonk, New York, USA).

Results

Participant characteristics

Baseline characteristics of participants who were included in the breathing exercises and endotoxemia studies are listed in Table 1. Flow diagrams of both studies are provided in Supplemental Figures 1 and 2.

Due to technical issues, the NASQ assessment could not be analyzed in one participant of the breathing exercises study. Data of all randomization groups in the breathing exercise study were combined, resulting in a total of 19 participants. We analyzed data of all groups together, as the increase in epinephrine levels in the breathing exercises study was similar in all four groups [2]. Also, no between-group differences were observed in any of the NASQ parameters (data not shown).

Learning effects

Learning effects were assessed in the breathing exercise study by comparing $NASQ_{PRE}$ with $NASQ_{CON1}$ (Figure 1). No significant differences between these measurements for any of the NASQ parameters were present (Table 2).

Acute and prolonged effects of the breathing exercise on NASQ parameters

The effects of acute and prolonged effects of the breathing exercise on pain perception in the absence of systemic inflammation were evaluated in the breathing exercise study by comparing $NASQ_{CON1}$ with $NASQ_{DuBR}$ and $NASQ_{CON2}$ with $NASQ_{AfBR}$, respectively (Figure 1).

For pressure pain thresholds (PPT), no differences were observed (Figure 2A). However, for electrical pain detection thresholds (EPDT), a significant increase in EPDT was observed both between $NASQ_{CON1}$ (14.6±1.9 mA) and $NASQ_{DuBR}$ (19.0±2.2 mA, p=0.001, Figure 2B), and between $NASQ_{CON2}$ (15.2±2.0 mA) and $NASQ_{AfBR}$ (17.5±2.2 mA, p=0.03, Figure 2B).

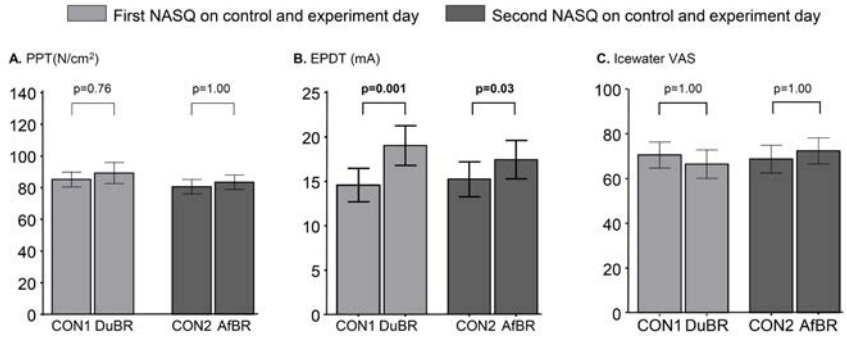

Figure 2. Acute and prolonged effects of the breathing exercise on pain perception.

A. Pressure pain threshold (PPT) **B.** Electrical pain detection thresholds (EPDT) **C.** peak VAS score during the icewater test. Data are presented as mean ± SEM of 19 participants per group. P-values were calculated using paired t-tests. CON1: The first NASQ measurement on the control day, time-matched (around 10am) with the first NASQ measurement on the experiment day (DuBR). CON2: The second NASQ measurement on the control day, time-matched (around 3pm) with the second NASQ measurement on the experiment day (AfBR). DuBR: The first NASQ measurement on the experiment day, time-matched with CON1, taken during conduct of the breathing exercise. AfBR: The second NASQ measurement on the experiment day, time-matched with CON2, taken 4 hours after cessation of the breathing exercise.

To explore possible mechanisms behind this effect, we correlated the sharp increases in plasma epinephrine levels and pH observed during the breathing exercises [2] with the increases in EPDT. No such relationships were identified (data not shown). For the ice water test, no significant differences were found in the proportion of participants that reached the maximum time of 180 seconds neither during the breathing exercise ($NASQ_{con1}$ 84% vs $NASQ_{DuBR}$ 74%, p=1.00), nor after the breathing exercise ($NASQ_{con2}$ 89% vs $NASQ_{AfBR}$ 79%, p=1.00). The peak pain score (VAS) during the ice water test was also similar between the different measurements (Figure 2C). Finally, conditioned pain modulation (CPM) parameters showed no change between the measurements, both for pressure and electrical stimulation (data not shown).

Influence of the different training regimens employed in the human endotoxemia study on NASQ parameters

The influence of the different training modalities used in the endotoxemia study (ie cold exposure [CEX], breathing exercise [BRT], cold exposure and breathing exercise [CBR], and the control group [CG]) on NASQ parameters were evaluated. $NASQ_{BeTR}$ and $NASQ_{T-1}$ measurements were compared, reflecting changes in pain perception caused by the training program, before induction of human endotoxemia (Figure 1).

For PPT, significantly lower PPT values post-training were observed in the CEX group (118.6±6.7 N/cm^2 vs. 94.8±6.8 N/cm^2, p=0.01), whereas no significant differences were found in the other groups (Figure 3A). For EPDT, no differences were observed in any of the groups (Figure 3B). Likewise, for the ice water test, no significant differences were found in the proportion of participants who reached the maximum time (CG: 33.3% vs. 16.7%, p=1.00; CEX: 66.7% vs. 81.8%, p=1.00; BRT: 41.7% vs. 50.0%, p=1.00; CBR: 58.3% vs. 83.3%, p=0.71). No effects of the training were observed for the highest reported pain score (VAS) during the ice water test in the control group (76.7±4.7 vs. 77.2±4.1, p=1.00) or the BRT group (72.0±7.5 vs 70.6±8.3, p=1.00, Figure 3C). However, significantly lower VAS scores were reported in both cold exposure groups after training (CEX group: 78.9±6.8 vs. 61.0±5.8, p=0.04; CBR group: 79.6±5.6 vs 59.8±5.7, p<0.001, Figure 3C). CPM parameters again showed no change between the measurements, both for pressure and electrical stimulation (data not shown).

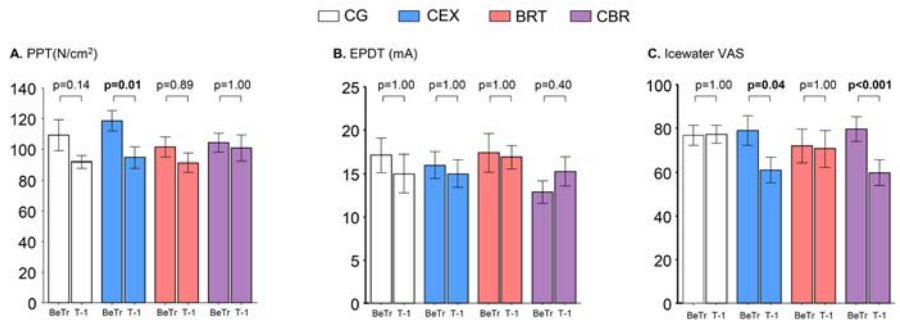

Figure 3. Influence of the different training regimens employed in the human endotoxemia study on pain perception. A. Pressure pain threshold (PPT) **B.** Electrical pain detection thresholds (EPDT) **C.** peak VAS score during the icewater test. Data are presented as mean ± SEM of 12 participants per group. P-values were calculated using paired t-tests. Bonferroni correction was applied to adjust for multiple testing. CG: untrained control group. CEX: cold exposure training group. BRT: breathing exercises training group. CBR: combined cold exposure and breathing exercises training group. BeTR: The baseline NASQ measurement in the endotoxemia study, taken immediately after inclusion and before randomization to the training groups. T-1: The first NASQ measurement on the endotoxemia experiment day, taken 1 hour before administration of LPS.

NASQ parameters during experimental endotoxemia in the different training groups

To assess the effects of endotoxemia on pain perception as well as the possible modulating effects of the different training modalities and, for the BRT and CBR groups, performing the learned breathing exercise, $NASQ_{T-1}$, $NASQ_{T2}$ and $NASQ_{T6}$ measurements were compared within the different groups (Figure 1).

In all groups, statistically significant lower values of PPT were found during human endotoxemia (CG p<0.001, CEX p=0.03, BRT p<0.001, CBR p<0.001, Figure 4A). Post-hoc testing revealed significant differences in all groups at time-points 2 and 6 hours (Figure 4A). For EPDT, no statistically significant changes were observed over time in the CG (p=0.52) or BRT (p=0.32) groups. However, significant changes were found in the CEX (p=0.04, post-hoc: increase at 6 hours post-LPS) as well as the CBR group (p=0.004, post-hoc: decrease at 2 hours post-LPS, Figure 4B).

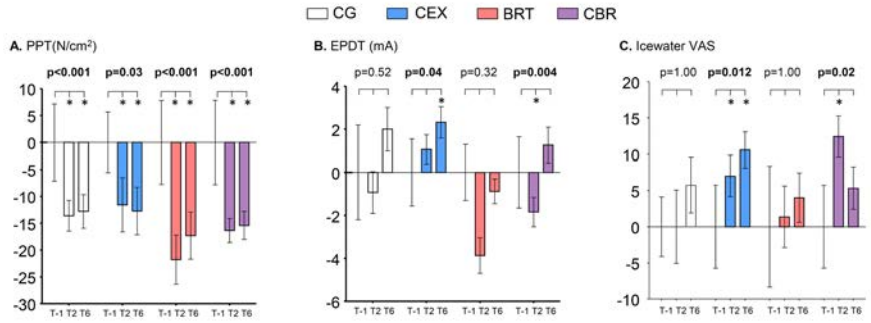

Figure 4. Influence of the different training regimens on pain perception during human endotoxemia. A. Pressure pain threshold (PPT) **B.** Electrical pain detection thresholds (EPDT) **C.** peak VAS score during the icewater test. Data are presented as mean ± SEM of 12 participants per group. All values are normalized to timepoint T-1 (which was set at 0). P-values represent within-group effects over time calculated using repeated measures one-way analysis of variance (ANOVA). *indicates p<0.05 vs. T-1 calculated using Tukey's post-hoc tests. Bonferroni correction was applied to adjust for multiple testing. CG: untrained control group. CEX: cold exposure training group. BRT: breathing exercises training group. CBR: combined cold exposure and breathing exercises training group. T-1: The first NASQ measurement on the endotoxemia experiment day, taken 1 hour before administration of LPS. T2: The second NASQ measurement on the endotoxemia experiment day, taken 2 hours after administration of LPS, during conduct of the breathing exercise in the groups trained in this exercise (BRT and CBR groups). T6: The third NASQ measurement on the endotoxemia experiment day, taken 6 hours after administration of LPS, 3.5 hours after cessation of the breathing exercise in the groups trained in this exercise (BRT and CBR groups).

For the ice water test, no differences in the percentage of participants that reached the maximum time of 180 seconds were observed in all groups across timepoints T-1, T2 and T6 (CG: 16.7%, 0%, 8.3%, p=0.34; CEX: 75.0%, 58.3%, 75.0%, p=0.59; BRT 50.0%, 25.0%, 50.0%, p=0.36; CBR: 83.3%, 66.7%, 83.3%, p=0.53). Across all timepoints, 8.3%, 69.4%, 41.7%, and 77.8% of participants reached 180 seconds in the CG, CEX, BRT, and CBR group, respectively (p=0.005). Nevertheless, peak VAS increased significantly over time in the CEX (p=0.01, post-hoc: increase at both 2 and 6 hours post-LPS) and CBR groups (p=0.02, post-hoc: increase at 2 hours post-LPS), whereas no significant changes were observed in the CG (p=1.00) or

BRT (p=1.00) groups (Figure 4C). Again, CPM parameters showed no significant changes over time throughout the endotoxemia experiment (data not shown).

Discussion

In this study, we first investigated the effects of a breathing exercise which was previously shown to result in profoundly increased plasma epinephrine levels [2] on pain perception in healthy volunteers. Our results indicate that the electrical pain threshold is increased during and several hours after this exercise, which is characterized by cycles of vigorous hyperventilation and prolonged breath retention. In the second part of this study, we evaluated the effects of different training regimens involving combinations of a similar breathing exercise and cold exposure on pain perception before and during experimental human endotoxemia, a standardized controlled model of systemic inflammation. Both training regimens that involve cold exposure resulted in lower pain perception during an ice water test before induction of endotoxemia. Systemic inflammation lowered the pressure pain threshold, whereas it nullified the decreased pain perception during the ice water test in participants trained in both regimens involving cold exposure.

No learning effects on any of the measured parameters were observed when performing repeated NASQ measurements in the absence of an intervention in the 'breathing exercise study' part of the current investigation. These results are in line with earlier studies on the short term test-retest reliability of specific QST parameters [21]. Also, the absence of a learning effect is further substantiated by the lack of differences between the first two NASQ measurements in the control group of the endotoxemia study, in which no intervention was applied as well. Collectively, these findings indicate that the results of our analyses into the effects of the different interventions can be interpreted without considering a relevant test-retest effect. This is an important finding, as not every QST battery has this property. For instance, in male patients with chronic pain, reproducibility of QST parameters was found to be insufficiently stable over a period of 10 days to be used in a clinical setting [22].

In the breathing exercise study, an increase in electrical pain threshold was observed during the conduct of the breathing exercise. Furthermore, this effect persisted for several hours after cessation of the breathing exercise. These findings are in line with an earlier study on the effects of voluntary breathing, in which an increase in electrical pain threshold was found in a group of healthy volunteers that was instructed to take effortful deep and fast inhalations [23]. The mechanism

behind this analgesic effect may involve pathways that are generally attributed to exercise-induced hypoalgesia [24]: activation of the endogenous opioid system and the autonomic nervous system. First, a direct effect on the endogenous opioid system, specifically an increase in the analgesic nociceptin/orphanin levels, has been documented during vigorous hyperventilation [25]. However, in the present study, we found no relationship between increased blood pH levels (as a measure of the extent of hyperventilation) and the increase in electrical pain thresholds. Second, activation of the autonomic nervous system may be involved; indeed the breathing exercise was previously shown to result in a strong increase in blood levels of epinephrine [2]. Epinephrine and other catecholamines are associated with an analgesic effects in the complex network of central pathways in the brain [26] and pain modulation effects [27]. Furthermore, in the above described study on the effects of vigorous hyperventilation, a relationship between norepinephrine and nociceptin/orphanin levels was identified [25]. However, similar as for pH, we found no correlation between increased epinephrine levels and changes in electrical pain thresholds. The absence of such correlations could also be due to the relatively low group size or a saturation effect (e.g. every subject was able to reach high pH and epinephrine levels).

When assessing the effects of the different training regimens used in the endotoxemia study, we observed a decrease in pressure pain threshold (PPT) before induction of endotoxemia in the group that was trained only in cold exposure. We do not have a clear explanation for this effect; furthermore, it was not observed in the group that was trained in both cold exposure and the breathing technique. Nevertheless, a consistent decrease in pain perception (VAS score) induced by cold exposure during the ice water test was found post-training in both cold exposure groups, whereas no change in the proportion of participants reaching the maximum immersion time of 180 seconds was present. This is in line with studies showing cold acclimation effects after repeated exposure to cold on several other parameters including less discomfort and cold sensation as well as greater heat retention and possible improvements in cognitive performance [28-30].

In all groups, the pressure pain detection threshold decreased during human endotoxemia, indicating a hyperalgesic change of pain perception during systemic inflammation. These results corroborate earlier work of our group, in which pressure and electrical pain thresholds were significantly decreased two hours after endotoxin administration [13]. No effects of any of the training regimens on pressure pain threshold (PPT) during endotoxemia were observed. Furthermore, the increase in electrical pain threshold (EPDT) observed in the breathing exercise study could not

be recapitulated during the endotoxemia study, as both groups that were trained in the breathing exercise (ie the BRT and CBR groups) did not display an increase during endotoxemia. We speculate that the hyperalgesic effects of endotoxemia as found in the current as well as our previous study [13] override the potential analgesic effects elicited by the hyperventilation exercise. This may be supported by the fact that clinical effects of endotoxemia are very dominant, for instance represented by an development of fever and significant flu-like symptoms [13]. Alternatively, the small difference in the breathing exercise employed between the studies (ie the lack of prolonged breath retention in the endotoxemia study) may be involved, which could implicate a role for hypoxia in modulation of the electrical pain threshold, as epinephrine induction by both exercises was similar [2]. Along these lines, hypoxia itself has been linked to hyperalgesia either through hormonal or inflammatory mediators [31], for instance in patients with nocturnal hypoxia due to sleep apnea [32].

In both groups that underwent cold exposure training, several changes in electrical pain thresholds were observed during endotoxemia. However, the two groups showed a contradicting pattern. After administration of endotoxin, in the CEX group, EPDT values were higher after 6 hours, whereas values in the CBR group were lower after 2 hours. As such, it is difficult to draw conclusions on the effects of cold exposure training on electrical pain thresholds. Also, in these two `cold exposure` groups, the reported VAS score during the ice water test increased following endotoxin administration. Conceivably, the systemic inflammatory response nullified the training-induced effects observed before endotoxin administration (ie lower VAS scores compared with the pre-training measurement in these two groups, see above), resulting in a significant increase afterwards. Despite this increase in VAS, a larger proportion of participants of these two groups reached the maximum ice water immersion period of 180 seconds across all timepoints on the endotoxemia experiment day, possibly indicating that participants in these groups were able to endure these higher VAS scores for a longer period of time.

A strength of the current work is that, compared with other QST studies that employed specific tests/elements, we used NASQ, a comprehensive battery of QST measurements used extensively in other studies [9-12]. Our study is limited by the fact that we only included male participants. This may be of relevance, because within the spectrum of QST parameters, the largest effect sizes for differences in gender were found in cold-induced pain and pain to blunt pressure [33]. However, as human endotoxemia experiments are very costly and labor-intensive studies, and for ethical reasons (we want to expose as few volunteers as possible to

endotoxemia), we only include male participants in virtually all of these studies. Another limitation is the potential to extrapolate the findings to clinical practice, as we studied healthy volunteers. For instance, exercise-induced hypoalgesia observed in pain-free adults may have opposing hyperalgesic effects in patients with chronic pain [24]. However, we feel this work has important value in the translation to clinical practice.

Conclusions

In conclusion, a breathing exercise characterized by cycles of vigorous hyperventilation and prolonged breath retention decreases pain perception induced by an electrical stimulus. Furthermore, training in cold exposure may decrease pain perception induced by hand immersion in ice water. Whether these effects translate to beneficial effects in patients, for instance those suffering from autoimmune diseases, remains to be determined.

Acknowledgements

The authors would like thank Jacky van Gemert for help with the QST measurements.

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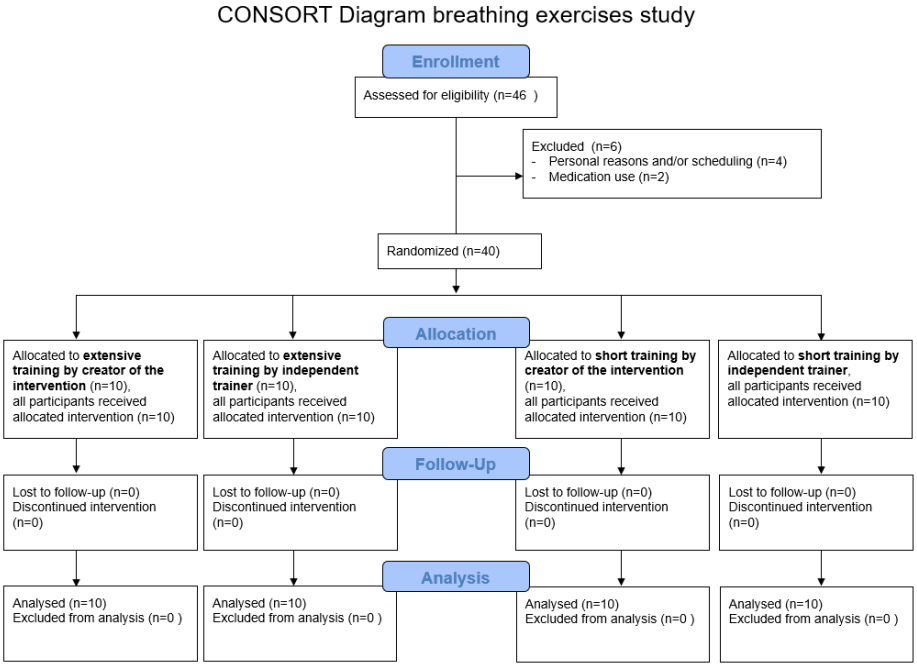
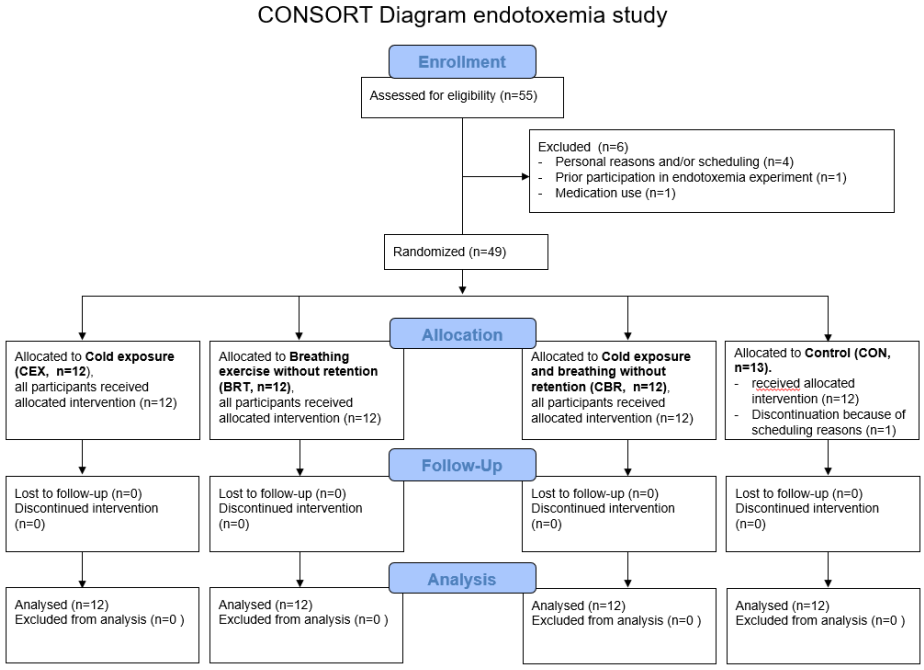
Supplemental figures



Supplemental Figure 1. Flow chart (Consort diagram) of the breathing exercises study.



Supplemental Figure 2. Flow chart (Consort diagram) of the endotoxemia study.

Chapter 7

Stewart analysis unmasks acidifying and alkalizing effects of ionic shifts during acute severe respiratory alkalosis

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Abstract

Purpose: Although both the Henderson-Hasselbalch method and the Stewart approach can be used to analyze acid-base disturbances and metabolic and respiratory compensation mechanisms, the latter may be superior in detecting subtle metabolic changes.

Materials and methods: We analyzed acid-base disturbances using both approaches in six healthy male volunteers practicing extreme voluntary hyperventilation. Arterial blood gas parameters were obtained during a breathing exercise consisting of approximately 30 cycles of powerful hyperventilation followed by breath retention for approximately 2 minutes.

Results: Hyperventilation increased pH from 7.39±0.01 at baseline to 7.74±0.06, $PaCO_2$ decreased from 34.1±1.1 to 12.6±0.7 mmHg, PaO_2 increased from 116±4.6 to 156±4.3 mmHg. Baseline apparent strong ion difference was 42.3±0.5 mEq/L, which decreased to 37.1±0.7 mEq/L following hyperventilation. The strong ion gap significantly decreased following hyperventilation, with baseline levels of 10.0±0.9 dropping to 6.4±1.1 mEq/L.

Conclusions: Henderson-Hasselbalch analysis indicated a profound and purely respiratory alkalosis with no metabolic compensation following extreme hyperventilation. The Stewart approach revealed metabolic compensation occurring within minutes. These results challenge the long-held axiom that metabolic compensation of acute respiratory acid-base changes is a slow process.

Introduction

Interpreting blood acid-base balance is a daily, but often challenging practice for physicians in the intensive care and emergency departments. Acid-base disturbances can either be analyzed by the traditional Henderson-Hasselbalch method or using the Stewart approach. Whereas the Henderson-Hasselbalch method mainly focusses on the buffer capacity of HCO_3^-, Stewart's model also takes into account ionic shifts of Cl^-, K^+, Na^+, and PO_4^{3-}, and the buffering capacity of albumin. Although both models can be used interchangeably [1], Stewart's approach may be superior in detecting subtle metabolic changes [2]. During acute hyperventilation, it is assumed that metabolic compensation for the induced respiratory alkalosis takes hours to days [3]. We investigated acute metabolic changes in healthy volunteers practicing extreme voluntary hyperventilation using the Stewart approach, and compared this method with the Henderson-Hasselbalch analysis.

Methods

Study procedures

The previously published study [4] in which the subjects participated was aimed at investigating the immunomodulatory effects of a training program encompassing exposure to cold, meditation, and different breathing exercises. The study was approved by the local ethics committee (2012-455/NL42337.091.12) and written informed consent was obtained from all subjects. On the experiment day, subjects were admitted to our research unit and a 20-gauge intra-arterial catheter (Angiocath; Becton Dickinson) was placed in the radial artery and connected to an arterial pressure monitoring set (Edwards Lifesciences) for arterial blood sampling. This sub-study was carried out in six volunteers of the trained group after completion of the regular experimental day procedures [4]. For this sub-study, subjects only practiced one of the breathing exercises described in [4], namely the exercise consisting of deep and powerful hyperventilation for an average of 30 breaths followed by an exhalation and retention of breath for approximately 2 minutes ("retention phase"). The duration of breath retention was entirely at the discretion of the subjects. Breath retention was followed by a deep inhalation breath, which was held for 10 seconds. Subjects performed several cycles of this breathing exercise for a total of approximately 30 minutes. Lithium heparin-anticoagulated blood was obtained from the arterial catheter before the start of hyperventilation (baseline), at the end of each of the hyperventilation cycles (after hyperventilation), and at the end of the retention phase (after retention). Blood gas parameters were determined by a point of care i-STAT blood gas analyzer using CG4+cartridges (both from Abbott). The same blood samples were centrifuged (2000g, 10 min, 4 °C), after which plasma was stored at -80 °C until determination of Na^+, K^+, Ca^{2+}, Mg^{2+}, PO_4^{3-}, and albumin using routine analysis methods to conduct the Stewart analyses [2, 5].

Calculations and statistical analysis

For each subject, the hyperventilation cycle during which the highest pH level was reached was used to calculate the apparent strong ion difference ($SIDa = [Na^+]+[K^+]+[Ca^{2+}]+[Mg^{2+}]-[Cl^-]-[Lactate^-]$, the effective strong ion difference ($SIDe = [HCO_3^-] + [albumin]x(0.123 \times pH-0.631) + [PO_4^{3-}] \times (0.309 \times pH - 0.469)$) and the strong ion gap ($SIG = SIDa - SIDe$). Henderson-Hasselbalch analyses were performed using the nomogram based on the equation: $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$. Data are expressed as mean±SEM after confirmation of normal distribution (calculated by Kolmogorov-Smirnov tests). Baseline values of one of the subjects were missing; these data were imputed using the mean baseline values of the other subjects. Comparisons were made using repeated measures one-way analysis of variance (ANOVA) followed

by Dunnet's post-hoc tests. 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).

Results

Age of the participants was 22.8±1.4 years, whereas weight, length, and body mass index was 75.0±3.6 kg, 182.8±2.8 cm, and 22.4±0.8 kg/m^2, respectively. No adverse events occurred during the study.

The hyperventilation exercise and subsequent retention of breath (Video 1) resulted in profound alterations of the blood gas parameters pH, $PaCO_2$, PaO_2, and HCO_3^- (Figure 1A-D).

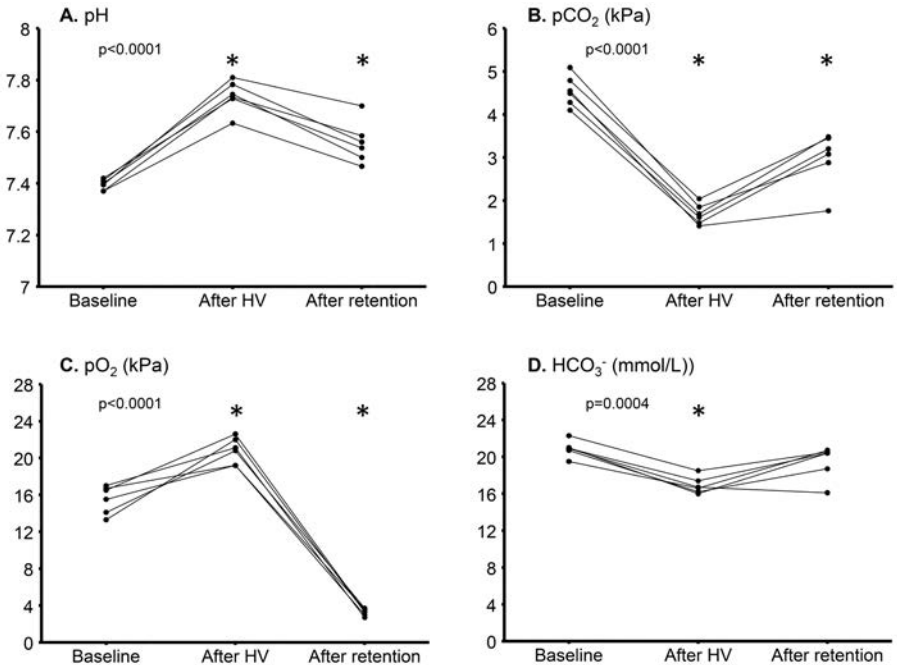

Figure 1: Blood gas parameters during the hyperventilation-retention exercise. A. pH**. B.** Carbon dioxide partial pressure ($PaCO_2$). **C.** Oxygen partial pressure (PaO_2). **D.** Bicarbonate (HCO_3^-). Data are presented as individual data points of 6 subjects. p-values depicted in the graphs represent the effect over time calculated using repeated measures one-way ANOVA. Asterisks represent significant difference at individual timepoints compared to baseline calculated using Dunnett`s post-hoc tests. Baseline: data obtained before the start of the hyperventilation exercise. After HV: data obtained after the cycle of hyperventilation during which the highest pH level was reached and before the start of the retention phase. After retention: data obtained at the end of the breath retention phase.

pH increased from 7.39±0.01 at baseline to 7.74±0.06 following hyperventilation, $PaCO_2$ decreased from 34.1±1.1 to 12.6±0.7 mmHg, PaO_2 increased from 116±4.6 to 156±4.3 mmHg, and HCO_3^- decreased from 20.9±0.4 to 16.9±0.4 mmol/L. Following the prolonged period of breath retention (on average 2 minutes), HCO_3^- was the only blood gas parameters that was fully restored to baseline levels (19.5±0.7 mmol/L). Based on the Henderson-Hasselbalch nomogram, these acid-base disturbances reflect acute and pure respiratory alkalosis with no metabolic compensation (Figure 2).

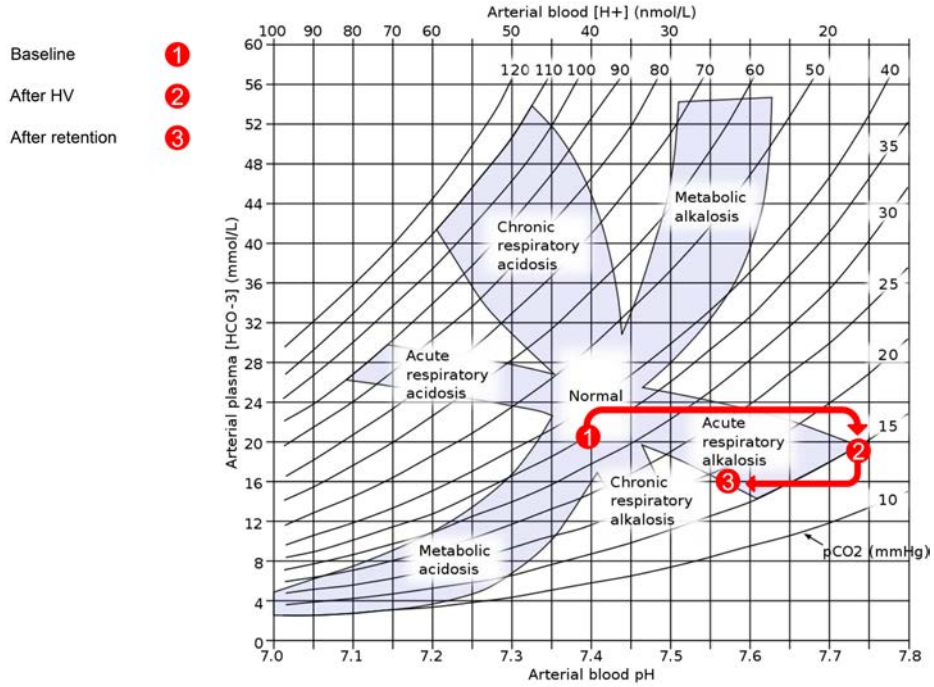

Figure 2: Nomogram of acid-base disturbances during the hyperventilation-retention exercise using the Henderson-Hasselbalch method. Baseline: data obtained before the start of the hyperventilation exercise. After HV: data obtained after the cycle of hyperventilation during which the highest pH level was reached and before the start of the retention phase. After retention: data obtained at the end of the breath retention phase. Figure adapted from the original figure obtained from https://commons.wikimedia.org/wiki/File:Acid-base_nomogram.svg.

The full dataset used for the Stewart analysis is available in Table 1 and an overview of the Stewart parameters is depicted in Figure 3A.

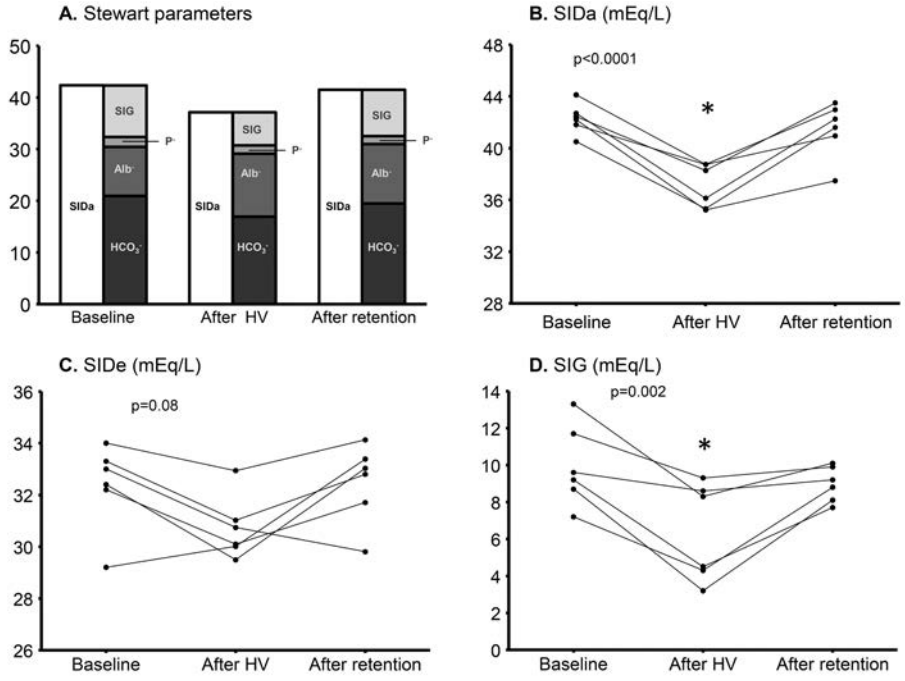

Figure 3: Overview of, and individual Stewart parameters during the hyperventilation-retention exercise. A. Overview of Stewart parameters. **B.** apparent strong ion difference (SIDa). **C.** effective strong ion difference (SIDe). **D.** strong ion gap (SIG). Data are presented as means (panel A) or individual data points (panels B-D) of 6 subjects. p-values depicted in the graphs represent the effect over time calculated using repeated measures one-way ANOVA. Asterisks represent significant difference at individual timepoints compared to baseline calculated using Dunnett`s post-hoc tests. Baseline: data obtained before the start of the hyperventilation exercise. After HV: data obtained after the cycle of hyperventilation during which the highest pH level was reached and before the start of the retention phase. After retention: data obtained at the end of the breath retention phase. HCO_3^- : bicarbonate. Alb^- : ionized albumin. P^- : ionized phosphate.

Baseline apparent strong ion difference was 42.3±0.5 mEq/L, which decreased to 37.1±0.7 mEq/L following hyperventilation, and reverted back to baseline levels after retention (41.5±0.9 mEq/L) (Figure 3B). Paired analyses of the individual components of the apparent strong ion difference (listed in Table 1) revealed that following hyperventilation, a significant decrease in Na^+, K^+, and Ca^{2+}, and a significant increase in lactate and Cl^- took place (repeated measurements one way ANOVA). Levels of Mg^+ did not change. Effective strong ion difference tended to decrease following the hyperventilation period (Figure 3C), changing from 32.4±0.7 at baseline to 30.7±0.5 mEq/L after hyperventilation, and returning back to levels comparable with those observed at baseline following breath retention (32.5±0.6 mEq/L). Analyses of the effective strong ion difference components

showed a significant decrease in HCO_3^- and an increase in albumin as well as a trend towards decreased PO_4^{3-} following hyperventilation (Table 1). The strong ion gap significantly decreased following hyperventilation (Figure 3D), with baseline levels of 10.0±0.9 dropping to 6.4±1.1 mEq/L. After retention, the strong ion gap was similar to baseline levels (9.0±0.4 mEq/L).

Discussion

Using Stewart's approach, we show that multiple metabolic processes are activated within minutes following acute vigorous hyperventilation. The resulting acute metabolic changes are not detected using the Henderson-Hasselbalch nomograms.

Whereas hyperventilation causes an expected decrease in HCO_3^-, our data reveal that it also results in a significantly decreased apparent strong ion difference (acidifying effect). Furthermore, this decrease in apparent strong ion difference is more pronounced than the reduction in effective strong ion difference, resulting in a decreased strong ion gap (alkalizing effect. Collectively, these findings point towards an increase of unmeasured cations (other than Na^+, K^+, Ca^{2+} and Mg^+) or a decrease in unmeasured anions (other than Cl^-, lactate, albumin, HCO_3^- and PO_4^{3-}) during hyperventilation.

A significant decrease of Na^+ and increase of Cl^- concentrations was observed following hyperventilation in a paired test, which mainly accounts for the decrease in apparent strong ion difference. In view of the very swift occurrence of these metabolic compensation effects, transcellular shifts of electrolytes due to changes in CO_2 pressure or pH is a plausible mechanism of action. For Cl^-, the "Hamburger shift" may be involved [6], in which low plasma CO_2 concentrations results in low HCO_3^- concentrations in erythrocytes. Subsequently, HCO_3^- is transported from plasma into erythrocytes in exchange for Cl^- by the anion exchanger protein Band 3 (SLC4A1), resulting in increased plasma Cl^- levels. For Na^+, several different transporters regulate intra- and extracellular Na^+ concentrations. It appears plausible that the decrease in Na^+ observed during extreme hyperventilation is caused by the activation of the HCO_3^-/Na^+ cotransporter family (NCBTs), which are directly controlled by intracellular and systemic pH [7], causing a flux of Na^+ into the cell. Also, a less pronounced, but still significant, decrease of plasma Ca^{2+} and K^+ was observed, further lowering the apparent strong ion difference. This is probably due to the well-known decrease in plasma H^+ concentrations as reflected by pH increases, resulting in exchange of intracellular H^+ with extracellular K^+ [8].

Free Ca^{2+} concentrations are directly influenced by pH, as protein binding of Ca^{2+} is higher at higher pH, leading to decreased free Ca^{2+} concentrations [9]. These acute electrolyte shifts from changes in pH and PaCO2 may induce acute metabolic partially compensatory disturbances. Further, ' slow' renal responses will add to this. Combined, these findings represent an interesting phenomenon to comprehensively understand acid-base phenomenology. Finally, we observed a significant increase in circulating lactate concentrations, further decreasing the apparent strong ion difference. Whether this truly represents a metabolic compensation mechanism or merely is a result of increased anaerobic metabolism caused by the powerful breathing exercise and/or decreased tissue oxygen delivery due to the extreme alkalosis remains elusive [10].

Conclusion

Stewart analysis reveals that, in healthy subjects, respiratory alkalosis due to acute vigorous hyperventilation is rapidly compensated by metabolic acidosis, involving decreases in Na^{+}, Ca^{2+} and K^{+} as well as an increase in Cl^{-}, resulting in a decreased apparent strong ion difference. Acute pH-induced changes in transcellular transport of electrolytes most likely account for these acute metabolic compensatory effects. These results challenge the long-held axiom that metabolic compensation of acute respiratory acid-base changes is a slow process.

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Chapter 8

A decrease in long-term cortisol in response to a training program involving meditation, exposure to cold, and breathing exercises

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Abstract

Determination of cortisol in scalp hair is a relatively novel technique which has been validated to represent long-term cortisol levels. Increased hair cortisol concentrations (HCC) have been associated with psychological stress, cardiovascular disease, and metabolic syndrome. We aimed to investigate changes in hair cortisol levels in response to a training intervention demonstrated to result in acute activation of the sympathetic nervous system, reflected by augmented levels of epinephrine, and attenuation of the endotoxin-induced inflammatory response. After randomization, 9 healthy young males underwent a ten-day training program consisting of meditation, cold exposure, and breathing exercises. Eight healthy young males served as controls. In all subjects, scalp hair was collected and hair samples were separated into segments, corresponding to the month before and the month during/after training. HCC decreased with 23±9% in subjects who underwent the training program (P=0.03), while no decrease was found in control subjects (7±5%, P=0.17).

We showed that an anti-inflammatory intervention comprising meditation, cold exposure, and breathing exercises is associated with a decrease in long-term cortisol levels measured in scalp hair. Furthermore, our results add to the accumulating evidence that measurement of cortisol in scalp hair is a promising technique to assess long-term cortisol levels in intervention studies.

Introduction

The acute stress response in humans is characterized by increases in two classes of hormones: catecholamines and glucocorticoids. The main glucocorticoid in humans is cortisol, which is produced in the adrenal cortex under the influence of the hypothalamus-pituitary-adrenal (HPA) axis. Long-term exposure to pathologically high levels of cortisol (i.e. Cushing's syndrome) is associated with deleterious effects including insulin resistance, dyslipidemia, hypertension, and abdominal adiposity [1]. Furthermore, subtle increases in long term cortisol have been associated with metabolically deleterious phenotypes as well [2].

A study in healthy volunteers demonstrated that the sympathetic nervous system can be voluntarily activated through a short training program consisting of meditation, cold exposure, and breathing exercises. Executing the exercises learned during this training program resulted in profound increases in circulating epinephrine levels and attenuation of the systemic inflammatory response elicited by intravenous administration of endotoxin [3]. Although short-term cortisol levels were not altered in these volunteers, repeatedly practicing these apparently stressful exercises might alter long-term cortisol levels. This is of clinical relevance, since observational studies suggest that lower long-term cortisol levels may be associated with beneficial health effects [2, 4]. Of interest in this respect, the above described training program was recently shown to result in attenuation of inflammatory parameters and disease activity, and improved quality-of-life measures in patients with axial spondyloarthritis [5].

Cortisol levels in serum, saliva and urine are highly variable due to pulsatile secretion, diurnal and day-to-day variations, as well as responses to acute stressors [6]. Therefore, cortisol levels in body fluids may not adequately reflect long-term cortisol exposure. Scalp hair analysis is increasingly being used as a technique to measure long-term cortisol. Scalp hair grows at a rate of approximately 1 centimeter (cm) per month and indeed, 1 cm of hair has been shown to reflect mean cortisol levels over a period of 1 month [7-9]. In addition to eliminating the limitations of time-point measurements, this technique can be used to obtain retrospective timelines of cortisol exposure by separating hairs into segments and analyzing them individually [10, 11]. Increases in hair cortisol concentrations (HCC) have been associated with perceived stress and mental disorders [12-14], metabolic syndrome [15] and cardiovascular disease [16]. The latter two findings indicate that interventions that decrease long-term cortisol levels may provide beneficial health effects.

The main objective of this study was to examine whether the previously described training program [3] affects long-term cortisol levels, as this glucocorticoid hormone is of major importance of many aspects of mental and physical health.

Methods

Study population and protocol

This sub-study was part of a randomized controlled study in healthy male volunteers, aimed at investigating the influence of a training program on the sympathetic nervous system and immune response *in vivo* [3]. Hair samples for this sub-study were collected if there was sufficient hair at the posterior vertex (≥4 cm). A detailed description of the training program is provided elsewhere [3]. Briefly, healthy male non-smoking volunteers, who did not use corticosteroids or any other prescription drugs, were randomized to either a 10-day training program (trained group) or no training (control group). Subjects were excluded before randomization if they had prior experience with any of the elements of the training intervention as well as with other breathing, meditation, or cold exposure techniques, mindfulness or yoga, or if they took (or had taken) regular cold showers. The training program was developed and provided by a Dutch individual, who holds several world records with regard to withstanding extreme cold. The training program consisted of three elements: i) meditation, so-called "third eye meditation", a form of meditation including visualizations aimed at total relaxation; ii) exposure to cold, including standing in the snow bare foot and lying bare chested in the snow for up to 20-30 minutes, daily dipping/swimming in ice-cold water, and hiking up a snowy mountain (elevation: 1590 m) bare chested, wearing nothing but shorts and shoes at temperatures ranging from -5 to -12°C (wind chill: -12 to -27°C); and iii) two different breathing exercises, consisting of hyperventilation with or without prolonged breath retention. In the first technique, participants performed multiple cycles of breath holding (2-3 minutes), followed by a deep inhalation that was held for 10 seconds. The second exercise consisted of deep inhalations and exhalations in which every in- and exhalation was followed by breath holding for 10 seconds, during which the subject tightened all his body muscles. There were no dropouts during the training program and it was well tolerated.

Subjects in the trained and control groups subsequently participated in human endotoxemia experiments (the training program lasted until the day of the endotoxemia experiment). During the endotoxemia experiment, trained individuals practiced the learned breathing exercises for 3 hours, starting 30 minutes before

administration of endotoxin. After the day of the endotoxemia experiment, subjects were free to practice the learned techniques at their own discretion and the amount of exercise after endotoxemia was not quantified.

Samples for the determination of serum cortisol were drawn at 8 AM on the endotoxemia experiment day. Serum for cortisol analysis was available for 8 subjects in the control group and 7 subjects in the trained group. Serum cortisol levels were analysed as described previously [3].

Hair sample collection and cortisol analysis

Scalp hair was obtained from 9 subjects of the trained group and 8 subjects of the control group four weeks after the endotoxemia experiment. A sample of approximately 150 hairs was collected from the posterior vertex of the scalp. Each hair sample was divided in two one-cm segments. The one cm of hair most proximal to the scalp was used as the post-intervention hair sample, roughly corresponding to the month partly during and after the training intervention. Then, 0.5 cm of hair was discarded to prevent carry-over effects, after which another cm of hair was used for analysis as the pre-intervention sample, corresponding to the one month before the training intervention.

HCC were determined as described previously [9] in a blinded fashion. Hair samples were coded and the investigator executing the laboratory procedures was not aware of group allocation. For each hair segment, approximately 20 mg was weighed and cut into small pieces in glass vials. Care was taken to keep the weights of the two hair segments similar within each subject. In 14 out of 17 participants, the difference between the two hair segments was below 1 mg. The mean absolute difference between the two hair samples was 1.07 mg. Extraction took place in 1 ml of methanol at 52˚C for 16 hours. After extraction, the methanol was transferred into a clean glass tube and evaporated under a nitrogen stream. The samples were then dissolved in 250µl of phosphate buffered saline (PBS). Thereafter, samples were vortexed for 1 minute, and prior to the analysis again for 30 seconds. Measurement of cortisol in hair extract was performed using a commercially available ELISA kit designed to measure cortisol in saliva (DRG Instruments GmbH, Marburg, Germany).

Statistics

Demographic data are presented as number (percentage) or median (range), all other data are presented as mean±SEM. HCC values were logarithmically transformed to attain normal distribution. Statistical tests used are indicated in the text and figure/table legends. Pearson correlation was used. A *P* value of <0.05 was

considered statistically significant. SPSS version 21 and GraphPad Prism version 5.01 were used for analysis.

Ethics statement

The trial was approved the local ethics committee of the medical center at which the experiments were conducted, carried out according to GCP standards and the declaration of Helsinki including current revisions, and registered at ClinicalTrials.gov.

Results

Baseline characteristics of the participants are summarized in Table 1.

Table 1: Baseline characteristics

	Trained group (n=9)	Control group (n=8)
Age (median, range)	22 (20 – 27)	22 (19 – 27)
BMI (median, range)	23.1 (18.6 – 24.0)	23.2 (19.9 – 25.7)
Hair treatment[a] (n, %)	0 (0%)	0 (0%)
Use of hair products (n, %)	3 (33%)	4 (50%)
Hair washing ≥3 times/week (n, %)	3 (33%)	4 (50%)

[a]Hair treatment includes coloring, bleaching and permanent straightening or curling. No significant differences between the trained group and the control group were present (all *P*-values >0.05, Mann-Whitney U-tests [age, BMI] and Fisher exact tests [other parameters]).

A significant decrease in HCC was found in the trained group post-intervention (23±9%, $t(8)=-2.611$, $P=0.03$, paired Student's t-test; Figure 1A), while no significant change was observed in control subjects (7±5%, $t(7)=-1.538$, $P=0.17$, paired Student's t-test; Figure 1B).

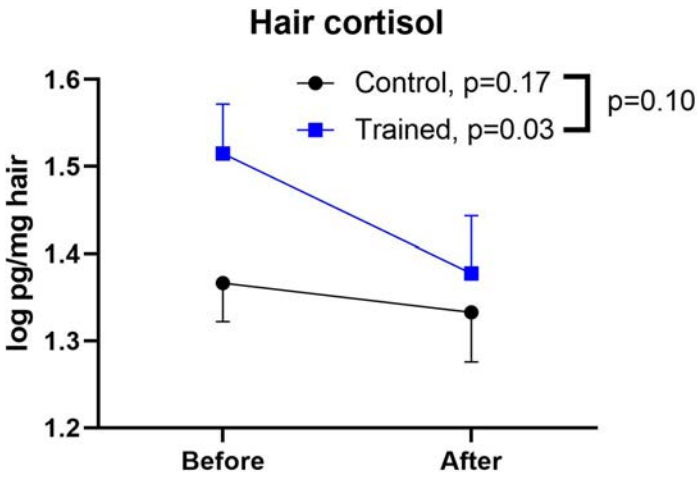

Figure 1: Changes in hair cortisol concentrations (HCC) in 9 subjects who underwent the intervention and 8 control subjects. HCC significantly decreased in the intervention group and remained stable in the control group. Data are presented on a logarithmic scale. Horizontal lines and error bars indicate mean±SEM. *P*-values were calculated using paired Student's t-tests (within-group comparisons over time) and two-way ANOVA (time * group interaction term, reflecting the between-group comparison over time

To test whether the change in HCC differed between the trained and control groups, a two-way ANOVA was performed, which revealed a trend towards a between-group effect over time (time * group interaction: F(1)=3.01, p=0.10).

Serum cortisol concentrations on the endotoxemia experiment day were similar in the trained and control groups (0.53±0.06 vs. 0.61±0.03 µmol/L, respectively; *t*(13)=1.301, *P*=0.22, unpaired Student's t-test).

No significant correlations were observed between serum and HCC within each group (*trained group:* pre-intervention hair vs. serum r=0.03, p=0.95; post-intervention hair vs. serum r=0.44, p=0.33. *control group:* pre-intervention hair vs. serum r=-0.10, p=0.81; post-intervention hair vs. serum r=-0.01, p=0.98). Furthermore, no correlations were found in subjects of both groups combined (pre-intervention hair vs. serum r=-0.17, p=0.54; post-intervention hair vs. serum r=0.22, p=0.44).

Discussion

In this study, we found that a short-term training program in young healthy male volunteers, consisting of meditation, breathing exercises and cold exposure, resulting in a short term increase in plasma epinephrine concentrations [3], is associated with a significant decrease in long-term cortisol levels measured in scalp hair. These effects were not observed in the subjects in the sex, age and BMI -matched control group, who did not participate in the training program.

This study provides preliminary evidence that long-term cortisol levels can be influenced by a behavioral intervention. Furthermore, the differences between the trained and control groups in HCC were not reflected in serum cortisol levels on the morning of the endotoxemia experiment day, and no correlations between HCC and serum cortisol were found. Point measurements in serum do not adequately reflect long-term cortisol levels, which can be well explained by the substantial physiological variability in cortisol production due to the circadian rhythm, pulsatile secretion, and environmental influences. Measurement of cortisol in scalp hair has previously shown to represent a valuable tool to reliably evaluate chronic cortisol levels and is not limited by the aforementioned short-term changes [8, 9]. Short et al convincingly showed that HCC are not correlated to frequently used time point measures of cortisol in serum, saliva or urine, but does strongly correlate the prior 30-day integrated cortisol production measure, as measured by average salivary cortisol area under the curve based on 3 samples collected per day [7]. This also supports the notion that HCC provide a reliable value long-term integrated free cortisol production. The present findings of a decrease in HCC after this behavioral intervention are an important addition to our previous report that HCC decrease after a cortisol-lowering medical or surgical therapy for Cushing's disease [11].

We found no reports on the influence on HPA axis activity of an intervention similar to that used in the present study. In a randomized controlled trial conducted in war-affected youth in northern Jordan, an eight-week program aimed at stress attunement was associated with a decrease in HCC [17]. One other small study demonstrated a decrease in HCC after one of two interventions for smoking cessation. However, whether the decrease in this study was an effect of the intervention (mindfulness based or cognitive behavioral therapy) or smoking cessation itself, is unknown [18]. Furthermore, others have shown that a stress-management cognitive behavioral program was associated with decreased urinary cortisol output and attenuated increases of cortisol in saliva in the Trier Social Stress test [19, 20]. Various aspects of the intervention used in the present study

may influence cortisol metabolism. Meditation techniques have been associated with lower circulating cortisol levels in the acute setting in multiple studies [21, 22]. However, lack of standardization of these techniques and the fact that the training investigated in the current study comprised two stressful components; exposure to cold and the breathing techniques, makes a comparison with other meditation techniques difficult. Although sudden cold exposure has been associated with an increase in circulating cortisol levels [23], it remains uncertain whether repeated exposure to cold affects basal cortisol levels. Along these lines, practicing the learned breathing exercises resulted in an acute increase in plasma epinephrine but not cortisol levels compared with control subjects during endotoxemia, indicating acute activation of the sympathetic nervous system [3]. Nevertheless, the HPA axis did not appear to be activated by these techniques, as cortisol peak levels were unaffected [3]. Together with the decrease in long-term cortisol in trained subjects, this may indicate a lower total cortisol production after training.

It was recently demonstrated that in patients with the chronic inflammatory disease axial spondyloarthritis, the training program described in the present work is safe and led to reductions in the inflammatory marker ESR and disease activity score ASDAS-CRP [5].. Furthermore, patients who followed the training program reported improved quality-of-life, reflected by increased SF-36 physical component score (PCS) and mental component score (MCS) [5]. Whether the decrease in long-term cortisol observed in the present study plays a role in possible health benefits conferred by the training program remains to be determined. Nevertheless, a behavioral/psycho-educational intervention in men chronically stressed from overworking also resulted in decreased salivary cortisol levels and improved SF-36 scores [24]. Furthermore, our group has shown an inverse correlation between hair cortisol levels and SF-36 PCS in patients with structural heart disease [25]. It has been shown that psychoneuroimmunology-based interventions (e.g cognitive behavior therapy, yoga, meditation, mindfulness, and physical exercises) are indeed associated with decreases in short-term or time point measurements of cortisol and decreases in inflammatory status in a wide variety of diseases [26]. However, to our knowledge no studies have been published showing the relation between long-term cortisol as measured in hair and inflammation before and after an intervention.

Several limitations of our study need to be taken into consideration. First, because we studied historic timelines of HCC in a repeated measures design, the stability of HCC over time has to be taken into account. Previously, we found that HCC remained stable across a hair length of at least 18 cm in female subjects, corresponding to a retrospective timeline of 18 months [9]. In contrast, Kirschbaum *et al.* found that

HCC gradually decreased in more distal hair segments and suggested that the most proximal 1-6 cm may be used to estimate systemic cortisol levels [10]. As we only used the proximal 2.5 cm of hair, this is unlikely to be a major confounder in our results. Furthermore, the gradual decrease observed by Kirschbaum *et al.* would result in lower HCC in the most distal segment, while we found a higher HCC in the distal segment in our trained group. Second, no pre-training serum cortisol levels were determined, so a before-after analysis as performed for HCC could not be performed. Third, this study was performed in a small number of participants comprising of men below the age of thirty, without obesity. Therefore our findings warrant replication in a larger sample of a more diverse group of individuals. Fourth, no sham intervention was employed in the control group, which impedes drawing conclusions about what aspect of the intervention may be responsible for the observed effects.

Finally, we did not obtain data on the subjects' activities between the end of the endotoxemia experiment day and final scalp hair collection four weeks later. Participants continued their normal daily activities with no guide or specific instructions. We did not record whether the trained subjects continued with (elements of) the training program during this period, which could have influenced our results.

In conclusion, we show that a behavioral intervention comprising meditation, exposure to cold, and breathing techniques is associated with a decrease in long-term cortisol levels, as measured in scalp hair. Furthermore, our study provides evidence that measurement of cortisol in scalp hair is a promising technique to assess long-term cortisol levels in intervention studies.

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Chapter 9

Summary

This thesis describes the *in vivo* immunomodulatory effects of two non-pharmacological interventions: (1) a training program devised by 'Iceman' Wim Hof, and (2) remote ischemic preconditioning (RIPC). In addition, several non-immunologic parameters influenced by the first intervention were explored: plasma metabolites, pain thresholds, changes in acid-base balance, and long-term cortisol levels. *Chapter 1* provides a general introduction into (modulation of) the inflammatory response in health and disease, the training program devised by Wim Hof, RIPC, and the experimental human endotoxemia model.

Part 1 – immunomodulatory effects of non-pharmacological interventions

The first intervention investigated in this thesis is a training program consisting of breathing exercises, exposure to cold, and meditation. This training program was devised by Wim Hof, who claimed that he could influence his immune system by applying these techniques. In an earlier n=1 study performed on Wim Hof, we observed that indeed he appeared to be able to mount a controlled stress response characterized by activation of the sympathetic nervous system and subsequent catecholamine release, associated with a remarkably mild inflammatory response during experimental human endotoxemia (administration of bacterial lipopolysaccharide [LPS]) compared to 112 healthy volunteers that participated earlier. However, being an n=1 study, no firm conclusions could be drawn on the causality of these findings.

In *chapter 2*, the effects of the abovementioned training program were therefore investigated in a randomized controlled study carried out in 24 healthy, male volunteers naïve to the training methods of Wim Hof. These subjects were randomized to a 10-day training program provided by Wim Hof (n=12) existing of breathing exercises, repeated exposure to cold, and meditation; or to the control group who received no training (n=12). Subsequently, both groups were subjected to experimental endotoxemia, during which hormones related to sympathetic nervous system activity and the magnitude of the systemic inflammatory response were assessed. The trained group practiced the breathing exercises during the endotoxemia experiment, which led to profound intermittent respiratory alkalosis and hypoxia as well as significantly increased plasma epinephrine concentrations. Furthermore, subjects in the intervention group displayed a much earlier and profound increase in plasma concentrations of the anti-inflammatory cytokine IL-10, whereas concentrations of proinflammatory cytokines were markedly lower compared to the control group. The increase in epinephrine concentrations correlated strongly with the enhanced IL-10 response, which, in turn, was associated

with the attenuation of pro-inflammatory mediators. Finally, flu-like symptoms and fever were less pronounced in the intervention group. This study demonstrates the training program allows individuals to voluntarily activate their sympathetic nervous system, illustrated by profound epinephrine release, and subsequently suppress their innate immune response. Up to the, the autonomic nervous system and innate immune response were considered to be systems that cannot be willingly influenced. This study, for the first time, demonstrated that both systems can nevertheless be voluntarily modulated through practicing techniques that are relatively easy to learn within a short time frame.

In *chapter 3*, the elements of the training program responsible for the observed effects and the underlying mechanisms were further explored in two phases. The first phase assessed which of the two breathing exercises (hyperventilation alternated with prolonged retention of breath or with normal breathing) employed in the training program induced the most profound increase in circulating epinephrine concentrations. Furthermore, it was explored whether training by an independent trainer instead of Wim Hof himself (to account for a possible `Guru` effect) and the longevity of the training influenced this parameter. To this end, 40 healthy male volunteers were randomized to either a short or an extensive training in both breathing exercises by either Wim Hof or an independent trainer (n=10 per group). The magnitude of the initial increase in epinephrine levels was shown not to depend on prolonged breath retention and associated periods of hypoxia. Furthermore, neither training by Wim Hof himself, nor a longer duration training program was required to attain the pronounced epinephrine response. Hence, in the second phase of the study, a short duration training by an independent trainer in the breathing exercise without prolonged retention of breath was employed. In this phase, a 2 × 2 design was used, in which the participants were randomized into four different groups (n = 12 per group): cold exposure only, the breathing exercises only, cold exposure and the breathing exercises, and a control group that did not receive any training. All participants were subjected to experimental endotoxemia, during which those that were trained in the breathing exercise practiced this technique during experimental endotoxemia. Participants of all groups, except the control group, were trained in the week leading up to the administration of endotoxin. Cold exposure training alone did not relevantly influence the inflammatory response, but did attenuate the reported endotoxemia-associated flu-like symptoms. Subjects trained in the breathing exercises showed increased plasma concentrations of IL-10, as well as an attenuated pro-inflammatory response. The addition of cold exposure training to the breathing exercises significantly enhanced these immunomodulatory effects.

As such, the combination of cold exposure training and a breathing exercises was found to be most effective in attenuating the inflammatory response during human endotoxemia. These results pave the way for clinical trials employing this intervention to treat inflammatory conditions.

The second intervention for which the immunomodulatory effects were evaluated is remote ischemic preconditioning (RIPC). RIPC is the application of short cycles of ischemia and reperfusion in either a limb or organ and has shown renal- and cardioprotective effects in multiple animal studies. These beneficial clinical effects may (at least in part) be mediated by modulation of the immune response. In humans, RIPC is achieved by using a tourniquet to temporarily cut off the blood supply to a limb, typically the forearm. In *chapter 4*, the hypothesis that RIPC attenuates the systemic inflammatory response during a subsequent endotoxin challenge was tested. To this end, 30 healthy male volunteers were randomized to receive either daily RIPC for 6 consecutive days including RIPC during the 40 min preceding the administration of endotoxin (n=10), RIPC only during the 40 min before administration of endotoxin (n=10), or no RIPC (control group, n=10). Both RIPC modalities resulted in an acute increase in urinary concentrations of renal cell-cycle arrest markers tissue inhibitor of metalloproteinases-2 [TIMP2]*insulin-like growth factor binding protein-7 [IGFBP7], which were previously also shown to be induced by RIPC. However, neither RIPC modality modulated the systemic cytokine response induced by endotoxin administration. Furthermore, experimental endotoxemia also induced an increase in [TIMP2]*[IGFBP7] but this increase was not influenced by RIPC treatment prior to endotoxemia. These results indicate that the putative beneficial effects of RIPC are not likely mediated by direct immunomodulatory effects.

Part 2 – effects of the Wim Hof training program on non-immunologic parameters

In view of the profound changes in O_2, CO_2, pH, symptoms, and stress hormones observed in subjects who followed the training program devised by Wim Hof, non-immunologic parameters related to cellular metabolism, pain perception, acid-base balance, and long-term cortisol were explored in trained subjects.

In *chapter 5,* results of metabolomic profiling of plasma samples obtained in the study described in chapter 2 are presented. This study was conducted to assess (1) how systemic inflammation alters the plasma metabolome and (2) whether the Wim Hof training program affects the plasma metabolome and if these changes are linked to the immunomodulatory effects observed. In total, 224 metabolites were

identified in plasma obtained from 24 healthy male volunteers at six timepoints, of which 98 were significantly altered following administration of endotoxin. Metabolite Set Enrichment Analysis (MSEA) revealed that the pathways most significantly affected by endotoxin administration included glutamate metabolism, oxidation of various fatty acids, pyrimidine metabolism, the urea cycle, and the Warburg effect. The plasma metabolome of trained and untrained individuals was largely similar before administration of endotoxin. However, trained subjects who practiced the breathing exercises during endotoxemia exhibited elevated concentrations of lactate and pyruvate, which correlated with enhanced plasma concentrations of IL-10. These findings were supported by *in vitro* validation experiments, in which co-incubation with lactate and pyruvate enhanced IL-10 production and attenuated production of pro-inflammatory cytokines by leukocytes stimulated with endotoxin. Therefore, this work uncovered an important role of lactate and pyruvate in the anti-inflammatory phenotype observed in trained subjects.

The volunteers that participated in the study described in chapter 2 reported less flu-like symptoms during endotoxemia. In *chapter 6*, it was explored whether these effects on symptoms are due to the mitigated inflammatory response or involved a direct tolerance/analgesic effects of (elements of) the training program. Therefore, during the conduct of the study described in chapter 3, putative modulation of pain sensitivity was objectively mapped using Nijmegen-Aalborg Screening Quantitative sensory testing (NASQ). First, NASQ parameters were evaluated in 20 healthy volunteers before, during, and after performing the hyperventilatory breathing exercise without profound retention of breath. Second, NASQ measurements were performed before and after 48 healthy volunteers followed different modalities of the training program: breathing exercise training, cold exposure training, the combination of both, or no training. Lastly, NASQ measurements were performed in these 48 subjects during experimental endotoxemia. The breathing exercise decreased pain perception induced by an electrical stimulus, both in the acute phase and four hours afterwards. Furthermore, cold exposure training decreased pain perception induced by hand immersion in ice water, but only in the period before administration of endotoxin.

In *chapter 7*, the effects of the hyperventilatory breathing exercise with breath retention on the acid-base balance in peripheral blood were assessed in a subgroup of 6 subjects that participated in the study described in chapter 2. To identify subtle metabolic changes that are generally not detectable by the classic Henderson-Hasselbalch method, the Stewart approach was used as well. This method takes

into account ionic shifts of Cl–, K+, Na+, and PO_4^{3-}, and the buffering capacity of albumin. Arterial blood gas parameters were obtained during a breathing exercise consisting of approximately 30 cycles of powerful hyperventilation followed by breath retention for approximately two minutes. Henderson-Hasselbalch analysis indicated a profound and purely respiratory alkalosis with no metabolic compensation following extreme hyperventilation. Using the Stewart approach, metabolic compensation that occurred within minutes was demonstrated, thereby challenging the traditional axiom that metabolic compensation of acute respiratory acid-base changes is a slow process that may take days.

Up to this point, this thesis only described acute effects of the training program devised by Wim Hof. These acute, profound and short-lived increases in stress hormones may nevertheless exert longer-term effects, for instance through feedback mechanisms. Therefore, in *chapter 8*, a relatively novel technique was used to determine cortisol levels in scalp hair of the participants of the study described in chapter 2. Increased hair cortisol concentrations have been associated with chronic psychological stress, cardiovascular disease, and metabolic syndrome. From the 12 healthy young males that underwent the aforementioned training program, nine provided hair samples. Eight participants from the untrained group served as controls. In all subjects, scalp hair was collected and hair samples were separated into segments, corresponding to the month before and the month during/after training. Hair cortisol concentrations decreased significantly in subjects who underwent the training program, but not in the control group. The finding that the short-lived increases in stress hormones in subjects who followed the Wim Hof training program are associated with a decrease in hair cortisol levels provides preliminary evidence that long-term cortisol levels can be attenuated by a behavioral intervention, putatively through negative feedback mechanisms.

Chapter 10

General discussion and future perspectives

In this final chapter we discuss the findings described in this thesis, draw overall conclusions and present future perspectives in light of recent studies in the field that were performed during or after the conduct of our own research.

Effects of the Wim Hof training program on acute inflammation

Immunomodulatory effects of the integral Wim Hof training program

The study described in *chapter 2* demonstrates that the training program of Mr. Wim Hof including all three elements (i.e. two slightly different breathing exercises, cold exposure, and meditation) allowed healthy, young males to voluntarily activate their sympathetic nervous system, illustrated by profound epinephrine release, and subsequently suppress their innate immune response during human endotoxemia. More specifically, we observed a strong release of epinephrine directly after the initiation of the breathing exercises. We hypothesized that the anti-inflammatory effects could be attributed to either a direct response to hyperventilation or to the distinct hypoxemia occurring after the cessation of breath during one of the breathing exercises.

A direct adrenergic response to hyperventilation has been described before and was attributed to a reduction in plasma bicarbonate concentrations during acute respiratory alkalosis [1, 2]. Indeed, during our study, the intense hyperventilation during the breathing exercises caused an acute severe respiratory alkalosis in which the plasma pH temporarily reaches extreme values up to 7.7. These profound effects on acid-base balance were further explored in *chapter 7* and discussed further down below in this chapter. The effects of epinephrine on the immune system have been found in several *in vitro* and *in vivo* studies. For instance, in *in vitro* studies using leukocytes obtained from healthy participants, epinephrine attenuates the production of the pro-inflammatory cytokine tumor necrosis factor (TNF) and enhances the production of anti-inflammatory cytokine interleukin (IL)-10 [3]. In accordance, *in vivo* studies in humans showed that exogenous infusion of epinephrine prior to induction of endotoxemia resulted in reduced proinflammatory and enhanced IL-10 responses [4, 5]. These results are in line with the results from *chapter 2*. Thus, these effects of epinephrine on immune system parameters in combination with the sharp increase of epinephrine during the hyperventilatory phase of the breathing exercises point towards a pivotal role of these breathing exercises in the observed effects of the training program during endotoxemia.

The prolonged breath retention and resulting bouts of hypoxia that characterized one of the breathing exercises could play an important role in the immunomodulatory effects observed. In this breathing exercise, subjects held their breath for up to several minutes, resulting in saturation levels as low as 50%, albeit for relatively short periods of time. This hypoxia may amplify the adrenergic response or exhibit direct anti-inflammatory effects, as previous data from our group showed clear anti-inflammatory effects of short-term hypoxia during experimental human endotoxemia [13]. In more detail, an air-tight helmet was used in this previous study to titrate the peripheral saturation of participants to 80-85% for a period of 3.5 hours during endotoxemia.

Next to the role of epinephrine and hypoxia, we identified a role of lactate and pyruvate in the anti-inflammatory phenotype observed in the trained participants, as described in *chapter 5*. More specifically, trained subjects who practiced the breathing exercises during endotoxemia exhibited elevated concentrations of lactate and pyruvate, which significantly correlated with enhanced plasma concentrations of IL-10. The interplay between these, and other, metabolites and the innate immune system has been established and expanded upon in recent years [6, 7]. In general, immune cell function is regulated extensively by metabolic pathways and, vice versa, metabolites can modulate inflammatory cell signaling pathways, regulate gene expression and act directly as signaling molecules to influence production of both pro-inflammatory and anti-inflammatory cytokines [7]. More specifically, in the context of this thesis, lactate was shown to attenuate LPS-induced production of pro-inflammatory cytokines by human mononuclear cells [8] and pyruvate attenuated mRNA expression and protein levels of pro-inflammatory cytokines in endotoxin-stimulated canine mononuclear cells, whereas production of the anti-inflammatory cytokine IL-10 was increased [8, 9]. In acute hyperinflammatory conditions, of which sepsis is a prime example, lactate levels are often profoundly increased, and it might be speculated that this acts as a negative feedback mechanism limiting an overwhelming and potentially damaging pro-inflammatory response [8].

Taken together, based on early studies, the immunomodulating effects of the Wim Hof training program appeared to be predominantly explained by the adrenergic response induced by the breathing exercises. Both employed vigorous hyperventilation, but only one of the breathing exercises involved prolonged breath retention and thus hypoxia. It was not clear which one had (the most prominent) effects on the adrenergic response and immune system. Furthermore, the other elements of the training program may play a direct or modulating role as well.

Effects of different training modalities

One of the main goals of the research project described in this thesis was to decipher the effects of the different elements making up the training program developed by Mr. Hof. For instance, the extended use of prolonged breath retention and the resulting in hypoxia is a potential safety concern, especially with future clinical trials with vulnerable patients in mind. Also, exposure to (extreme) cold may be dangerous, for instance causing hypothermia and (stress-induced) cardiac problems in vulnerable individuals. Furthermore, especially exposure to cold may be practically challenging to implement in studies or daily life. We chose to not study the potential impact of meditation in our studies. One of the reasons for this is that data on the effects of meditational techniques on immunological parameters are tentative and heterogeneous, and do not point to a specific mechanism [10]. Although some studies have suggested a reduction of pro-inflammatory markers in participants using meditational techniques such as mindfulness [11, 12], the `third-eye meditation` employed in the training program differs significantly from mindfulness. Also, the volunteers participating in the study described in *chapter 2* reported that the meditation part was relatively vague, with many stating that they used it to relax or even to fall asleep. All in all, we felt that the mediation techniques incorporated in the training program are not well-enough defined and not likely to exert immunomodulating effects.

In the first part of *chapter 3* ("breathing exercises study", without endotoxemia), we demonstrated that the increase in plasma epinephrine was comparable between the breathing exercises with and without hypoxemia and therefore conclude that prolonged retention of breath is not an essential part of the breathing exercises required to induce the strong adrenergic response observed in *chapter 2*. Consequently, the breathing exercises without prolonged retention we employed in the follow-up human experimental endotoxemia study described in the second part of *chapter 3*. Our conclusion was based on the observation that the magnitude of the initial increase in epinephrine was similar between the exercise with prolonged breath retention and that without. However, it needs to be acknowledged that there were notable differences in the longevity of the epinephrine response. While the two modalities led to similar increases in epinephrine in the first hour, the exercise with prolonged retention of breath and subsequent hypoxia showed a more sustained elevation of epinephrine concentrations afterwards, up to 1.5 hours. Furthermore, when we compare the epinephrine responses between the first study described in *chapter 2* and the breathing exercises study described in the first part of *chapter 3*, there are also notable differences. At baseline, the epinephrine concentrations were twice as high in *chapter 2* than in the breathing exercises study in *chapter 3*.

Possibly, the volunteers who participated in the endotoxemia study had a higher stress levels, knowing that a demanding experimental day was ahead of them. Furthermore, the peak adrenaline concentrations were also roughly twofold higher in *chapter 2* compared with *chapter 3*. Finally, a comparison of the epinephrine responses between the study in described in *chapter 2* and those observed in the endotoxemia study described in the second part of *chapter 3* also reveals differences. For instance, baseline concentrations were again notably lower in *chapter 3* than in *chapter 2*. Furthermore, although peak epinephrine concentrations were similar between the two studies, the rate of increase was significantly slower in *chapter 3*, peaking at 2 hours after the initiation of the breathing exercise (1.5 hours post-LPS administration), whereas peak levels in *chapter 2* were already reached 30 minutes after initiation of the breathing exercise. Also, an important difference which may provide an explanation for these discrepancies, is that Mr. Hof was present in the experimentation room in the study described in *chapter 2*, whereas he was absent during the conduct of the experiments described in *chapter 3*. The presence and motivational speeches delivered by Mr. Hof ("firing the volunteers up for the study") may have led to increased baseline epinephrine concentrations, and possibly also to a swifter increase after initiation of the breathing exercises. These differences may explain why the magnitude of the anti-inflammatory effects of the intervention was somewhat less pronounced in *chapter 3* compared to *chapter 2*. This especially pertains to the IL-10 response, which we showed to be highly correlated with the early epinephrine response in *chapter 2*. IL-10 concentrations were increased by 194% in *chapter 2*, whereas a 44% increase was observed in the combined cold exposure and breathing exercise group in *chapter 3*, which was most comparable to the intervention group studied in *chapter 2*. Furthermore, IL-10 kinetics were also different, with a much steeper rate of increase observed in the original study with subjects trained in Poland with Mr. Hof.

As briefly alluded to before, the prolonged breath retention and the resulting bouts of hypoxia may also exert direct immunomodulatory effects. The previous study by our group that investigated the effects of short-term hypoxia during experimental human endotoxemia showed a doubling of the anti-inflammatory cytokine IL-10 compared to the control group, accompanied by attenuation of pro-inflammatory cytokines by up to 50% [13]. This cytokine response is in line with the results from *chapter 2*. However, the suggested underlying mechanism of enhanced adenosine release by hypoxia was not explored in the studies presented in this thesis. Furthermore, it needs to be acknowledged that the role of hypoxia in regulating the inflammatory response is far from clear, especially in the longer term, as other studies have shown pro-inflammatory effects of prolonged hypoxic exposure [14].

Taken together, including prolonged retention of breath in the breathing exercises probably has additional value in mitigating inflammation. However, especially for future clinical studies in vulnerable patients, the risks of repeated periods of hypoxia must be weighed against a potential benefit.

In our endotoxemia study described in *chapter 3*, cold exposure training alone did not relevantly influence the inflammatory response, but it did potentiate the anti-inflammatory effects of the hyperventilatory breathing exercise. Several other studies have evaluated the effects of cold exposure on markers of the sympathetic nervous system and inflammation. In a small study in seven healthy men who sat in a climatic chamber at 5 degrees Celsius for 2 hours, plasma norepinephrine concentrations as well as plasma IL-6 concentrations increased [15]. In another study in nine healthy volunteers exercising for 60 minutes in climactic chamber of either 22 or 0 degrees Celsius, a more pronounced pro-inflammatory cytokine response was found in participants that were subjected to pre-exercise cooling to induce shivering thermogenesis [16]. As may become clear from these examples, the definition of `exposure to cold` is not uniform and different types, durations, and protocols are used throughout the literature. In our studies, we used a multitude of exposures, ranging from cold showers to standing/lying in the snow and full body immersion (excluding the head) in ice baths with water temperatures of 0-2 degrees. To the best of our knowledge, all other studies used only one modality, such as cold showers [17] or a climatic chamber [15]. For future studies, only having to instruct participants to use cold showers is obviously the more practical solution and may be the most feasible way. However, it is difficult to objectify and standardize the temperature, length and compliance of this intervention. From our data, we hypothesize that exposure to cold itself is primarily a stressor for the human body, which may even enhance the inflammatory response in the acute setting (which we did not assess). However, when exposure to cold is used as a `preconditioning` modality (the way it was used in our studies), it may mitigate inflammation and have additional analgesic effects, as discussed in the next section.

Other potential benefits and effects of the Wim Hof training program

Analgesic effects

During the endotoxemia studies described in *chapters 2 and 3*, trained participants reported less flu-like symptoms, and cold exposure appears to play a major role in mitigation of these symptoms (*chapter 3)*. However, it should be noted that these

studies were primarily designed to evaluate immunological parameters and flu-like symptoms are subjective by nature and were not evaluated with a validated instrument. There are several mechanisms that may be involved in these possible analgesic effects of the training program. First, a direct effect of the mitigated inflammatory response must be considered. As pro-inflammatory cytokines like TNF and IL-6 are directly linked to response of nociceptive neurons [18], and the subjects that were exposed to cold showed both lower amount of circulating TNF and IL-6, they might have had some benefit from a reduced activation and sensitization of the peripheral neurons leading to dampened pain sensitivity [18]. Second, exercise-induced hypoalgesia (defined as a decrease in sensitivity to painful stimuli approximately 30 minutes after a single bout of exercise) may also play a role in the analgesic effects of breathing exercise, which can be considered a form of exercise (for instance evidenced by the increased lactate concentrations, demonstrated in *chapter 5*) [19, 20]. The mechanisms behind this effect are debated, but activation of the endogenous opioid system is most often selected as the most prominent hypothesis [19]. Repeated cold exposure may cause a habituation effect by repeatedly activating the nociceptive system [21]. For instance, in a study in 37 healthy male volunteers, performing a cold pressor tests consisting of immersing a hand in ice-cold water for 7 consecutive days led to a gradual increase in pain thresholds and tolerance [22].

In *chapter 6*, we aimed to shed more light on the putative analgesic effects of the training program using the Nijmegen-Aalborg Screening Quantitative sensory testing battery (NASQ) in subjects enrolled in the study described in *chapter 3*. Results indicated that the hyperventilatory breathing exercise (irrespective of prolonged retention of breath) increased the threshold for an electrical pain stimulus, both in the acute phase (i.e during the exercise) and several hours later, all in the absence of systemic inflammation. Furthermore, we evaluated the effects of the four training regimes (i.e. only breathing exercise without retention of breath, only cold exposure training, the combination of both modalities, and no training in the control group) on pain perception before and during human endotoxemia. Interestingly, the two training regimens that involved exposure to cold resulted in lower pain perception induced by immersion of a hand in ice-cold water (so analogous to the above described cold pressor test), but only before induction of systemic inflammation by LPS administration. These results suggest that there is indeed an habituating analgesic effect of the repeated exposure to cold in the week prior to the experiments. These were however not observed during systemic inflammation, nor could we replicate the aforementioned effects of the breathing exercise on electrical pain threshold. This may be caused by overriding hyperalgesic

effects of the LPS-induced inflammatory response, shown in this chapter as well as before by our group [23].

Taking a step back from these results, the goal of objectifying experienced pain and possible analgesic effects of any intervention in inflammatory diseases is highly relevant, as it represents a major burden of disease. For instance, 94% of patients with difficult-to-treat rheumatoid arthritis are on pain killers [24]. The use of the NASQ has the potential to represent a somewhat objective manner to evaluate pain perception, especially as we found no significant test-retest effect (i.e. no learning effect). However, carrying out this testing battery is very labor- and time-intensive and requires specifically trained personal. Furthermore, the accuracy of quantitative sensory testing and its objectivity is debated. Although it might be able to identify patients with osteoarthritis who are susceptible to chronic pain and therefore offer a more personalized effective treatment [25], there was no added value of using quantitative sensory testing in identifying patients with neuropathic pain over standard bedside examination [26]. Also, different testing methods and definitions are used around the world, hampering evaluation and implementation [27].

Stress reduction

Next to catecholamines such as epinephrine, other hormones also play a central role in the human stress response, with cortisol being the most prominent. Cortisol levels in serum, saliva and urine are highly variable and may not adequately reflect long-term cortisol exposure. A possible solution is scalp hair analysis, which is increasingly being used to assess cortisol concentrations over longer periods of time. In *chapter 8*, we provide preliminary data on cortisol levels in scalp hair obtained from a subset of healthy volunteers who participated in the study described in *chapter 2*. Our results suggest an association between participation in the training program and a decrease in long-term scalp hair cortisol levels, and thus possibly stress reduction. We have clearly shown that the breathing exercises of the training program elicit acute stress. It is likely that the cold exposure elements may have a similar effect. Therefore, it could be hypothesized that certain feedback mechanisms may be involved in the reduction of chronic stress reflected by lower levels of scalp hair cortisol. Interestingly, in a recent study in 86 German individuals from the general population, perceived stress was measured using two validated questionnaires before and after being trained in only the breathing exercises, only cold exposure (by cold showering), the combination of both modalities, or no training (control group) [28]. Similar to what we observed in *chapter 3* pertaining to the immunomodulatory effects of (elements of) the intervention, the training program that combined the breathing exercises and cold exposure led to the

most pronounced decrease in perceived stress. Comparable results were found in an observational study in members of an Antarctic expedition who followed an 8-week Wim Hof training program or no training [29]. After the training program, a reduction in depressive symptoms was found using psychometric questionnaires. Furthermore, a non-significant reduction in hair cortisol was observed in this group as well. However, similar to our study, the groups were very small (n=6 and n=7 for the intervention and control groups, respectively). Furthermore, the group assignment was not randomized, but based on their interest in the training program. This introduces a major selection bias that impedes firm conclusions. To date, there are no studies investigating the effects of the Wim Hof training program on both subjective parameters (e.g. perceived stress) and objective parameters (e.g. hair cortisol) of stress in a scientifically sound manner.

Changes in acid-base balance due to the breathing exercise

The effects of the hyperventilatory breathing exercise developed by Mr. Hof on the acid-base balance of the blood are striking and profound (*chapters 2 and 3)*. In *chapter 7*, we describe the acute respiratory changes as well as the immediate metabolic changes using the Stewart method, an approach to assess acid-base analysis using a combination of chemistry and physiology [30]. In daily clinical practice, the Henderson-Hasselbalch nomograms are used almost exclusively in favor of the Stewart method. In the context of the hyperventilatory breathing exercise, Henderson-Hasselbalch analysis indicated a profound and purely respiratory alkalosis with no acute metabolic compensation following extreme hyperventilation. However, the more detailed Stewart method revealed metabolic compensation occurring within minutes, characterized by decreases in Na^+, Ca^{2+} and K^+ as well as an increase in Cl^- [30]. In the future, the use of automated assessment of acid-base disorders might favor the use of the Stewart method. However, a recent study in which computer algorithms were developed and tested on 100 randomly chosen blood gas results from ICU patients still concluded that the use of the Stewart method was cumbersome and that it proved difficult to develop a working algorithm based on this method [31].

Potential clinical application of the Wim Hof training program

A role for this intervention in the treatment of inflammatory diseases?

Although many individuals have provided testimonials praising the effects of the Wim Hof training program on their personal health and quality of life, scientific evidence is largely lacking, although there may be potential for specific patient groups.

First of all, we see no place for the training program in the context of acute severe illness and/or infections. Patients in the emergency ward and on the ICU are severely ill and not able to perform any of the elements of the training program. Moreover, attenuation of the immune system is potentially harmful in infectious diseases, where a robust pro-inflammatory response is required to clear the infection. Patients undergoing major surgery are also not a plausible target group for this intervention. Although a proportion of these patients also suffer from the consequences of an overzealous inflammatory response, the use of anesthetics precludes practicing elements of the training program.

However, there might be potential for this intervention in a group of patients with non-infectious inflammatory diseases, in particular for those with auto-immune diseases, like rheumatoid arthritis. There is some preliminary evidence to support this. The Wim Hof training program was investigated in 24 patients with moderately active axial spondylarthritis in a randomized cross-over study with safety as the primary endpoint [17]. In this cross-over study, patients either started with the 8-week training program right away or started 8 weeks later. The 8-week waiting period was used as the control condition (which therefore consisted of roughly half of the patients compared with the training condition). No differences in adverse events were found between the intervention and control conditions. Secondary biological outcomes included changes in C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR) and calprotectin concentrations over the 8-week training or control periods. ESR, an important marker for inflammation and disease activity in rheumatoid arthritis [32], significantly decreased over time during the training period: from a median of 16 (range 9-27) mm/hour before start of the training to a median of 9 (range 5-23) mm/hour after 8 weeks of training ($p=0.04$). No differences were observed during the control period: 14 (range 8-27) mm/hour at baseline vs. 16 (range 5-37) mm/hour after 8 weeks ($p=0.41$). Similar, but statistically not significant reductions were observed for CRP and calprotectin in the training group. In addition, several exploratory patient-reported outcome measures (PROMs)

pointed towards beneficial effects of the training as well, including significant improvement of the ankylosing spondylitis disease activity score (ASDAS), the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) and quality of life using the Short Form 36 questionnaire (both physical and mental domains). This is a significant finding, as PROM are important end-points in clinical studies into inflammatory diseases such as rheumatoid arthritis [33] and inflammatory bowel disease (IBD) [34]. However, relatively few patients were included in the study, limiting the robustness of the results. Furthermore, the cross-over design introduces the possibility of `period bias` in the control condition, resulting from the natural changes of the patients` condition over time. Finally, the relevance of the different elements in the training program remains unclear from this study. For instance, exercise in general is known to be a pivotal part of the treatment of axial spondylarthritis and also part of the training program (strength exercises).

Recommendations for future studies into the Wim Hof training program

Taking all results on (elements of) the Wim Hof training program presented in this thesis into account, future (clinical) studies investigating this intervention ideally include both exposure to cold and the hyperventilatory breathing exercise with prolonged breath retention. However, as alluded to before, possible risks of certain elements (e.g. repeated periods of hypoxia, cold exposure) must be weighed against potential benefits, and leaving out elements for certain (patient) groups should be carefully considered. In any case, it is imperative to acknowledge that there is a paucity of studies into the safety aspects of the training program. Therefore, safety should remain the primary focus, especially in early future clinical trials in patients with auto-immune disease such as spondylarthritis or rheumatoid arthritis. For instance, it is of utmost importance to practice the breathing exercise lying down, as this exercise can lead to a temporary lowered state of consciousness. Preferably, the breathing exercise should also be supervised by another individual who does not practice it. Next, as patients are much more fragile than the healthy volunteers we studied and carry an increased risk for cardiovascular diseases [35], we advise to stratify patients into cardiovascular risk groups and to advise the participants in the high risk group to monitor the peripheral arterial blood saturation to a safe, predetermined level. Furthermore, we advocate the use of supervised immersions in ice baths in line with the protocol described in *chapter 3* as the preferred way to objectively and thoroughly expose participants to cold. In short, this constitutes of immersion of the whole body without the head (sitting or swimming) in ice-cold water (0-2 degrees Celsius) for a period up to 3 minutes (or less, always at the discretion of the participant). We want to acknowledge that the use of ice baths

is controversial in the context of safety, and emphasize that the immersion in water should never be combined with the performance of the breathing exercises, and should not be performed alone.

In terms of outcome parameters, it is of pivotal importance to incorporate patient reported outcome measurements (PROM) in the study protocol, next to inflammatory parameters. Furthermore, objectifying potential analgetic effects of the training program devised by Wim Hof could be considered in these studies as well, for instance using quantitative sensory testing such as the NASQ method employed in the current thesis. Another outstanding question which was not addressed in this thesis concerns the required intensity and the longevity of the training program's effects, i.e. how often do you have to practice the techniques, for how long, what temperatures should be used for cold exposure and, related to the above, how long do the effects persist? Future studies may come up with an answer to this question, possibly show a 'dose-effect' relationship and may thereby reduce the effort that patients/subjects have to make to achieve beneficial effects. This may also mitigate some of the safety concerns described above.

Future clinical trials in patients will not have the tightly controlled, highly homogenous, and thus inherently artificial circumstances that we created during our studies in young, healthy subjects. Furthermore, as outlined above, these clinical studies will need to have more subjective outcome parameters (for instance PROMs). Therefore, especially for early clinical studies into this intervention which will likely have relatively small sample sizes, the included patient population should be as homogenous as possible. In the context of rheumatic disease, this can for instance be achieved by including only patients with relatively new onset of disease, stable medication use, and few comorbidities.

Finally, an important factor in designing future clinical trials concerns the sham/control group that should be used. We chose not to include a sham training in our studies, instead we used untrained controls. This was a deliberate choice, as the studies described in this thesis were designed as proof-of-principle studies primarily aimed at identifying immunomodulatory effects, which are for the most part objective (i.e. cytokines, fever etc.), rendering a sham training of less importance. Inclusion of a sham training in these studies would also have represented a risk, as it may have immunomodulatory effects too. For upcoming clinical trials, we do however feel that an appropriate sham training should be used. We have several deliberations concerning what this sham training should look like. First, as briefly touched on before, a sham intervention must not have any documented

or suspected influence on the immune system. This is relevant for a seemingly obvious sham intervention like Mindfulness-Based Stress Reduction (MBSR), which includes meditation/relaxation techniques, but not the hyperventilatory breathing exercises and exposure to cold. As alluded to before in this chapter, several studies found significant effects of MBSR on the specific immune parameters investigated in the studies described in this thesis [10]. Therefore, it is not suitable as a true sham training, although it might be argued that a comparative study of MBSR versus the Wim Hof training program is also of interest. Second, the sham training must encompass a comparable time investment compared to the Wim Hof training program and should include somewhat comparable activities. A suggestion for a sham training is listening to relaxing music for the duration of the breathing exercise in the training program combined with normal temperature showers instead of cold showers/cold exposure in the training program.

Remote ischemic preconditioning

The other intervention we studied in this thesis is remote ischemic preconditioning (RIPC). As we observed both hypoxia (due to breath retention) and peripheral vasoconstriction (due to cold exposure and epinephrine release) during practicing of the different elements of the Wim Hof training program, we hypothesized that ischemic preconditioning is a possible part of the physiology involved in the observed effects of the training program. RIPC as an intervention has been described since the 1980`s and has shown reno- and cardioprotective effects in multiple animal studies, which were suggested to be (at least partially) mediated by modulation of the immune response. This suggested RIPC to be an easy, low-cost, and quick therapeutic strategy for patients undergoing major surgery. We demonstrated that there was indeed an effect on tubular cell-stress, as illustrated by an acute increase in urinary concentrations of renal cell-cycle arrest markers tissue inhibitor of metalloproteinases-2 [TIMP2]*insulin-like growth factor binding protein-7 [IGFBP7] following RIPC. However, we demonstrated that RIPC does not modulate the systemic cytokine response induced by endotoxin administration. Based on our study in a highly controlled and reproducible model, the putative beneficial effects of RIPC are therefore not likely to be mediated by direct immunomodulatory effects.

Over time, the beneficial effects of RIPC in humans have become less convincing. The translation of positive effects of RIPC from animal models to clinical trials has been very challenging. Beneficial effects of RIPC in humans were demonstrated in a

study in 648 patients undergoing hip fracture surgery, where RIPC reduced plasma troponin concentrations (a marker of myocardial injury) [36]. Another study in 240 patients at high risk for acute kidney injury (AKI) who underwent cardiac surgery found that RIPC reduced the rate of AKI [37]. Furthermore, a small interventional pilot study in 47 patients undergoing abdominal surgery demonstrated that RIPC was a significant independent predictor for lower overall postoperative morbidity [38]. However, larger multicentre studies showed no effects on important clinical outcome parameters. For instance, in 1612 patients undergoing coronary artery bypass grafting (CABG), RIPC did not improve clinical outcomes [39]. Similar results were obtained in a trial in 1403 patients undergoing elective cardiac surgery [40]. Furthermore, a large prospective, single-blind, randomized trial in 5401 patients with suspected myocardial infarction that were randomized to RIPC or sham-RIPC before an percutaneous coronary intervention demonstrated no differences in mortality or hospitalization over the following year [41]. Therefore, RIPC cannot, at least for now, be recommended as a standard peri-operative procedure. It may be that subgroups of patients that benefit from RIPC can be identified, primarily those at increased risk for AKI. Efficacy of RIPC to mitigate renal injury may be evidenced using TIMP2*IGFBP7 [37]. However, recent data also questions the usefulness of this new biomarker in the context of AKI. For instance, in 65 patients undergoing cardiac surgery with cardiopulmonary bypass, there was no evident added value of TIMP2*IGFBP7 over early minimal postoperative changes in plasma creatinine [42]. Furthermore, in 93 patients undergoing an open or endovascular aortic repair, early assessment of TIMP2*IGFBP7 failed to accurately predict the occurrence of postoperative AKI [43].

Taken together, we conclude that future studies should focus on the use of RIPC in a subgroup of patients at risk for post-operative AKI and evaluate this using classic parameters such as plasma creatinine. At this point, the predictive value of biomarkers such as TIMP2*IGFBP7 is not well defined. In addition, elucidating the underlying mechanisms by which RIPC exerts its effects can aid in identifying the groups of patients most likely to benefit from this easy to implement, low cost intervention. In addition, the diverging results obtained in the experimental human endotoxemia studies that explored the effects of the Wim Hof training program compared to the RIPC study, illustrate that the effects of the former do not involve an RIPC-related mechanism.

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Chapter 11

Research data management

Ethics and privacy

This thesis is based on the results of medical-scientific research with human participants, which were conducted in accordance with the latest version of the Declaration of Helsinki and guidelines for good clinical practice (GCP). The studies were approved by the medical and ethical review board Committee on Research Involving Human Subjects Region Arnhem-Nijmegen, Nijmegen, the Netherlands. All participants gave written informed consent to participate.

Data collection and storage

All data presented in this project are stored according to the FAIR principles (Findable, Accessible, Interoperable, and Reusable). All paper data are stored in the department archive (Radboudumc, room M340 -1.124). Human trial data were recorded in encoded form in the GCP-compliant data management system Castor (Castor EDC). Raw and processed data are stored digitally on the department of Intensive Care share on a local server of Radboudumc, which is backed up daily. The privacy of the participants in this thesis is warranted by use of encrypted and unique individual subjects codes.

Availability of data

All published studies in this thesis are freely available (open access). Study data will be stored for 15 years after termination of the study. Pseudonymized datasets are available from the corresponding author on reasonable request.

Chapter 12

Nederlandse samenvatting

Dit proefschrift beschrijft de effecten van twee niet-farmacologische interventies op het immuunsysteem: (1) een trainingsprogramma ontwikkeld door 'Iceman' Wim Hof en (2) de effect van `remote ischemic preconditioning` (RIPC). Daarnaast werden verschillende niet-immunologische parameters onderzocht: metabolieten in het bloed, pijndrempels, veranderingen in het zuur-base-evenwicht en cortisolspiegels in het bloed. Het proefschrift start met een algemene introductie over modulatie van de ontstekingsreactie bij gezondheid en ziekte, het trainingsprogramma bedacht door Wim Hof, RIPC, en het experimentele endotoxinemiemodel (toediening van bacterieel lipopolysaccharide [LPS, ook wel endotoxine genoemd] aan gezonde vrijwilligers).

Deel 1 – Effecten van niet-farmacologische interventies op het immuunsysteem

Hoofdstuk 2 beschrijft de resultaten van het onderzoek naar de eerste interventie die in dit proefschrift wordt onderzocht. Het betreft een trainingsprogramma bestaande uit ademhalingsoefeningen, blootstelling aan kou en meditatie. Dit trainingsprogramma werd bedacht door Wim Hof, die beweert dat hij zijn autonome zenuwstelsel en immuunsysteem kan beïnvloeden door deze technieken toe te passen. In een eerder onderzoek bij Wim Hof zelf (n=1) die geen deel uitmaakt van dit proefschrift namen we waar dat hij inderdaad in staat leek een gecontroleerde stressreactie op te wekken, gekenmerkt door activering van het sympathische zenuwstelsel en daaropvolgende afgifte van het acute stresshormoon adrenaline. Dit alles was, vergeleken met 112 gezonde vrijwilligers die eerder deelnamen aan een vergelijkbaar endotoxinemie-experiment, geassocieerd met een opmerkelijk milde afweerreactie van het immuunsysteem. Omdat het echter een n=1-onderzoek betreft, konden er geen harde conclusies worden getrokken over de causaliteit van deze bevindingen.

In ons onderzoek evalueerde we de effecten van het bovengenoemde trainingsprogramma in een gerandomiseerde gecontroleerde studie uitgevoerd bij 24 gezonde, mannelijke vrijwilligers die niet bekend waren met een van de trainingsmethoden van Wim Hof. Deze proefpersonen werden gerandomiseerd naar een 10-daags trainingsprogramma verzorgd door Wim Hof (n=12) bestaande uit ademhalingsoefeningen, herhaalde blootstelling aan kou, en meditatie; of naar de controlegroep die geen training kreeg (n=12). Vervolgens werden beide groepen onderworpen aan experimentele endotoxinemie, waarbij hormonen (die de activiteit van het sympathische zenuwstelsel) en cytokines (die de mate

van de afweerreactie weergeven) werden bepaald. De getrainde groep voerde de ademhalingsoefeningen ook tijdens het endotoxinemie-experiment uit, wat leidde tot een sterke intermitterende respiratoire alkalose, forse hypoxie en aanzienlijk verhoogde concentraties van adrenaline in het bloedplasma. Bovendien vertoonden proefpersonen in de getrainde groep een veel eerdere en sterkere toename van de plasmaconcentraties van het ontstekingsremmende cytokine IL-10, terwijl concentraties van ontstekingsbevorderende cytokines aanzienlijk lager waren vergeleken met de controlegroep. De toename in adrenaline correleerde bovendien sterk met de verhoging van IL-10, die op haar beurt geassocieerd was met de lagere spiegels van ontstekingsbevorderende cytokines. Ten slotte waren griepachtige symptomen en koorts veel minder uitgesproken aanwezig in de getrainde groep. Deze studie laat zien dat het trainingsprogramma van Hof proefpersonen in staat stelt om willens en wetens hun sympathische zenuwstelsel te activeren, gekenmerkt door sterke adrenaline afgifte, met als resultaat het onderdrukken van het immuunsysteem. Het autonome zenuwstelsel en het immuunsysteem werden altijd beschouwd als systemen die niet willens en wetens beïnvloed konden worden. Deze studie toont voor het eerst aan dat beide systemen desondanks vrijwillig beïnvloed kunnen worden door het beoefenen van technieken die relatief gemakkelijk aan te leren zijn binnen een kort tijdsbestek.

In **hoofdstuk 3** onderzochten we de verschillende elementen en de onderliggende mechanismen binnen het trainingsprogramma die de waargenomen effecten zouden kunnen verklaren. In de eerste fase werd beoordeeld welke van de twee ademhalingsoefeningen uit het trainingsprogramma de meest uitgesproken toename van adrenaline veroorzaakt (hyperventilatie afgewisseld met ofwel langdurig inhouden van de adem danwel met doorademen). Verder werd onderzocht of training door een onafhankelijke trainer in plaats van Wim Hof zelf (om een mogelijk 'Goeroe'-effect uit te sluiten) en de duur van de training invloed had op de mate van stijging van de adrenalinespiegels. Om dit te onderzoeken zijn 40 gezonde mannelijke vrijwilligers gerandomiseerd naar een korte of een uitgebreide training in beide ademhalingsoefeningen door ofwel Wim Hof, ofwel een onafhankelijke trainer (n=10 per groep). We toonden aan dat de stijging van de adrenalinespiegel niet afhankelijk is van langdurig inhouden van de adem en de daarmee samenhangende perioden van hypoxie. Bovendien blijkt noch training door Wim Hof zelf, noch een langdurig trainingsprogramma vereist om de uitgesproken adrenalinepiek te bereiken. In de tweede fase van het onderzoek is daarom gebruik gemaakt van een korte training door een onafhankelijke trainer in de ademhalingsoefening zonder langdurig inhouden van de adem. In deze fase werden 48 nieuwe gezonde vrijwilligers gerandomiseerd naar vier

verschillende groepen (n = 12 per groep): alleen blootstelling aan kou, alleen de ademhalingsoefening, blootstelling aan kou en de ademhalingsoefening, en een controlegroep die niet getraind werd. Een week later deden alle deelnemers vervolgens mee aan hetzelfde endotoxinemie-experiment, waarbij degenen die waren getraind in de ademhalingsoefening deze techniek ook uitvoerde tijdens het experiment. De afweerreactie van deelnemers die alleen getraind hadden in blootstelling aan kou was vergelijkbaar met die van de ongetrainde groep, alleen de zelf gerapporteerde griepachtige klachten na toediening van endotoxine waren duidelijk minder uitgesproken. Deelnemers die waren getraind in de ademhalingsoefeningen vertoonden verhoogde plasmaconcentraties van IL-10, evenals een verminderde aanmaak van ontstekingsbevorderende cytokines. De toevoeging van de koudetraining aan de ademhalingsoefeningen versterkte deze effecten op het immuunsysteem aanzienlijk. De combinatie van training in de kou met de training in de ademhalingsoefeningen blijkt dus het meest effectief te zijn in het onderdrukken van het immuunsysteem tijdens experimentele endotoxinemie. Deze resultaten kunnen zeer goed gebruikt worden in de voorbereiding van klinisch onderzoek naar de effecten van de interventie bij patiënten met ontstekingsziekten.

RIPC is de tweede interventie waarvan we de effecten op het immuunsysteem onderzochten. RIPC bestaat uit het toepassen van korte periodes van ischemie (geen doorbloeding) en reperfusie (op gang brengen van doorbloeding) in een ledemaat of orgaan, een behandeling die in meerdere dierstudies hart- en nierbeschermende effecten liet zien. Het precieze werkingsmechanisme achter deze gunstige effecten is onduidelijk, maar beïnvloeding van de afweerreactie zou een deel van de verklaring kunnen zijn. Bij mensen wordt RIPC bereikt door met een tourniquet de bloedtoevoer naar een ledemaat, meestal de onderarm, korte tijd afwisselend af te sluiten en weer op gang te brengen. In de literatuur zijn aanwijzingen voor zowel een acuut effect (minuten na de RIPC) als een lange termijn effect (uren tot dagen na de RIPC). In **hoofdstuk 4** testten we de hypothese dat RIPC gunstige effecten sorteert door het induceren van immunologische tolerantie. Met andere woorden: leidt RIPC tot een verminderde afweerreactie veroorzaakt door toediening van endotoxine? Hiervoor randomiseerden we 30 gezonde mannelijke vrijwilligers naar 3 verschillende groepen: dagelijks RIPC gedurende 6 opeenvolgende dagen inclusief RIPC gedurende de 40 minuten voorafgaand aan de toediening van endotoxine (n=10), of RIPC alleen gedurende de 40 minuten voorafgaand aan de toediening van endotoxine (n=10), of geen RIPC (controlegroep, n=10). Beide RIPC varianten resulteerden in een acute toename van een eiwitten in de urine dat vrijkomt als de niercellen onder stress staan (tissue inhibitor of metalloproteinases-2*insulin-like growth factor binding

protein-7 [TIMP2]*[IGFBP7]). Eerder werd al aangetoond dat het vrijkomen van deze eiwitten wordt veroorzaakt door RIPC. We konden hiermee dus aantonen dat we een RIPC juist hadden toegepast. Geen van beide RIPC-varianten had echter effecten op de afweerreactie na toediening van endotoxine. Verder veroorzaakte experimentele endotoxinemie zelf ook een toename van [TIMP2]*[IGFBP7], dus los van de RIPC. Echter, de mate toename werd niet beïnvloed door RIPC-behandeling voorafgaand aan de toediening van endotoxine. Deze resultaten geven aan dat de vermeende gunstige effecten van RIPC waarschijnlijk niet worden veroorzaakt door beïnvloeding van het immuunsysteem.

Deel 2 – Effecten van het Wim Hof-trainings-programma op niet-immunologische parameters

Gezien de extreme veranderingen in het zuurstof- en kooldioxide (CO_2)-gehalte in het bloed, zuurgraad, griepachtige symptomen en stresshormonen die we vonden bij proefpersonen die het trainingsprogramma van Wim Hof volgden, hebben we een aantal niet-immunologische parameters onderzocht bij getrainde proefpersonen en vergeleken met de ongetrainde controlegroep. Dit zijn verschillende metingen gerelateerd aan cellulair metabolisme, pijnbeleving, zuur-base-evenwicht en het stresshormoon cortisol.

In **hoofdstuk 5** beschrijven we de resultaten van metabolietmetingen in bloedmonsters van de deelnemers aan de studie beschreven in hoofdstuk 2. Deze studie werd uitgevoerd om te beoordelen (1) hoe de afweerreactie het metabolisme beïnvloedt en (2) of het trainingsprogramma van Wim Hof invloed heeft op het metabolisme en of deze mogelijke veranderingen verband houden met de waargenomen effecten op het immuunsysteem. Er werden op zes tijdstippen in totaal 224 verschillende metabolieten geïdentificeerd in de bloedmonsters van de 24 gezonde mannelijke deelnemers aan de studie beschreven in hoofdstuk 2. Daarvan veranderden er 98 significant na toediening van endotoxine. Analyse via `Metabolite Set Enrichment Analysis (MSEA)` liet een aantal metabole routes zien die die het meest relevant werden beïnvloed door de toediening van endotoxine: het glutamaatmetabolisme, de oxidatie van verschillende vetzuren, het pyrimidinemetabolisme, de ureumcyclus en het Warburg-effect. De samenstelling van de metabolieten in het bloed van getrainde en ongetrainde individuen was grotendeels vergelijkbaar voorafgaand aan toediening van endotoxine. Getrainde proefpersonen die de ademhalingsoefeningen uitvoerden tijdens endotoxinemie vertoonden echter verhoogde concentraties lactaat en pyruvaat, wat verband

hield met verhoogde aanmaak van IL-10. Deze bevindingen werden kracht bijgezet door de resultaten van *in vitro* validatie-experimenten. Witte bloedcellen werden in het laboratorium gestimuleerd met endotoxine, waarbij co-incubatie met lactaat en pyruvaat de productie van IL-10 verhoogde en de productie van ontstekingsbevorderende cytokines verminderde. Dit betekent dat lactaat en pyruvaat mogelijk een belangrijke rol spelen met betrekking tot de remming van het immuunsysteem bij getrainde proefpersonen.

De vrijwilligers die het trainingsprogramma van Hof volgden en deelnamen aan het onderzoek beschreven in hoofdstuk 2 rapporteerden veel minder griepachtige symptomen tijdens het endotoxine-experiment ten opzichte van de ongetrainde proefpersonen. In **hoofdstuk 6** werd onderzocht of deze effecten op de symptomen voornamelijk te wijten zijn aan de afgezwakte afweerreactie of dat er sprake is van directe beïnvloeding van de pijnbeleving door (elementen van) het trainingsprogramma. De pijngevoeligheid werd in dit onderzoek objectief in kaart gebracht met behulp van een specifieke pijnmeting volgens de Nijmegen-Aalborg Screening Quantitative Sensory Testing (NASQ). Eerst werden deze NASQ-parameters vastgelegd bij 20 gezonde vrijwilligers vóór, tijdens en na het uitvoeren van de hyperventilatie-ademhalingsoefening (zonder langdurig inhouden van de adem). Daarna werden pijnmetingen uitgevoerd voordat en nadat 48 gezonde vrijwilligers verschillende elementen van het trainingsprogramma volgden: ademhalingsoefeningen, training in blootstelling aan kou, de combinatie van beide, of geen training. Ten slotte werden bij deze 48 proefpersonen pijnmetingen uitgevoerd tijdens experimentele endotoxinemie. De ademhalingsoefening verminderde de pijnbeleving veroorzaakt door een elektrische prikkel, zowel tijdens het uitvoeren van de oefening als vier uur daarna. Bovendien verminderde koudetraining de waargenomen pijn veroorzaakt door onderdompeling van de handen in ijswater, maar alleen in de periode voorafgaand aan toediening van endotoxine.

In **hoofdstuk 7** werden de effecten van de hyperventilatie ademhalingsoefening met vasthouden van de adem op het zuur-base evenwicht nauwkeuriger onderzocht in een subgroep van 6 proefpersonen die deelnamen aan het onderzoek beschreven in hoofdstuk 2. Specifiek keken we naar de zuur-base evenwicht in het bloed verkregen uit een slagader. Om subtiele metabolische veranderingen te identificeren die doorgaans niet waarneembaar zijn met de klassieke Henderson-Hasselbalch-methode, werd ook de Stewart-aanpak toegepast. Deze methode houdt rekening met ionische verschuivingen van chloor, kalium, natrium, fosfaat en de buffercapaciteit van albumine. Bloedgasmetingen werden uitgevoerd

tijdens een ademhalingsoefening bestaande uit ongeveer 30 cycli van krachtige hyperventilatie gevolgd door ongeveer twee minuten inhouden van de adem. Henderson-Hasselbalch-analyse duidde op een diepe en puur respiratoire alkalose zonder metabolische compensatie na extreme hyperventilatie. Met behulp van de Stewart-benadering vonden we een metabole compensatie die reeds binnen enkele minuten plaatsvond. Hiermee stellen we het traditionele axioom ter discussie, waarin word verondersteld dat metabolische compensatie van acute respiratoire zuur-base-veranderingen een langzaam proces is dat dagen kan duren.

Dit proefschrift beschrijft vooral de acute effecten van het trainingsprogramma van Wim Hof. De acute, extreme en kortstondige stijgingen van acute stresshormonen zoals adrenaline kunnen niettemin via verschillende feedback mechanisme effecten op de langere termijn hebben. Daarom werd in **hoofdstuk 8** een relatief nieuwe techniek gebruikt om de hoeveelheid van het stresshormoon cortisol in het hoofdhaar van de deelnemers aan het onderzoek beschreven in hoofdstuk 2 te bepalen. Cortisolconcentraties in het haar zijn een afspiegeling van de spiegels in het bloed die langdurig aanwezig zijn. Verhoogde waarden zijn in verband gebracht met chronische psychologische stress, hart- en vaatziekten en het metabool syndroom. Van de twaalf gezonde jonge mannen die het eerder beschreven trainingsprogramma ondergingen, onderzochten we van negen deelnemers de haarmonsters. Acht deelnemers uit de ongetrainde groep dienden als controles. Bij alle proefpersonen werd hoofdhaar verzameld en werden de haarmonsters in segmenten verdeeld, corresponderend met de periode van circa maand vóór en de maand tijdens/na de training. De concentratie van haarcortisol daalde significant bij proefpersonen die het trainingsprogramma ondergingen, maar niet bij de deelnemers in de controlegroep. We concluderen daarmee dat de kortstondige toename van stresshormonen bij proefpersonen die het Wim Hof-trainingsprogramma volgden geassocieerd is met een afname van de cortisolspiegels voor een langere periode. Het zou dus kunnen zijn dat cortisolspiegels op de langere termijn kunnen worden verlaagd door een gedragsinterventie zoals die ontwikkeld door Wim Hof, vermoedelijk door middel van negatieve feedback mechanismen.

Het zwaartepunt van dit proefschrift ligt bij onderzoek naar het Wim Hof-trainingsprogramma, ook omdat, in tegenstelling tot RIPC, deze interventie duidelijke en reproduceerbare effecten op het immuunsysteem liet zien. Als we al onze resultaten naar de effecten van (elementen van) dit programma in ogenschouw nemen, is het advies om in toekomstige (klinische) studies naar deze interventie zowel blootstelling aan kou als hyperventilatie-ademhalingsoefeningen

met langdurig inhouden van de adem op te nemen. In de beschreven studies hebben we de acute effecten van training op het immuunsysteem onderzocht *bij jonge gezonde vrijwilligers* middels een *experimenteel model*, de toediening van endotoxine. Dit betekent dat deze resultaten niet 1-op-1 kunnen worden geëxtrapoleerd naar de *chronische effecten* bij patiënten *op leeftijd* die een *bepaalde inflammatoire aandoening* hebben. Het is daarom essentieel dat de mogelijke risico's van bepaalde elementen (bijvoorbeeld herhaalde periodes van hypoxie door het langdurig inhouden van de adem en acute effecten op het hart door koudetraining) worden afgewogen tegen de potentiële voordelen. Het is ook van belang te benadrukken dat de uitvoer van de trainingen tijdens onze studies onder directe supervisie gebeurde. Uit veiligheidsoverwegingen is het aan te bevelen de oefeningen niet alleen uit te voeren. Voor bepaalde (patiënten)groepen kan dit betekenen dat elementen van de training moeten worden weggelaten. Er is simpelweg nog te weinig onderzoek gedaan naar de veiligheidsaspecten van het trainingsprogramma bij potentieel kwetsbare groepen. Daarom moet veiligheid voorop blijven staan in eventuele toekomstige patiëntstudies. Tenslotte willen we benadrukken dat het onderzoek in dit proefschrift is gericht op het leggen van een basis voor deze patiëntonderzoeken. Gezien onze resultaten liggen studies naar (reumatische) auto-immuunziektes ons inziens het meest voor de hand. Er kunnen op basis van ons onderzoek dus geen conclusies worden getrokken over eventuele algemene, langere-termijn gezondheidsvoordelen van het trainingsprogramma bij gezonde mensen en/of patiënten.

Chapter 13

Dankwoord

Dankwoord

Geachte Prof. dr. Pickkers, beste **Peter**, wat is het prachtig geweest om onderdeel te zijn van jouw groep. Ik herinner me nog goed het oude kantoor waar we samen een experimentele meting aan het doen waren tijdens de experimenten van Mirrin. Ik kwam net kijken, maar je nam me meteen mee en vertrouwde me direct een aantal belangrijke handelingen toe. Het typeert je succesvolle manier van werken: veel vertrouwen geven. Ik heb me altijd veilig gevoeld om te zeggen wat ik dacht. Sterker nog: ik kom graag bij je op gesprek om mijn hart te luchten omdat ik er altijd 'lichter' uit kom dan dat ik erin ga. Dank voor het leggen van een stabiele basis voor dit proefschrift.

Geachte dr. Kox, beste **Matthijs**, bizar om in enkele zinnen te formuleren waar ik je voor wil bedanken. Het zou een enorm addendum worden als ik daar een concrete lijst van zou maken. Het lijkt me van grotere waarde om het gevoel te omschrijven. In mijn eerste jaren als promovendus knalde ik lekker door: het plannen, uitvoeren en verwerken van mijn onderzoeken ging soepel. Je gaf me natuurlijk ondersteuning bij het schrijven van de nodige aanvragen en protocollen, maar mijn autonomie groeide gestaag. Het was in de zomer van 2016 toen ik voelde dat die steile lijn omhoog aan het afvlakken was. Zeker toen het echte schrijfwerk begon heb ik je gewoon ontzettend nodig gehad, kort gezegd: het was zwaar. Toch heb je me de afgelopen jaren nooit scheef aangekeken. Je hebt me nooit boos opgebeld of me laten voelen dat iets slecht was. Er zijn meerdere momenten geweest dat ik vastliep of me schaamde voor wat ik gemaakt had. Jij analyseerde dan de situatie en wist op een neutrale en oordeelvrije manier de boel weer op de rit te krijgen. Daarbij ben je ook nog eens heel empathisch en betrokken bij mij als persoon. Buitengewoon en indrukwekkend. Zowel op vorm als op inhoud ben je onmisbaar voor het tot stand komen van dit proefschrift. Als ooit een van mijn achterkleinkinderen vraagt naar dat boek met mijn naam erop in de kast zal ik direct jouw stem in mijn achterhoofd horen: 'fraai, paar aanvullingen'. Dank Matthijs, voor alles.

Geachte dr. Dorresteijn, beste **Mirrin**, besef even dat wij tijdens een endotoxine experiment hoorden van het overlijden van Michael Jackson. Reken dan maar eens even uit hoe lang het geleden is dat ik je mocht helpen met jouw atazanavir-studie. Je leerde me natuurlijk bloed afnemen, pipetteren, de centrifuge bedienen en alle kneepjes van het endotoxinevak. Maar belangrijker nog de passie en plezier die te vinden was daar op H35 en in het kantoor van Peter. Ik voelde me meteen welkom in de groep. Dank daarvoor.

Geachte dr. van Eijk, beste **Lucas**, ik leerde je kennen als ervaren onderzoeker en als inspiratiebron voor mijn eigen carrière. Toen jij stopte met de opleiding tot internist heb ik je kort daarna uitgenodigd voor een avondje bier drinken. Dit is jaren geleden, maar maakte op mij veel indruk. Je sprak met respect en overtuiging over het proces dat je doormaakte, je toonde je kwetsbaar en emotioneel. Ik vond je al een epische baas, maar toen nog meer. Mijn 'internistenvlam' brandde op dat moment nog hevig, ik twijfelde zelf niet, toch heb je me laten inzien dat het goed is om soms van het gebaande pad te wijken. Dat heb ik zeker meegenomen.

Beste **anonieme, gezonde vrijwilligers**, jullie interesse, inzet en passie voor ons onderzoek heeft me altijd verbaasd. Natuurlijk was er een geldelijke vergoeding, maar omgerekend naar een uurloon (wat niemand doet) is het echt niet bizar veel. Jullie waren vrijwel allemaal veel meer betrokken dan strikt noodzakelijk was. Jullie opmerkzame vragen hielden ons scherp en maakten de experimentdagen tot de mooiste uit mijn hele onderzoekstijd.

Beste kamergenootjes van het eerste uur: lieve **Esther Peters, Kim Timmermans, Dorien Kiers, Rebecca Koch**. Wat delen we toch een bizarre tijd in ons leven. Elk een eigen specifiek project, maar ook zoveel overeenkomsten. Dezelfde worstelingen met aanvragen doen, voorstellen schrijven, gedoe met clinicaltrials.gov of de LPS die weer eens door iets of iemand goedgekeurd moet worden. Wat is het toch heerlijk om dit met elkaar te delen en dan aan het eind van de week luidkeels het vrijdagmiddaglied in te zetten. Onbetaalbaar.

Geachte (inmiddels de meeste niet meer) studenten, beste **Elise Saager, Femke van der Brug, Gijsbert Schuur, Hidde Heesakkers, Jon van Broekhoven, Kim Cortenbach, Mara Wiegerink, Peter Schmitz, Riemer Been, Thijs Kerstens, Thijs Kunz**. Dank voor al jullie inzet en kritische vragen. Jullie zijn allemaal ergens op de route een stukje met mij meegereisd, we hebben van elkaar geleerd en veel mooie momenten gedeeld. Dank voor jullie hulp, ik hoop dat ik jullie wat heb kunnen leren!

IC Research collega's! Beste **Annelies Wassenaar, Aron Jansen, Christopher Geven, Dirk van Lier, Esther Brull, Emma Kooistra, Guus Leijte, Harmke Duindam, Jeanette Vreman, Joeke Nollet, Jonne Doorduin, Judith van den Brule, Lex van Loon, Lisanne Roesthuis, Marcel Houwer, Marloes Witjes, Marieke Zegers, Mark van den Boogaard, Niek Kok, Nienke Peters van Ton, Niklas Bruse, Nina Wubben, Nori Smeets, Paul Rood, Quirine Habes, Pleun Hemelaar, Ruud van Kaam, Sjef van der Velde, Stan Hartman, Wytske Geense**

en alle anderen die tot de groep behoren, jullie belichamen voor mij het concept: 'staan op de schouders van reuzen'. Terugkijkend is het bizar te beseffen hoeveel denkkracht en expertise er nodig is om al die proefschriften op de muur van de afdeling te krijgen. Elk van jullie met een eigen persoonlijkheid, een eigen rol en een eigen manier van werken. Het is een grote eer om van jullie groep onderdeel te mogen zijn.

Geachte dr. van Groenendael, beste **Rogier**, wat hebben wij jouw in een mooi project mee kunnen slepen zeg, haha. Jij was de onafhankelijke onderzoeker die zo strak mogelijk onze protocollen uitlegde aan de vrijwilligers. Je deed dat met volle overtuiging, maar bleef zeer professioneel en gedisciplineerd. Je werd uiteindelijk een echte expert en een vraagbaak voor de deelnemers. Heel gaaf om te zien en enorm veel dank daarvoor. Ik heb uiteraard nog veel meer epische herinneringen aan onze tijd samen, maar die zijn minder geschikt voor gedrukt papier. Laten we die verhalen maar bewaren voor onze kleinkinderen.

Geachte dr. Beunders, beste **Remi**, je hebt jezelf ongetwijfeld gezocht bij de studenten, bij de collega`s of bij de kamergenootjes (waren we dat ooit??), maar jij krijgt natuurlijk je eigen dankstukje. Je kwam als student mij helpen met de RIPC studie en hebt tijdens mijn huwelijksreis met verve die studie overeind gehouden. Totaal terecht natuurlijk dat je daarna een eigen promotietraject kon starten. Ik kan niet goed benoemen waarom je nou zo`n fijne en relaxte vent bent, maar dat is natuurlijk precies het punt. Jouw krachtige persoonlijkheid maakt dat iedereen in de ruimte zich prettig voelt en met je wil werken aan iets moois. Laten we elkaar op de hoogte houden de komende jaren!

Lieve verpleegkundigen en secretariaat: **Hetty van der Eng, Noortje Roovers, Marieke van der A, Hellen Wezel, Chantal Luijten, Margreet Klop-Riehl, Yvonne Kaspers**, nog voordat ik zelf onderzoek deed en alleen maar als student mijn vergoeding kwam ophalen als deelnemer aan de studie van Bart Ramakers was het mij al duidelijk: zo'n afdeling als deze draait op jullie ondersteuning. Talloze keren ben ik binnengestormd voor praktische vragen en ik kon altijd rekenen op een vriendelijk woord en oprechte hulp. Heel erg veel dank daarvoor.

Alle internisten in Nederland, in het bijzonder: **Gerald Vervoort, Jacqueline de Graaff, Jaap Deinum, Jenneke Leentjens, Anneke Bech, Marcel Hovens, Hushang Monajemi, Danielle van den Berg, Lars Valke, Thomas Wierema**. Een jarenlange zekerheid waarmee ik door het ziekenhuis liep was de gedachte: 'ik word internist'. Dat pad, die route was een vast punt waar ik altijd naar kon terugkeren

als ik even niet wist wat ik moest doen, een klassieke stip op de horizon. Ik zag de opleiding tot onderzoeker (waar dit proefschrift het eindpunt van is) als een mooie aanvulling en verdieping op die opleiding tot specialist. Dat was naïef, voor mij heeft dat niet goed gewerkt. Tegen de tijd dat ik mijn laatste jaar wilde gaan afronden was er iets veranderd. Er is iets veranderd in mijzelf en mijn relatie met de interne geneeskunde. Iets dat die laatste stap van ervaren AIOS naar autonoom functionerende internist in de weg zat. De stip bleek een kruispunt. Dank voor jullie begeleiding en flexibiliteit om het onderzoek af te wisselen met de opleiding. Jullie nieuwsgierigheid en analytisch vermogen hebben mij veel geleerd. Dit heeft zeker geholpen in het afronden van dit proefschrift.

Geachte paranimfen, lieve **Yoen en Jakob**, wat geweldig om met jullie aan mijn zijde dit proefschrift te mogen verdedigen. Ik kijk met bewondering naar hoe jullie allebei in het leven staan, intens sociaal en empathisch met een hele rugzak vol heftige ervaringen die jullie meenemen in de keuze`s die jullie maken. Vol zelfvertrouwen, maar niet egocentrisch. Vol interesse in de ander, maar bewust van je eigen grenzen. Met alle drie een huis vol temperament spreken we elkaar niet vaak, maar ik reken jullie tot de meest waardevolle mensen die ik ken. Voor mijzelf, voor jullie naasten, maar ook voor de hele samenleving.

Geachte mijnheer Zwaag, lieve **Luuk**, ik had echt nooit gedacht dat jij ooit voor de klas zou staan als leraar. Toch past dit ontzettend goed bij je en ik hoop dat je er de komende jaren veel bevrediging uithaalt. Gek dat onze levens enerzijds compleet verschillen, maar ook zo vergelijkbaar zijn. Jouw carrièremove viel ongeveer gelijk met mijn verschuivende perspectief op het leven, en het is mooi om dat met je te delen. Dank voor je rust en kritische vragen, dat houd mij scherp. Oja, en dat abonnement op de sportschool stel ik voorlopig nog even uit.

Geachte agent Zwaag, lieve **Giel**, de diplomauitreiking van de politieacademie waar jij officieel als agent werd benoemd zal ik niet snel vergeten. Een hele rij aan collega`s die zich van de dienst hadden afgemeld om met jouw op de foto te kunnen maakte op mij indruk. Het toont dat je nog steeds een enorme sociale spin in het web bent, onafhankelijk van je leeftijd of je rol. Precies zoals ik je heb leren kennen tijdens de studentenfeestjes in Nijmegen waar ik je altijd graag mee naartoe nam. Dank voor je interesse en empathie, dat houd onze band stevig.

Pap en Mam, wat hebben jullie inmiddels een bijzondere rol in ons leven. Jarenlang keken jullie op afstand naar mij en Joyce, hoe wij ons gezin aan het opbouwen waren. Nu wonen jullie op fietsafstand en zijn jullie een integraal onderdeel

geworden van ons leven. Ik kijk er heel erg naar uit hoe dit zich de komende jaren gaat ontwikkelen. Dank voor jullie onaflatende steun en liefde. De basis die jullie legden voor de keuze`s die ik heb gemaakt is van onschatbare waarde.

Jacob, jij zoekt als enige van de broers regelmatig het 'grote bed' op om tussen ons in de nacht te vervolgen. Ik vind dat heel gezellig en geniet van die enorme glimlach waarmee je dan wakker wordt. Ik heb geen idee van wie je dit hebt, maar mijn ochtenden worden er beter van. Dank je voor al die vrolijkheid ondanks die twee intense oudere broers waar je tegen opbokst. Meestal letterlijk.

Ruben, jouw rol als middelste is onmiskenbaar: jij wil iedereen bij elkaar houden en zoveel mogelijk verbinding maken. Gelukkig weet je ook heel goed jezelf te blijven. Het is geweldig om te zien hoe jij soms op eigen initiatief jezelf afzondert. Even bijkomen van alle emoties en prikkels die op je afkomen (zowel intern als extern). Dank voor je passie en je hartstocht!

David, ik kijk met verdriet terug op de keren dat ik je ophaalde bij je oude school en jij eerst een kwartier moest huilen in een poging je emoties enigszins te reguleren. Sinds je van school bent gewisseld heb ik het gevoel dat je meer en meer jezelf kan zijn. Ik ben supertrots op hoe jij nu met spanning en onzekerheid omgaat. Je praat erover en wij kunnen dan hopelijk wat last van je overnemen. Dank voor al je grote vragen, scherpe analyses en woordgrappen. Wat betreft dat laatste zit je nog niet op het niveau van Joyce, nòg niet.

Joyce, ik kan nu oprecht zeggen dat "mijn boekje af is". Je moet daar de afgelopen jaren toch met verbazing naar hebben gekeken. Wekenlang hoor je er niks over, en dan moet ik opeens urenlang tot `s avonds laat een artikel submitten of nog naar het Radboud 'om redenen'. Nu dan toch 'the end of an era' en tijd voor een nieuwe fase. Wij moeten inmiddels erkennen dat we echt volwassen zijn, maar in de kern zijn we volgens mij nog steeds die twee lamme studenten die genieten van de hele dag in bed liggen, grappen maken en films kijken. De realiteit is natuurlijk anders, we hebben een intens gezin opgebouwd wat ons meer dan full-time bezig houd. Het lukt ons gelukkig steeds beter om elkaar op de hoogte te houden van alle gedachte`s en gevoelens in onze hoofden. Dat maakt onze band sterker dan ooit en die groeit ook nog eens elke dag. Ik hou van je.

Chapter 14

Curriculum Vitae & Portfolio

Jelle Zwaag werd geboren op 7 augustus 1986 aan de Dijkweg 47 in Andijk, West-Friesland en groeide op in Hoogkarspel. Hij was de eerste van wat uiteindelijk drie zoons zouden worden van James Zwaag en Marja Kuijk. Hij behaalde in 2004 zijn Atheneum diploma (profiel NT&NG) aan het Martinuscollege te Grootebroek. In die zomer verhuisde hij naar Nijmegen om Geneeskunde te gaan studeren aan de Radboud Universiteit.

Na het behalen van zijn propedeuse werd hij actief bij onder andere de medische faculteitsvereniging, faculteitskroeg De Aesculaaf, het Medisch Heerendispuut Ferus Ebrius en later de studenten volleybalvereniging Heyendaal. Naast dit ruime sociale leven ontwikkelde hij zich breder door te werken als student-assistent voor de dienstdoende artsen bij de interne geneeskunde ('STUART'). Voorafgaand aan het starten van de Masterfase kwam hij in contact met het onderzoeksteam van Peter Pickkers en ging hij als student-assistent ondersteunen bij het onderzoek van Mirrin Dorresteijn. Aan het einde van de masterfase (coschappen) kwam hij op dezelfde afdeling terecht voor zijn onderzoeksstage bij het onderzoek van Matthijs Kox en Lucas van Eijk onder leiding van Prof. dr. Peter Pickkers.

Na het behalen van zijn artsenbul in de zomer van 2013 startte hij direct aan de opleiding tot internist in het Elisabeth-Tweesteden Ziekenhuis (ETZ, Tilburg). Gedurende dit eerste jaar werd hij benaderd om het eerder genoemde onderzoek verder door te zetten met een promotieonderzoek. Dit startte in 2014 en resulteerde in de publicaties afgedrukt in dit proefschrift. Gedurende de jaren daarna wisselde hij zijn opleiding tot specialist af met het promotieonderzoek. In zijn persoonlijke leven veranderde veel: hij trouwde met Joyce en er kwamen drie kinderen bij in het gezin: David (2017), Ruben (2019) en Jacob (2021).

Na een forse burn-out hervatte hij in 2023 het laatste jaar van de opleiding tot internist in het Rijnstate ziekenhuis Arnhem. In die periode heeft hij besloten deze opleiding niet af te ronden en zijn pad te verleggen. Vanaf 2024 werkt hij als arts voor het bedrijf Meditel en richt zich op preventie, keuringen en reisvaccinaties. Naast de zorgtaken binnen het gezin en het huishouden is hij actief bij het Vrijwillig Landschapbeheer Beuningen.

PhD portfolio of Jelle Zwaag

Department: **Radboudumc - intensive Care Research**
PhD period: **01/10/2014 – 2/12/2024**
PhD Supervisor(s): **Prof. R.P. Pickkers**
PhD Co-supervisor(s): **Dr. M. Kox**

Training activities	**Hours**
Courses	
- Radboudumc - eBROK course (2015)	42.00
- RIMLS - Introduction course "In the lead of my PhD" (2016)	15.00
- Mediatraining Radboudumc (2016)	10.00
- Radboudumc - Scientific integrity (2016)	20.00
- Radboudumc - Re-registration BROK (2019)	5.00
Seminars	
- Science Day Internal Medicine, poster presentation (2015)	8.00
- Cytokinemeeting, oral presentation (2015)	6.00
- Open Mind STW meeting, oral pitch (2015)	12.00
- Cytokinemeeting, oral presentation (2016)	6.00
- Symposium Molecular Life Sciences, oral presentation (2016)	4.00
- Science Day RCI, poster presentation (2018)	6.00
- PhD Retreat, poster presentation (2019)	12.00
- Science Day Internal Medicine, oral presentation (2019)	8.00
Conferences	
- Lustrum meeting UMCU, oral presentation (2015)	8.00
- PhD Retreat, poster presentation (2016)	12.00
- NVA Science Day, oral presentation (2016)	12.00
- ESICM Milan, oral presentation (2016)	16.00
- Intensivistendagen, oral presentation (2017)	12.00
- Intensivistendagen, oral presentation (2019)	12.00
- ESICM Berlin, oral presentation (2019)	16.00
Other	
- Research meeting IC (2014)	3.00
- Radboudumc - General Radboudumc introduction for research personnel (2014)	9.00
- Research meeting IC (2015)	3.00
- Research meeting IC (2015)	6.00
- Research meeting IC (2016)	6.00
- Peer review (2019)	22.00
- Weekly attendence cytokine meeting (2019)	120.00
- Research meeting IC (2019)	160.00
Teaching activities	
Lecturing	
- Journal Club (2015)	6.00
- Research meeting Surgery (2015)	4.00
- Datapresention for study team (2015)	6.00
- Research meeting Anesthesiology (2016)	8.00
- Researchproject 2nd year medical students (2020)	45.00
Supervision of internships / other	
- Supervision of medical students (other than master internship) (2019)	80.00
- Supervision of medical master student internship (3 students (2019)	84.00
Total	**804.00**

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