Cytokine responses to repeated, prolonged walking in lean *versus* overweight/obese individuals

RUNNING TITLE: Cytokine responses to repeated exercise

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Abstract

Objectives. Obesity is characterized by a pro-inflammatory state, which plays a role in pathogenesis of metabolic and cardiovascular disease. An exercise bout causes a transient increase in pro-inflammatory cytokines, whilst training has anti-inflammatory effects. No previous study examined whether the exercise-induced increase in pro-inflammatory cytokines is altered with repeated prolonged exercise bouts and whether this response differs between lean and overweight/obese individuals.

Design. Lean (n=25, BMI 22.9±1.5kg/m^2) and age-/sex-matched overweight/obese (n=25; BMI 27.9±2.4kg/m^2) individuals performed walking exercise for 30, 40 or 50 km per day on four consecutive days (distances similar between groups).

Methods. Circulating cytokines (IL-6, IL-10, TNF-α, IL-1β and IL-8) were examined at baseline and <30 minutes after the finish of each exercise day.

Results. At baseline, no differences in circulating cytokines were present between groups. In response to prolonged exercise, all cytokines increased on Day 1 (IL-1β: P=0.02; other cytokines: P<0.001). IL-6 remained significantly elevated during the 4 exercise days, when compared to baseline. IL-10, TNF-α, IL-1β and IL-8 returned to baseline values from exercise day 2 (IL-10, IL-1β, IL-8) or exercise day 3 (TNF-α) onward. No significant differences were found between groups for all cytokines, except IL-8 (Time*Group Interaction P=0.02).

Conclusion. These data suggest the presence of early adaptive mechanisms in response to repeated prolonged walking, demonstrated by attenuated exercise-induced elevations in cytokines on consecutive days that occurs similar in lean and overweight/obese individuals.

Keywords: obesity, inflammation, training, adaptive response

Introduction

In individuals with obesity, a chronic state of low grade-inflammation is present which is characterized by elevated circulating levels of cytokines.[1] This chronic inflammation is associated with the pathogenesis of cardiovascular and metabolic diseases, which are strongly associated with obesity.[2, 3] Exercise training represents a potent non-pharmacological intervention with strong anti-inflammatory effects, leading to lower levels of circulating pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines.[4] Paradoxically, an acute exercise bout elicits a pro-inflammatory response, characterized by a transient rise of pro-inflammatory cytokines.[5, 6] The response of cytokines to acute exercise seems dose-dependent, as higher cytokine levels are observed after exercise of higher intensity and/or longer duration.[6] To support these observations, flu-like symptoms have been reported in relation to an exhaustive acute exercise bout, such as a marathon, which are accompanied by a (transient) rise in circulating cytokines.[6] Even exercise bouts of lower intensity have shown to cause a rise in pro-inflammatory cytokines.[5]

The pro-inflammatory effects of acute exercise *versus* the anti-inflammatory effect of regular exercise training imply the presence of an adaptive mechanism. Repeated exposure to the pro-inflammatory effects of acute exercise may induce an adaptive response, leading to an attenuated exercise-induced release of cytokines, as was previously demonstrated for Interleukin-6 (IL-6) in trained cyclists performing repeated exercise bouts of prolonged duration and moderate intensity (~72% of maximal heart rate).[7] In recent years an increasing number of voluntary exercise events, characterized by repeated prolonged exercise on consecutive days (e.g. walking, swimming, hiking, cycling), is organized. Since the release of cytokines in response to acute exercise seems to increase with longer duration and higher intensity,[6] it is highly relevant to examine physiological responses of cytokines to repeated prolonged exercise during such events.

Obesity is characterized by the presence of low grade inflammation.[3] Accordingly, the acute changes in cytokines in response to prolonged exercise may be affected in overweight individuals because of the presence of higher circulating cytokine levels in resting conditions.

Therefore, the aim of this study is to examine differences in the effect of repeated moderate-intensity prolonged exercise (i.e. prolonged walking 30, 40 or 50km on four consecutive days during the Nijmegen Four Day Marches, a voluntary walking event) on circulating cytokine levels (IL-6, IL-10, Tumor necrosis factor (TNF)-α, IL-1β, and IL-8) and between lean and overweight/obese individuals. We hypothesize that the presence of low-grade inflammation at baseline in overweight/obese subjects leads to exaggerated increases in pro-inflammatory cytokines in response to prolonged exercise when compared to lean individuals.

Methods

A total of 50 adult participants of the Nijmegen Four Days marches were included. Subjects were recruited form a cohort of participants in the Nijmegen 4 Day Marches that filled out a questionnaire as part of the Nijmegen Exercise Study. Subjects with a chronic inflammatory disease (e.g. inflammatory bowel disease, rheumatoid arthritis) and participants that used anti-inflammatory drugs (non-steroidal anti-inflammatory drugs, corticosteroids) were excluded from participation since these conditions can cause a change in circulating cytokines independent from overweight/obesity. All participants completed a distance of 30, 40 or 50 km per day on four consecutive days at a self-selected pace. Every participant was assigned to an individual distance (30, 40 or 50 km) and completed the same distance on the four consecutive exercise days. To answer our research question, subjects were allocated either to a lean (BMI <25 kg/m^2) or overweight/obese (BMI $\geq$25 kg/m^2) cohort. Furthermore, subjects were individually matched based on age, sex and walking distance and were selected for recruitment accordingly. Since exercise intensity is known to influence cytokine levels, participants were also matched based on exercise intensity, calculated based on individually recorded heart rate during the walking event. Written informed consent was obtained from all participants prior to the start of the study. This study was approved by the Medical Ethical Committee of the Radboud University Nijmegen Medical Center, and was conducted in accordance with the declaration of Helsinki.

Baseline data (subject characteristics and blood sample; day 0) were collected 1 or 2 days prior to the start of the event after a minimum resting period of 24 hours. During Day 1, exercise intensity was assessed with the use of a 2-channel chest band system (Polar Electro Oy, Kempele, Finland). At baseline, height and body weight (Seca 888 scale, Hamburg, Germany) were measured to calculate body mass index (BMI). Waist and abdominal circumference were measured with a measuring tape (Seca 201, Chino, USA) to calculate waist-to-hip ratio. A four-point skinfold thickness measurement (biceps, triceps, sub-scapular, supra-iliac) was obtained by a well-trained, experienced researcher to calculate the body fat percentage as previously described.[8] Resting heart rate and blood pressure were measured in supine position, after a 5 minute rest period.

Habitual daily energy intake, macronutrient and anti-oxidant intake were assessed with use of an online validated 180-item semi-quantitative Food Frequency Questionnaire (FFQ).[9] The FFQ reference period was one month, and portion sizes were estimated using standard portions. Intake of total energy and nutrients was calculated using the Dutch Food Composition Database.[10]

At baseline, physical activity levels were assessed with the use of the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH), a validated tool to assess physical activity levels in the Dutch population.[11]

Heart rate was measured with a 2-channel ECG chest band system (Polar Electro Oy, Kempele, Finland) at every 5 km point during Day 1. Exercise intensity was calculated for each measurement by dividing the mean heart rate during exercise by the maximal predicted heart rate (208-0.7*age).[12] By calculating the mean of these percentages of maximal heart rate, the mean intensity for the exercise bout was recorded for each participant.

Venous blood was sampled at baseline (between 9.00 AM to 4.00 PM after a minimum resting period of 24 hours) and at each walking day within 30 minutes after completion of the exercise bout by venepuncture. Blood was centrifuged at 3000 RPM for 15 minutes and plasma was stored at -80ºC until analysis. Cytokines (IL-6, IL-10, TNF-α, Il-1β and IL-8) were simultaneously analyzed using the ultrasensitive MesoScale Discovery (MSD) QuickPlex SQ 120 Instrument with Multi-spot assay (Human Proinflammatory Panel 1, K15049D, MSD) according to the manufacturer's recommendations. The lower detection limit varied per plate and was 0.029–0.159 (IL-6), 0.025–0.051 (IL-8), 0.021–0.042 (IL-10), 0.008–0.061 (IL-1β), and 0.034–0.079 (TNF-α) pg/ml. 34 of the 250 (<15%) samples for IL-1β were below the lower detection limit. These samples were excluded from further analysis. The other cytokines were all above the detection limit.

Statistical Analysis. Data were checked for normality with use of the Shapiro-Wilk test and visual inspection of Q-Q plots. Baseline characteristics were normally distributed and therefore assessed with use of a one-way ANOVA. Cytokine data that was not normally distributed was transformed with use of square root transformation (IL-6 and TNF-α) or inverse transformation (IL-10). Cytokine data were analyzed using a time (exercise day) X group (lean vs. overweight) linear mixed model analysis. Post hoc analysis (Bonferroni) per group was performed when a significant effect was found. The level of statistical significance was defined at $\alpha=0.05$. Data are presented as mean±SD, unless stated otherwise. The statistical analyses were conducted in SPSS 25 (Statistical Package for Social Sciences 25.0, SPSS Inc., Chicago, Illinois, USA)

Results

Subject characteristics are presented in Table 1. We found significant differences between the lean and overweight/obese subgroups for weight, BMI, body fat percentage and waist-hip-ratio, whilst no differences in age and sex distribution were present due to selective matching. Furthermore, the groups reported comparable habitual physical activity levels, daily energy intake and intake of macronutrients and anti-oxidants (Table 1).

All subjects successfully completed the four exercise days. No group differences were present for exercise intensity and exercise duration (Table 1). At baseline, circulating levels of IL-6, IL-10, IL-8, IL-1β and TNF-α were not significantly different between the lean and overweight groups (Figure 1).

Repeated prolonged exercise resulted in a significant change of all cytokines (Figure 1). For all cytokines, except for IL-8 (interaction effect $P=0.02$), we found no differences in the post-exercise levels between lean and overweight/obese subjects (all $P>0.05$, Figure 1). Specifically, IL-6 showed a significant increase that remained elevated on all exercise days ($P<0.001$), with no differences between groups. In contrast, IL-10 increased significantly on exercise day 1(lean group: $P = 0.005$; overweight/obese group: $P=0.003$), but post-exercise levels were similarly declined to baseline on subsequent exercise days in both groups (interaction-effect $P>0.05$). For TNF-α, a significant effect of exercise was only present at exercise day 1 and 2 in the overweight/obese group ($P<0.001$ day 1, $P = 0.02$ day 2), whilst the lean group exhibited no change after exercise on any of the exercise days. IL-1β was significantly higher on day 1 ($P=0.04$) in the overweight/obese group, whilst no post-exercise increases were found in the lean group. For IL-8 a significant Time*Group Interaction effect ($P=0.02$) was found. Both groups showed an increase in IL-8 on day 1 that returned to baseline on subsequent days. The lean group demonstrated a significantly larger decline resulting in below-baseline levels on day 4 ($P=0.001$). (Figure 1)

Discussion

This study presents the following findings. First, prolonged exercise induced an immediate increase in pro- and anti-inflammatory cytokines, and the magnitude of this response was not different between lean and overweight/obese individuals. The exercise-induced elevation in cytokine levels was attenuated following exercise on consecutive days. Except for IL-8, no differences in cytokine responses between lean and overweight/obese individuals were found. Our data suggest the presence of early adaptive mechanisms in inflammatory cytokines in response to repeated prolonged exercise bouts performed on consecutive days, which did not markedly differ between lean and overweight/obese individuals.

In contrast to our hypothesis, no differences in plasma cytokines between the lean and overweight/obese group were present at baseline. In this study, relatively fit subjects were included since all subjects participated in a 4-day walking event. Previous work has shown that overweight and obese subjects with higher cardiorespiratory fitness levels, as a result of higher levels of physical activity, demonstrate lower levels of circulating pro-inflammatory cytokines, compared to unfit individuals.[13] Furthermore, we included subjects with only modest obesity (range BMI: 25-32.9 kg/m^2). Higher levels of BMI are significantly related to higher levels of inflammation.[14] Last, the individuals in the overweight/obese cohort report similar caloric and macronutrient intake when compared to the individuals in the lean cohort, despite being overweight/obese. It can be hypothesized that the reported dietary intake of the overweight/obese cohort is relatively healthy because these are fit individuals who perform exercise on a regular basis. Therefore, the relatively high level of fitness, modest level of obesity and similar dietary intake when compared to lean controls in our study may explain the absence of differences in baseline levels of cytokines between the overweight/obese and lean group.

To our knowledge, this is the first human study that examined responses of different cytokines to repeated exercise bouts on subsequent days and whether these responses differ between lean and overweight/obese individuals. We found no differences between lean and overweight/obese individuals in responses of IL-6, IL-10, TNF-α and IL-1β to repeated exercise. Exercise caused a subsequent rise in circulating IL-6 across the four consecutive exercise days in both groups. Of all known cytokines, IL-6 shows the largest response to exercise.[15] This might explain why IL-6 plasma levels remain elevated throughout the four-days of walking. Furthermore, previous work has shown that expression and circulating levels of IL-6 remain elevated at least 24 hours after cessation of an exercise bout, which might also have contributed to the persistent rise of circulating IL-6 in our study and why no group differences were found.[16] Anti-inflammatory IL-10 showed a significant rise after the first exercise day. The release of IL-10 into the circulation is induced by the presence of IL-6, which was previously observed in both in vitro and in vivo work.[17, 18] This might explain the rise in IL-10 we observed after the first exercise day in both groups. However, IL-10 returns to baseline levels after the subsequent exercise days in both groups, despite the elevated levels of IL-6 on all 4 exercise days. It has been hypothesized previously that IL-6 levels have to reach a certain threshold to cause IL-10 production by leukocytes.[17] Possibly this threshold was not reached on exercise day 2-4, since IL-6 levels are lower on exercise day 2-4 when compared to exercise day 1, which may explain the return to baseline of IL-10 levels from exercise day 2 onwards.

We observed a significant change in cytokines on day 1 (IL-8, TNF-α and IL-1β) and day 2 (TNF-α in the overweight/obese cohort) when compared to baseline, that was no longer present on the consecutive exercise days. This suggests an attenuated acute response to exercise of pro-inflammatory cytokines (TNF-α, IL-8 and IL-1β) after repeated bouts of prolonged exercise. In discordance with our hypothesis, we found no differences in this attenuation between lean and overweight/obese individuals, except for IL-8. Our time-effects results show a transient rise in IL-1β on day 1 in the overweight/obese group, whilst IL-1β in the lean group shows no change. The modest response of IL-1β to exercise might relate to the presence of a persistent rise in IL-6. Previous work postulated that under influence of IL-6, the presence of IL-1receptor antagonist (IL1-ra) in the circulation is induced,[4, 19] which subsequently causes a decrease in IL-1β by competitively binding to the IL-1receptor.[19] The presence of elevated levels of IL-6, therefore, may contribute to the attenuated exercise-induced increase in IL-1β in the overweight/obese group.

IL-8 is a cytokine involved in chemotaxis and phagocytosis. IL-8 is elevated in individuals with obesity and related to constitutes of the metabolic syndrome, such as waist circumference and insulin resistance (i.e. HOMA-IR). [20] The difference between the lean and overweight/obese group in IL-8 response to repeated prolonged exercise seems to be caused by the decrease in IL-8 in the lean cohort on exercise day 4 when IL-8 decreases below baseline. This attenuated response of IL-8 suggests the presence of early adaptations to repeated bouts of prolonged exercise. This is in line with previous work that found a decrease in IL-8 after exercise training, although the exercise stimulus in our study is different due to the prolonged duration.[21]

Based on our data, one may speculate that the shift from the pro-inflammatory effects of a single bout of prolonged exercise to the known anti-inflammatory effects of exercise training is mediated by a change in cytokine secretion in response to repeated prolonged exercise bouts. During acute prolonged exercise, cytokines are secreted from adipose tissue[22] and skeletal muscle.[23] Exercise training is known to change gene expression in these tissues, which eventually results in altered secretion patterns of cytokines.[24-27] Gene expression in skeletal muscle is altered during each prolonged exercise bout because of altered contractile activity,[23] but is also believed to be influenced by the increased respiratory capacity in skeletal muscle that occurs by aerobic exercise training.[28] These adaptive responses, where responses to acute bouts of exercise relate to subsequent adaptation, have been referred to as *hormesis*: a biological process in which exposure to a low amount of a damaging factor leads to an adaptive beneficial effect in the organism.[29] Pro-inflammatory cytokines, i.e. the pro-inflammatory state which occurs during and after a single bout of exercise could be classified as a "hormesis stimulus", where the acute responses to exercise mediate an adaptive response contributing to health benefits when performed repeatedly.[30] The attenuated response of cytokines we observed in our study fits well in this hypothesis. This is further supported by a study that examined responses of IL-6 mRNA expression in skeletal muscle after a 3-h exercise protocol, before and after 10 weeks of exercise training in untrained men. A decrease in IL-6 mRNA expression levels in response to prolonged exercise from 76-fold (before training) to 8-fold (after the training period) was observed.[2727] Although it is important to emphasize that our design does not resemble the typical exercise training response, our data support the presence of an attenuated magnitude in exercise-induced changes in circulating cytokines when subjects repeat the same exercise stimulus on subsequent days.

Some limitations must be considered. Due to practical reasons, it was impossible to measure cytokines directly before the start of the walking exercise on the four consecutive days. Baseline levels were measured one or two days prior to the start of the walking event. Therefore, we were unable to assess potential adaptations in resting levels of cytokines (prior to each exercise bout). However, the primary goal of this study was to investigate differences between overweight and lean individuals in cytokine responses to repeated prolonged exercise bouts, which were therefore assessed immediately after cessation of such a bout. In our study, a prolonged exercise stimulus was used to examine cytokine responses to repeated exercise. Because of the duration of the exercise bouts (8.6 ± 2.1 hours) this design is not intended as a training study but rather as a model to examine physiological changes in response to repeated exercise stimuli.

Conclusion

This study demonstrated that prolonged exercise induces an immediate increase in pro- and anti-inflammatory cytokines in lean and overweight/obese individuals while repeated bouts of prolonged exercise lead to an attenuated exercise-induced cytokine response. Our data suggest that overweight/obese subjects, when matched for sex, age and fitness, largely show comparable exercise-induced changes in levels of cytokines across consecutive days of prolonged walking exercise. Therefore, our data suggest the presence of early adaptive mechanisms in circulating cytokines in response to repeated exercise bouts..

Practical Implications

- Cytokines are circulating factors that play a role in inflammation in the human body. Inflammation contributes to the development of metabolic and cardiovascular disease. Our study reveals that a prolonged walking exercise results in a rise in these cytokines that attenuates when this exercise bout is repeated.

- Our study demonstrates that both lean and overweight individuals largely show comparable exercise-induced changes of cytokines across four days of repeated prolonged walking.
- The attenuation of cytokine IL-8 occurs delayed in overweight individuals when compared to lean controls.

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Table 1. Physiological characteristics of the study groups

	Lean subjects (n=25)	**Overweight/Obese subjects (n=25)**	**P-value***
Baseline characteristics			
Age (years)	56.4 ± 14.4	58.4 ± 11.9	0.60
Male sex (%)	56%	56%	1.00
Weight (kg)	69.3 ± 7.7	84 ± 12.6	<0.0001
Body mass index (kg/m²)	22.9 ± 1.5	27.9 ± 2.4	<0.0001
Body fat percentage (%)	27.3 ± 6.6	33.5 ± 6.7	0.002
Waist-to-hip ratio	0.89 ± 0.1	0.95 ± 0.1	0.02
Systolic blood pressure (mmHg)	139 ± 21	142 ± 16	0.59
Diastolic blood pressure (mmHg)	86 ± 12	89 ± 9	0.92
Resting heart rate (bpm)	62 ± 8	63 ± 7	0.54
Daily physical activity levels			
Total SQUASH score	6342 ± 3974	7397 ± 4687	0.41
METmin/day	968 ± 522	1130 ± 629	0.32
Habitual dietary intake			
Caloric intake (kJ)	9592 ± 2516	9570 ± 3441	0.98
Total protein (g)	82 ± 21	87 ± 30	0.48
Total fat (g)	93 ± 34	89 ± 35	0.72
Saturated fat (g)	31 ± 13	31 ± 15	0.87
Total carbohydrates (g)	249 ± 64	244 ± 101	0.86
Fibre (g)	27 ± 7	25 ± 10	0.34
Dietary anti-oxidant intake			
Retinol (µg)	616 ± 369	655 ± 446	0.74
Vitamine E (mg)	16 ± 5	16 ± 7	0.71
Vitamine C (mg)	121 ± 54	115 ± 59	0.71
Exercise characteristics			
Exercise intensity (%HR$_{max}$)	66 ± 5	69 ± 5	0.11
Exercise distance • **30 km (n)** • **40 km (n)** • **50 km (n)**	 5 15 5	 5 15 5	 - - -
Exercise duration day 1 (minutes)	510 ± 129	444 ± 167	0.12
Exercise duration day 2 (minutes)	534 ± 83	522 ± 98	0.64

Exercise duration day 3 (minutes)	508 ± 140	509 ± 114	0.97
Exercise duration day 4 (minutes)	565 ± 112	540 ± 124	0.46

*One-way ANOVA between lean and overweight subgroups

Figure Legends

Figure 1 Mean circulating cytokine levels of IL-6 (A); IL-10 (B); TNFα (C); IL-1β (D) and IL-8 (E) at baseline and after each exercise day, with data being presented for lean subjects (◯) and overweight/obese subjects (■). Error bars represent the standard error of the mean. * Significantly different from baseline in lean group (P <0.05); # Significantly different from baseline in overweight/obese group (P <0.05)

Figure 1