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Research Paper

Immobilization increases interleukin-6, but not tumour necrosis factor-α, release from the leg during exercise in humans

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New Findings

- **What is the central question of this study?**
  Does physical inactivity influence the exercise-induced release of tumour necrosis factor-α and interleukin-6 in healthy humans? In young, healthy subjects, we immobilized one leg for 2 weeks, followed by 45 min two-legged exercise where one leg served as the control and the other was the previously inactive leg.
- **What is the main finding and its importance?**
  We found that prior physical inactivity enhances interleukin-6 release during exercise, and it is released in the blood from the legs during exercise much faster than previously known. However, tumour necrosis factor-α is not released in the blood with exercise, even from a previously inactive leg.

Data on interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) release during acute exercise are not conclusive, and information is lacking about the impact of physical inactivity. Some studies have shown an increase, but others report no changes in IL-6 and TNF-α release during exercise. We have now studied the temporal relationship of leg IL-6 and TNF-α release before and during isolated two-legged exercise after 14 days of one-leg immobilization (IM) while the other leg served as the control (CON) leg. Fifteen healthy male subjects (mean ± SEM age, 23 ± 1 years; body mass index, 23.6 ± 0.7 kg m^{-2}; and maximal oxygen uptake, 46.8 ± 1.4 ml kg^{-1} min^{-1}) performed 45 min of two-legged dynamic knee-extensor exercise at 19.6 ± 0.8 W. Arterial and femoral venous blood samples from the CON and the IM leg were collected every 15 min during exercise, and leg blood flow was measured with Doppler ultrasound. The arterial plasma IL-6 concentration increased ($P < 0.05$) with exercise (rest, 1.3 ± 0.1 pg ml^{-1}; 15 min, 1.9 ± 0.2 pg ml^{-1}; 30 min, 2.4 ± 0.2 pg ml^{-1}; and 45 min, 3.1 ± 0.3 pg ml^{-1}). Interleukin-6 release occurred after 15 min of exercise, and the release from the IM leg was significantly greater compared with the CON leg after 45 min (1114 ± 152 *versus* 606 ± 14 pg min^{-1}, respectively, $P < 0.05$). Tumour necrosis factor-α release did not differ between the CON and the IM leg, and arterial concentrations remained unchanged during exercise ($P > 0.05$). In conclusion, prior immobilization enhances release of IL-6 from the leg during exercise at a moderate workload, and the release is already present in the early phase of exercise. Neither immobilization nor exercise had an effect on TNF-α release in the working legs.

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Cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), are low molecular weight proteins and peptides that work in a hormone-like fashion, exerting specific endocrine effects by mediating interactions among immune and non-immune cells, organs and organ systems throughout the body (Nieman *et al.* 2001; Gomez-Merino *et al.* 2006).

In response to exercise, circulating levels of IL-6 increase by up to 100-fold, when long duration and high intensity exercise, e.g. marathon running, is performed (Ostrowski *et al.* 2000; Suzuki *et al.* 2003). Interleukin-6 is considered to be a marker of inflammation and an immunomodulatory cytokine produced predominantly by leucocytes in response to exercise-induced local damage in the working muscles, especially after eccentric exercise (Bruunsgaard *et al.* 1997). It has been suggested that skeletal muscle expresses the *IL-6* gene and IL-6 protein during continuous contractile activity (Hiscock *et al.* 2004; Steensberg *et al.* 2002). The release of IL-6 can be affected by bioavailability of carbohydrates (Nieman *et al.* 2003). However, a recent study showed that the release of IL-6 from the leg was not correlated to release or uptake of exogenous substrates or to muscle glycogen content or utilization during whole-body exercise, and therefore the relationship of IL-6 to carbohydrate turnover could not be confirmed (Helge *et al.* 2011). Thus, the importance and possible role of IL-6 release from the exercising leg remain open questions. Moreover, the data describing kinetics of IL-6 release from working legs during acute exercise are also not conclusive and lack information about the impact of training status. Some studies have shown an increase (Febbraio *et al.* 2003), whereas others report no changes (Steensberg *et al.* 2002) in leg IL-6 release during the first hour of exercise.

Data on venous plasma concentrations of TNF-α during physical activity vary. Thus, no change (Steensberg *et al.* 2002), an increase (Jammes *et al.* 2009) or a decrease (Gokhale *et al.* 2007) in response to exercise has been demonstrated.

Some studies have shown that neither circulating monocytes (Starkie *et al.* 2001) nor skeletal muscles (Steensberg *et al.* 2002; Febbraio *et al.* 2003; Helge *et al.* 2011) release TNF-α during exercise, while others report an increase in muscle TNF-α mRNA content (Nieman *et al.* 2005). Thus, the importance of TNF-α release during exercise and the effect of physical inactivity are not yet fully elucidated.

In this study, our aim was to investigate leg IL-6 and TNF-α release during moderate intensity exercise, and to test whether there were differences in the release of these cytokines between a previously immobilized (IM) and a control (CON) leg.

Methods

Ethical approval

The study was performed according to the latest revision of the Declaration of Helsinki and was approved by the Ethical Committee of Copenhagen (H-4-2010-85). All subjects were informed about the possible risks and discomfort involved before written consent to participate was obtained.

Subjects

Fifteen healthy male subjects (age, 23 ± 1 years; and body mass index, 23.6 ± 0.7 kg m^{-2}) volunteered to participate in the study. There are no data for the control leg in two subjects, because insertion of the venous catheter was not successful.

Prestudy preparation

Prior to randomization and immobilization of the selected leg by a Donjoy cast for 14 days with a knee angle of 60 deg, subjects were familiarized with the exercise protocol using a custom-made two-legged knee-extension ergometer. Subsequently, the value of W_{max}, defined as the point where the slope of the whole-body O_2 uptake, heart rate and pulmonary ventilation with increasing workload changed from a linear relationship to an exponential relationship, was determined using a graded exercise test for each leg separately. At that point the quadriceps femoris is no longer able to sustain the workload, and therefore additional muscles are recruited during exercise, leading to the non-linear relationship (Andersen *et al.* 1985).

The subjects had a maximal oxygen uptake of 46.8 ± 1.4 ml kg^{-1} min^{-1}, assessed during an incremental cycle test to exhaustion (Jaeger ER800, Ergoline, Hochberg, Germany). The maximal oxygen consumption was achieved when two of the following three criteria were met: (i) plateau in oxygen consumption; (ii) achievement of a minimum of 85% of predicted maximal heart rate; (ii) respiratory exchange ratio above 1.15 (the average value over 20 s was used). The maximal heart rate during the maximal oxygen uptake test was measured with a Polar RS400 (Polar Electro Oy, Kempele, Finland).

Experimental protocol

After immobilization of one leg for 2 weeks, the subjects reported to the laboratory in the morning after an overnight fast. Catheters were placed into the brachial artery (20 gauge arterial cannula; Becton Dickinson a/s, Albertslund, Denmark) and both femoral veins (14 gauge

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catheter; Arrow International, ViCare Medical, Birkeroed, Denmark) under local anaesthesia using an aseptic technique. The catheters were intermittently flushed with sterile sodium chloride to maintain patency.

After resting for at least 1 h, the subject was positioned in a two-legged knee-extension ergometer in a semi-supine position. The ergometer is a modified version of the one-legged knee extension ergometer (Andersen *et al.* 1985), which has been used previously (Stallknecht *et al.* 2007). Subsequently, at rest, blood was sampled simultaneously from the brachial artery and femoral veins at −15 min and immediately before exercise. Thereafter, 45 min of exercise with both legs was started. The absolute leg workload was set to 50% of the W_{max} determined before the immobilization. During exercise, blood was sampled after 15, 30 and 45 min. Femoral venous blood flow was measured at all time points in both legs by Doppler ultrasound (Acuson S2000; Siemens Healthcare, Ballerup, Denmark). Heart rate was recorded continuously during exercise with a Polar RS400. Whole-body oxygen consumption was measured from the 20th to the 27th minute of exercise using an Oxycon Pro (CareFusion GmbH, Hoechberg, Germany). Throughout the experiment, subjects had free access to water.

The day after the exercise experiment, the subjects reported to the laboratory in order to determine changes in their W_{max} after immobilization. This could not be done before the exercise experiment, because this test would interfere with the immobilization protocol. The relative workload for both legs during the exercise experiment was calculated.

Analytical procedures

Blood was sampled anaerobically and distributed into tubes containing heparin or Trasylol/EDTA. The heparinized samples were immediately analysed for haematocrit (ABL800 Flex, Radiometer, Copenhagen, Denmark). Plasma for determination of cytokines was separated by centrifugation, frozen on dry ice, and stored at −80°C until further analysis. Plasma IL-6 and TNF-α were measured with high-sensitivity enzyme-linked immunosorbent assay kits from R&D Systems (Minneapolis, MN, USA), Human IL-6 Immunoassay (HS600B) and Human TNF-α Immunoassay (HSTA00D), respectively.

Calculations

The Fick principle was used to calculate IL-6 and TNF-α release across exercising legs using brachial arterial and femoral venous plasma concentration differences multiplied by plasma flow [calculated as blood flow × (1 − haematocrit)].

Statistical analysis

Data were analysed by SigmaPlot 11.0 software (Systat Software Inc., San Jose, CA, USA). Two-way repeated-measures ANOVA and paired Student's *t* test was performed as appropriate. In the case of significant main effects or interactions, the Holm–Sidak *post hoc* test was performed to discern statistical differences. Data are expressed as means ± SEM. A value of $P < 0.05$ was considered to be significant in two-tailed testing.

Results

The average heart rate during the exercise was 114 ± 0.3 beats min^{-1} (59 ± 2% of maximal heart rate). Whole-body oxygen uptake during the exercise was 1.10 ± 0.02 l O_2 min^{-1}. During the exercise experiment, the absolute workload was identical for the two legs (19.6 ± 0.8 W). Subsequently (i.e. the day after), W_{max} was determined for both legs and it tended to be higher in the CON leg compared with the IM leg, 42.2 ± 3.1 *versus* 37.6 ± 3.6 W, respectively ($P = 0.06$). Thus, the relative workload for the IM leg was higher than for the CON leg, 58 ± 3 *versus* 49 ± 2% of W_{max}, respectively ($P < 0.05$).

There was a significant increase in thigh plasma flow after 15 min of exercise (Fig. 1; $P < 0.05$); thereafter, it remained unchanged and was similar between the two legs at all time points. Throughout the exercise, the arterial plasma IL-6 concentration increased significantly at each time point (Fig. 2; $P < 0.05$), whereas the venous plasma IL-6 concentration in the CON and the IM leg was unchanged during the first 30 min of exercise and increased only after 45 min (Fig. 2; $P < 0.05$). At

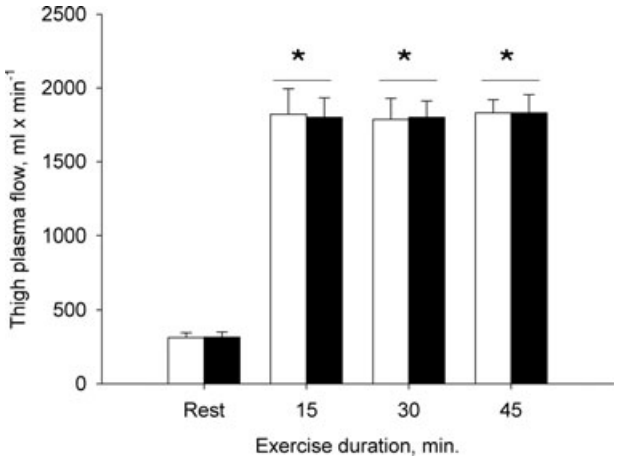

Figure 1. Thigh plasma flow during rest and two-legged knee-extension exercise
*Significant difference compared with rest ($P<0.05$). Filled and open bars represent the immobilized and the control leg, respectively. Values are shown as means + SEM.

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rest and throughout exercise, the venous plasma IL-6 concentrations exceeded arterial concentrations, and after 45 min the plasma IL-6 concentration in the IM leg was higher than in the CON leg (Fig. 2; $P < 0.05$). The venoarterial plasma IL-6 difference was significantly decreased at 15 min compared with rest in both legs and remained unchanged until the end of exercise (Fig. 3*A*; $P < 0.05$). There was a significant difference between the CON and the IM leg venoarterial plasma IL-6 difference at 45 min (Fig. 3*A*; $P < 0.05$). There was a twofold increase in IL-6 release from both legs during the first 15 min of exercise (Fig. 3*B*; $P < 0.05$), and IL-6 release from the immobilized leg continued to increase throughout the exercise. As a result, there was a significant difference between the CON and the IM leg IL-6 release after 45 min (Fig. 3*B*; $P < 0.05$). Throughout the exercise, there were no changes in arterial or venous plasma TNF-α concentrations (at rest and at 45 min, respectively: average for both legs, artery, 1.0 ± 0.2 *versus* 1.0 ± 0.2 pg ml^{-1}; CON, 1.1 ± 0.1 *versus* 0.9 ± 0.1 pg ml^{-1}; and IM, 0.9 ± 0.1 *versus* 0.9 ± 0.1 pg ml^{-1}; $P > 0.05$). Consequently, there was no significant release or uptake observed from the working legs (at rest and at 45 min, respectively: CON, 2 ± 30 *versus* -346 ± 272 pg min^{-1}; and IM, -42 ± 37 *versus* -315 ± 314 pg min^{-1}; $P > 0.05$).

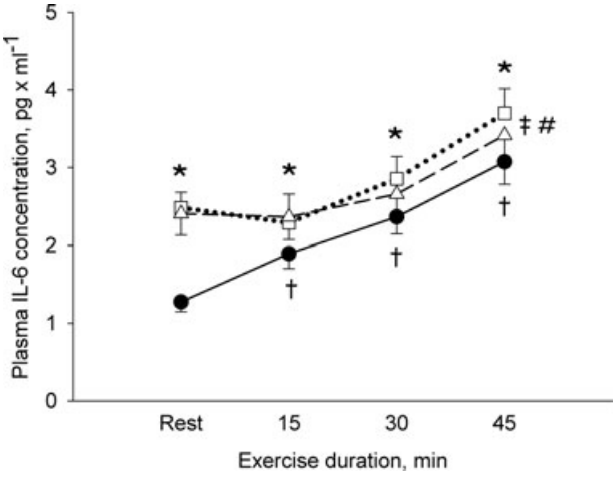

Figure 2. Brachial arterial and femoral venous plasma interleukin-6 (IL-6) concentrations at rest and during two-legged knee-extension exercise
At rest, the average of measurements from samples obtained at two time points (−15 and 0 min) was used. Filled circles (continuous line), open triangles (dashed line) and open squares (dotted line) represent IL-6 plasma concentrations in the artery, femoral venous control leg and femoral venous immobilized leg, respectively. * $P < 0.05$, artery *versus* both femoral veins; # $P < 0.05$, femoral veins, CON *versus* IM; † $P < 0.05$, artery, *versus* previous time point; and ‡ $P < 0.05$, both femoral veins, rest *versus* 45 min. Values are shown as means ± SEM.

Discussion

The main finding of this study is that immobilization for 14 days increases IL-6 release from the working leg during submaximal exercise when compared with the control leg. However, neither immobilization nor exercise had an effect on TNF-α release from the working legs. Furthermore, IL-6 release had already increased by 15 min after onset of exercise.

The data describing the impact of training status on cytokine responses to acute exercise are not conclusive. It has been shown that athletes have an attenuated cytokine response to acute exercise, because they had smaller change in the venous plasma IL-6 and TNF-α concentrations

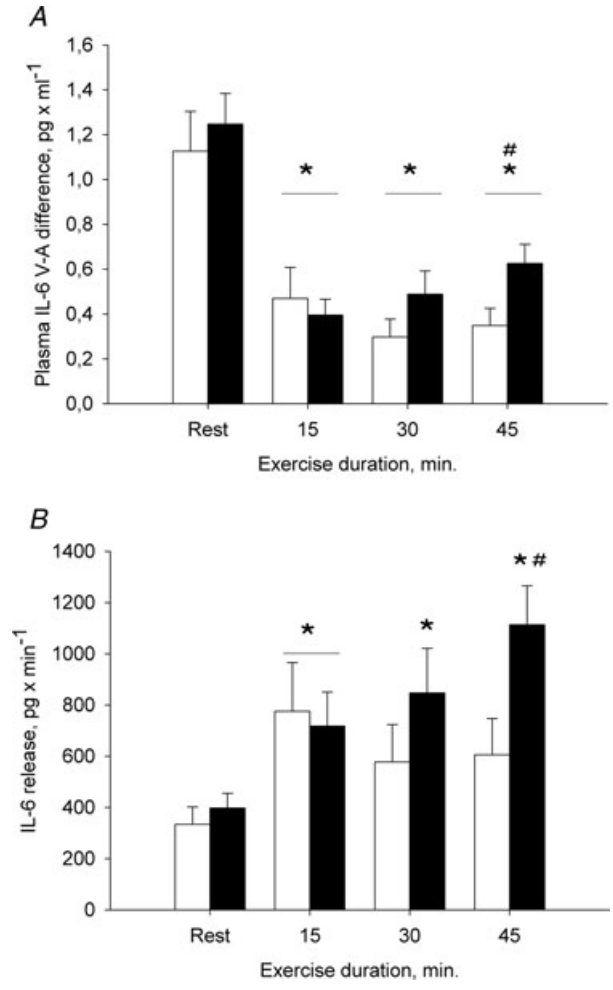

Figure 3. Venoarterial (V–A) plasma IL-6 difference (*A*) and thigh IL-6 release (*B*) during rest and two-legged knee-extension exercise
* Significant difference compared with rest ($P<0.05$); # significant difference between legs ($P<0.05$). At rest, the average of measurements from samples obtained at two time points (−15 and 0 min) was used. Filled and open bars represent the immobilized and the control leg, respectively. Values are shown as means + SEM.

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following an intermittent running exercise in comparison to non-athletes (Gokhale *et al.* 2007). In contrast, acute exercise induced a 30 min postexercise increase in venous plasma IL-6, but not TNF-α, concentrations with training (male rowers; Mäestu *et al.* 2010). Finally, the arterial IL-6 concentration has been shown to increase after 3 h of exercise in the trained, but not in the untrained, state (Fischer *et al.* 2004). After additional 2 h postexercise, however, the arterial IL-6 concentration was also increased in the untrained state (Fischer *et al.* 2004). This increase in arterial IL-6 in the trained state was contrasted by the fact that Fischer *et al.* (2004) found that acute exercise-induced skeletal muscle IL-6 mRNA expression was markedly lower after endurance training (despite fivefold higher absolute workload). Overall, there is a complex picture in the previous literature regarding the effect of training on plasma IL-6 concentration during acute exercise.

Instead of a training programme, we have now used immobilization to test the influence of physical activity on IL-6 release during an acute bout of exercise. Our data showed that the exercise-induced IL-6 release was significantly greater across the IM leg compared with the CON leg at the same absolute workload. These findings are in agreement with other studies that show a positive relationship between IL-6 release and relative exercise intensity (Helge *et al.* 2003), because the relative workload for the IM leg was significantly higher than for the CON leg. Even though the physical inactivity imposed by the immobilization may not be regarded as directly opposite to training, it appears that findings in the present study are in agreement with the data of Gokhale *et al.* (2007), but not those of Mäestu *et al.* (2010) and Fischer *et al.* (2004). The strength of the present study is the fact that we have measured the IL-6 concentrations in plasma samples simultaneously obtained directly from the two legs.

Data describing the kinetics of IL-6 release during acute exercise are not conclusive. It has been shown that IL-6 release from the working legs does not increase until after at least 1 h of knee-extensor exercise at ∼50% W_{max} (Steensberg *et al.* 2000, 2002). In contrast, one study found that IL-6 was released from the working legs after 10 min of cycling exercise when pre-exercise muscle glycogen stores were low (MacDonald *et al.* 2003). However, plasma IL-6 concentrations in both low and high glycogen content trials where similar after 60 min of exercise at a relative workload of 70% of peak oxygen uptake (MacDonald *et al.* 2003), suggesting either that IL-6 is released mainly from organs other than muscle during exercise of this duration or that clearance of plasma IL-6 is affected differently. Our data showed a significant increase in IL-6 release from both working legs after the first 15 min of exercise.

Data on the plasma concentration and muscle mRNA expression of TNF-α during physical activity vary, and the mechanisms involved in TNF-α activation during exercise are still unknown. Similar to our data, previous studies evaluating the TNF-α response to acute exercise have shown no changes in plasma TNF-α release from working legs (Helge *et al.* 2011) or in TNF-α mRNA expression in muscles (Steensberg *et al.* 2002; Febbraio *et al.* 2003). It has been proposed that the increase in plasma TNF-α concentrations after prolonged running, e.g. marathon (Toft *et al.* 2000; Starkie *et al.* 2001), was the result of systemic endotoxaemia induced by a decrease in blood flow to the splanchnic bed (Steensberg *et al.* 2002). An exercise-induced increase in TNF-α concentrations may also be related to the exercise intensity, as previously shown (Kimura *et al.* 2001). It cannot be excluded that the moderate exercise intensity applied in this study was not of sufficient intensity to elicit changes in plasma TNF-α concentration. Furthermore, it has been proposed that upregulation of *IL-6* gene expression in skeletal muscle during muscle contraction may inhibit an increase in TNF-α production (Steensberg *et al.* 2002) in order to maintain glucose homeostasis (Youd *et al.* 2000), and this link between IL-6 and TNF-α cannot be excluded in the present study.

Some potential limitations of the study should be mentioned. First, the relative workload for the two legs was not similar. An earlier study has shown that IL-6 increases with increasing exercise intensity (Ostrowski *et al.* 2000), implying that the slightly, but significantly, higher workload by the immobilized leg would cause a greater IL-6 release. However, a later study has shown that this workload dependence operates only at the high end (>65% W_{max}) of the workload. Secondly, the 2 week immobilization would be expected to induce insulin resistance in the skeletal muscle, which in the resting situation would change substrate and fuel metabolism and thereby potentially influence IL-6 release. During exercise, however, substrate uptake and release are not influenced by insulin resistance (Dela *et al.* 1996).

In conclusion, 2 weeks of unilateral immobilization enhances leg IL-6 release during exercise at a moderate workload, and the release is already present in the early phase of exercise. Neither immobilization nor exercise had an effect on TNF-α release in the working legs.

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