IT IS WELL DOCUMENTED THAT exercise induces marked increases in several plasma cytokines (20–22, 25, 26, 38). These cytokine responses are often closely linked; thus an increase in plasma tumor necrosis factor is accompanied by a subsequent increase in interleukin (IL)-6, which is followed by an increase in IL-1 receptor antagonist (IL-1ra) (22, 39). In relation to concentric as well as eccentric exercise, IL-6 is produced in larger amounts than any other cytokine (27). Recent studies have demonstrated that IL-6 is produced locally in the working muscle, as opposed to IL-1ra, which does not appear to be originating from muscle cells but from mononuclear and polymorphonuclear leukocytes (23, 27, 39). Moreover, IL-6 produced in contracting skeletal muscles is released in large amounts into the circulation. This can account for the exercise-induced increase in plasma IL-6 (34). Additionally, it has been demonstrated that the production rate and total production of IL-6 are further enhanced when muscle glycogen content is low (14, 32). It is well known that IL-6 stimulates the production of IL-1ra, which binds to and blocks the IL-1 receptor, thus exerting strong anti-inflammatory effects (6, 11, 39). With exercise, peak IL-1ra is found 1–2 h after peak IL-6 (17, 22). From this, it is assumed that the level of IL-1ra reflects the production of IL-6.

Most elite athletes perform more than one training session per day. Recently, it has been shown that the second bout of exercise on the same day induces more pronounced changes in leukocyte subsets and stress hormones, especially in epinephrine and growth hormone, compared with a single bout of identical exercise (29, 28). Although it has been suggested that epinephrine plays a mechanistic role in the exercise-induced cytokine production (5, 24), a recent study did not lend much support to this idea (33). However, during a second bout of endurance exercise, muscle glycogen content may be compromised by the previous exercise bout (35). This may induce an energy crisis in the working muscle, affecting both carbohydrate and fat metabolism, if the recovery period between the exercise sessions is short and the work intensity is high. Muscle-derived IL-6 has recently been suggested to work in a hormone-like fashion, mediating glucose homeostasis during exercise (27, 35–37, 40).

Several investigations have studied pro- and anti-inflammatory cytokine responses to various protocols of a single bout of exercise (17, 19, 21, 22, 38), but there is very limited information on how repeated bouts of exercise on the same day affects IL-6 and IL-1ra (18). Thus we designed a study that used two identical bouts of exercise but with a different duration of rest between the exercise sessions. We hypothesized that a second bout of exercise performed after only 3 h of rest and with incomplete glycogen resynthesis after the first bout would induce more pronounced increases in plasma IL-6 and IL-1ra levels compared with a single bout performed immediately after the first bout. Such findings would illustrate that exercise does affect cytokine responses during repeated exercise, and this may be interesting for the training of elite athletes.
bout of exercise. Furthermore, we wanted to examine whether extending the rest period between the exercise sessions from 3 to 6 h would attenuate the IL-6 and IL-1ra responses.

METHODS

Subjects. Nine male, elite endurance athletes [four triathletes and five speed skaters, age 21–27 yr, weight 74.7 ± 5.4 kg, maximal O2 uptake (VO2 max) 69.1 ± 3.7 ml·min⁻¹·kg⁻¹] from the respective Norwegian national teams participated. All subjects were accustomed to two daily training sessions as part of their normal exercise schedule, including cycling as one of the training modalities. A medical examination was performed on each subject before he entered the study, and they were thoroughly informed about the purposes and procedures of the study before a written consent was obtained. The protocol was approved by the Regional Committee for Ethics in Medical Research, Norway.

Design. All subjects participated in four trials, each lasting from 0700 to 0800 the following day: 1) complete bed rest (Rest), 2) one bout of exercise from 1515–1630 (One), 3) two bouts of exercise (1100–1215 and 1515–1630) with 3 h of rest in between (Short), and 4) two bouts of exercise (0800–0915 and 1515–1630) with 6 h of rest in between (Long) (Fig. 1). The trials were separated by 12–17 days to ensure complete recovery among trials and were randomized in a counterbalanced order with each subject serving as his own control. The triathletes were tested between January and March, and the speed skaters between April and June, i.e., outside the competitive season for both groups. Except for the last 2 days before each trial, when exercise was regulated by the study protocol, the subjects completed their regular training program without any interruption during the study period.

Pretrial procedures. Approximately 2 wk before the start of the study, the subjects performed an incremental exercise test on a cycle ergometer (Lode, Groningen, The Netherlands), starting at a workload of 175 W with a subsequent increase of 25 W every 5 min until they had reached a workload of 275 W. The subjects then rested for 10 min before a continuous ramp test was used to estimate VO2 max, starting at 275 W, with a subsequent increase of 25 W every 30 s until volitional exhaustion (i.e., the subject could not sustain the workload for a period of >30 s). A respiratory exchange ratio (RER) of >1.1 was used as an additional criterion that VO2 max had been reached. The results were used to estimate a workload corresponding to 70% of VO2 max for each subject based on the regression line of O2 uptake vs. workload from the incremental exercise test. However, in the next experimental trials, performing at the individually estimated workload resulted in a mean O2 uptake of ~75% of VO2 max.

No medication or nutritional supplements were allowed the last week before or throughout the study period. Serum ferritin and whole blood hemoglobin concentrations were measured 2 wk before the first trial and at the end of each trial. Iron supplementation was given if serum ferritin concentration was <30 µg/l but was discontinued 7 days before each trial. Hemoglobin concentration was measured again in the morning before each trial, and the trial was postponed for 1 wk if the concentration was reduced by >1.0 g/dl from the previous trial. If a subject had an episode of illness with fever or malaise, the trial was postponed until he had been without symptoms or medication for at least 5 days.

High-intensity exercise was not allowed during the last 2 days before trials, and no exercise was permitted the last day before each trial. A dietary record was obtained for the last 24 h before the first trial, and the subjects were instructed to consume an identical diet the day before each subsequent trial. A standardized meal of cereal and milk was served at 9:00 PM the evening before each trial, and the subjects spent the night at the National Sports Centre next to the laboratory.

Trial procedures. The subjects arrived in the laboratory at 7:00 AM, emptied their bladder, had their body weight measured, and were subsequently put to bed. A flexible temperature probe was inserted in the rectum, and the subjects were connected to a temperature, electrocardiogram, and

Fig. 1. Study design for the 4 separate 24-h trials (top): 1) Rest, complete bed rest; 2) One, 1 bout of exercise in the afternoon (Ex-A); 3) Short, 2 bouts of exercise, morning (Ex-M) and Ex-A, separated by 3 h of rest; and 4) Long, 2 bouts of exercise, Ex-M and Ex-A, separated by 6 h of rest. All exercise bouts lasted 75 min and are shown as shaded bars. Meals were given 4 times in each trial at the hours indicated by arrows within each trial. Bottom: time schedule for blood sampling during the 24-h trials is illustrated by arrows.
heart rate monitor (Siemens SC 6000 P, Siemens Medical Systems, Danvers, MA). A flexible intravenous catheter (Venflon 1.2; 32 mm, BOC Health Care, Helsingborg, Sweden) was inserted into an antecubital vein and kept there for the whole trial.

The morning (Ex-M) and afternoon exercise (Ex-A) bouts were equal in intensity and duration and consisted of a 10-min warm-up period at 50% of $V_{\text{O}2\text{max}}$, immediately followed by 65 min at the subjects' predetermined workload with a cadence of 90–100 rpm. All subjects completed all of the exercise sessions, but the workload had to be reduced temporarily on five occasions to avoid premature exhaustion. Subjects who had to have their workload reduced were excluded from the data analysis of $O_2$ uptake during exercise. The $O_2$ uptake was measured for 60 s after 15, 30, 45, 60, and 70 min of exercise, as well as continuously during the first hour postexercise and for 10 min every hour during the subsequent recovery period. Blood for cytokine analysis was sampled at the start of all trials, 15 min before Ex-A, at the end of exercise, and 1, 2, 4, and 14 h postexercise. The subjects rested in bed at all hours when they did not exercise and spent the following night sleeping in the laboratory until 0700 the next morning. The subjects were allowed to read and thus had a 45° headrest, except for the last hour postexercise and for 10 min every hour during the subsequent recovery period. Blood for catecholamine analysis was collected in a 3-ml precooled 143-USP heparinized tube, kept on ice for 15 min before centrifugation at 6°C for 10 min at 3,000 rpm, frozen immediately, and stored at −70°C. Plasma epinephrine was analyzed by HPLC with electrochemical detection, according to a previously described method (28).

Statistical analyses. In three of the nine subjects, IL-6 concentrations >4 pg/ml were measured in the resting state. These were considered outliers and were, therefore, excluded from the statistical analysis. The same subjects were also excluded from the IL-1ra analysis. The plasma glucose data were analyzed by using all subjects ($n = 9$). A two-way ANOVA procedure for repeated measures, testing for main effects of trial and time, was used to compare the individual trials, including measurements from 1500 to 2030 h. Student's $t$-test and Pearson correlation test were used for between-trial comparisons at the same time point (peak values) or time period (delta values). $P$ levels < 0.05 were considered significant, but specific $P$ values are generally given. Results are presented as means ± SE, unless otherwise noted.

**RESULTS**

**IL-6.** Plasma levels of IL-6 increased in all exercise trials during the Ex-A compared with trial Rest (time effect; $P < 0.001$) and remained elevated for at least 2 h (Fig. 2). Peak IL-6 was significantly higher in trial Short (8.8 ± 1.3 pg/ml) compared with trial One (5.2 ± 0.7 pg/ml; $P = 0.014$) but not compared with trial Long (5.9 ± 1.2 pg/ml; $P = 0.160$). When concentration is compared during the entire period from 1500 to 2030 h, the increase in concentrations of IL-6 was more pronounced in trial Short compared with trial One ($P = 0.025$). However, there was no statistical difference between changes in IL-6 in trials Short and Long ($P = 0.084$). After 4 h of recovery, IL-6 had reached baseline levels.

![Fig. 2. Concentrations of interleukin (IL)-6 measured in the morning, before and at the end of Ex-A, and during the subsequent recovery period in the 4 trials (Rest, One, Short, and Long). Values are means ± SE; $n = 6$ subjects.](http://jap.physiology.org/DownloadedFrom/http://jap.physiology.org/ by 10.220.33.5 on June 10, 2017)
levels in all exercise trials. There was no correlation between pre- to post-Ex-A changes in IL-6 and plasma glucose concentrations in trials Short \( (r = 0.48) \) or Long \( (r = 0.03) \). When the pre- to post-Ex-A changes in IL-6 are compared with the corresponding changes in epinephrine (Table 1) \( (28) \), we found a significant correlation in trial Short \( (r = 0.91, P < 0.02) \) but not in trial Long \( (r = 0.79, 0.05 < P < 0.10) \).

**IL-1ra.** As shown in Fig. 3, the plasma level of IL-1ra was elevated before the Ex-A in trial Short compared with Rest \( (P = 0.022) \), but no significant change in IL-1ra was observed from before to after Ex-A in the three exercise trials. However, IL-1ra increased in all exercise trials during the first hour of recovery (time effect; \( P < 0.02 \)). Peak IL-1ra was higher in trial Short \( (1774 \pm 373 \text{ pg/ml}) \) compared with trial One \( (454 \pm 109 \text{ pg/ml}; P = 0.015) \) but was not statistically higher compared with trial Long \( (1276 \pm 451 \text{ pg/ml}) \). When concentration is compared during the entire period from 1500 to 2030, the increase in concentrations of IL-1ra was more pronounced in trial Short compared with trial One \( (P = 0.002) \). There was no statistical difference between IL-1ra changes in trials Short and Long \( (P = 0.219) \) during this period. After 4 h of recovery, IL-1ra was still elevated in trial Short compared with One \( (P = 0.031) \) but not compared with trial Long \( (P = 0.137) \).

**Plasma glucose and epinephrine.** Compared with trial Rest, there was a significant decrease in plasma glucose during Ex-A in trials One \( (P = 0.030) \), Short \( (P = 0.011) \), and Long \( (P = 0.001; \text{Fig. 4}) \). Furthermore, during Ex-A, there was a trend toward a larger decrease in trial Short compared with trial One \( (P = 0.059) \), but there was no difference in the magnitude of decrease between trials Short and Long \( (P = 0.865) \).

During the recovery period after Ex-A, plasma glucose showed a larger magnitude of changes in all three exercise trial compared with trial Rest \( (\text{trial} \times \text{time effect}; P < 0.001) \); however, there were no differences in the magnitude of change between trials One and Short \( (P = 0.523) \) or trials Short and Long \( (P = 0.112) \). Concentrations of epinephrine in the four trials during the time period for Ex-A and the first hour of recovery (1500–1730) are given in Table 1. Peak epinephrine after the end of Ex-A was higher in trial Short \( (9.1 \text{ nmol/l}) \) compared with trial One \( (1.6 \text{ nmol/l}; P < 0.0005) \) and trial Long \( (6.1 \text{ nmol/l}; P < 0.005) \).

**\( O_2 \) uptake and RER.** Mean \( O_2 \) uptake during exercise was 3.7 \( \pm 0.1 \text{ l/min} \) in trial One, 3.9 \( \pm 0.1 \text{ l/min} \) during the second bout in trial Short, and 3.8 \( \pm 0.1 \text{ l/min} \) in trial Long. Mean \( O_2 \) uptake during Ex-A in trial Short was 0.21 \( \pm 0.04 \text{ l/min} \) higher than in trial One \( (P = 0.040) \) and 0.09 \( \pm 0.03 \text{ l/min} \) higher than in trial Long \( (P = 0.025) \). The average RER during Ex-A was lower in trial Short \( (0.84 \pm 0.02) \) compared with trial One \( (0.87 \pm 0.02; P = 0.005) \) and trial Long \( (0.86 \pm 0.02; P = 0.010) \). During the first 5 h of recovery, RER was lower in trial Short than trial One \( (P = 0.006) \), but there was no difference between trials Short and Long \( (P = 0.242) \).

**DISCUSSION**

The main finding in the present study was that the second bout of exercise in trial Short provoked more pronounced increases in plasma levels of IL-6 and IL-1ra compared with the first (single) bout in trial One. Furthermore, the increased levels of these cyto-

---

**Table 1. Plasma epinephrine concentrations 15 min before and at the end of the afternoon exercise and during the first 60 min of recovery**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Ex-A</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
</tr>
<tr>
<td>Rest</td>
<td>0.1±0.1</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>One</td>
<td>0.0</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>Short</td>
<td>0.1±0.1</td>
<td>9.1±2.8*</td>
</tr>
<tr>
<td>Long</td>
<td>0.2±0.1</td>
<td>6.1±2.8</td>
</tr>
</tbody>
</table>

Values are means ± SE in nmol/l. Ex-A, afternoon exercise; Pre and Post, before and after Ex-A, respectively. Rest, complete bed rest; One, 1 bout of exercise; Short, 2 bouts of exercise with 3 h of rest in between; Long, 2 bouts of exercise with 6 h of rest in between.

*Greater than trial One, \( P > 0.001 \). †Greater than trial Long, \( P > 0.001 \).
kines during and after the second bout of exercise were attenuated when the period of rest between the exercise sessions was extended from 3 h in trial Short to 6 h in trial Long (Figs. 2 and 3). Plasma glucose decreased during Ex-A in trials One, Short, and Long, but there was no significant difference in magnitude of change among the three exercise trials.

To our knowledge, there is only one published study examining the effect of repeated bouts of exercise on plasma levels of IL-6 (18). Using a protocol with three bouts of exhaustive rowing, each lasting 6 min and separated by 4 h of rest, Nielsen et al. (18) showed a trend toward augmented peak values after each consecutive bout of exercise. Despite the differences in exercise protocol, the findings of Nielsen et al. correspond with the results of the present study. The more pronounced IL-6 and IL-1ra responses observed on the second bout after only 3 h of rest in the present study were according to our hypothesis. There may be several explanations for this finding, but we propose incomplete resynthesis of muscle glycogen during recovery between the two bouts of exercise as the most plausible explanation (27).

Earlier studies have observed a 60–80% reduction in glycogen content of working muscles after a single bout of exercise with similar intensity and duration as in the present study (2–4, 8, 13, 15, 41). Furthermore, these and other studies have demonstrated that restoring muscle glycogen completely after strenuous endurance exercise may take as long as 24 h and that the rate of resynthesis is, to a great extent, dependent on the glucose availability during the postexercise period. We did not obtain muscle biopsies in the present study, but, on the basis of the aforementioned investigations, a substantial reduction in glycogen stores could be expected at the end of the first bout of exercise. By analyzing multiple muscle biopsies after exhaustive work of 78–113 min at 75% \( \dot{V}O_2_{\text{max}} \), Blom et al. (3) estimated a maximal rate of glycogen synthesis of \( \sim 6 \) mmol·kg\(^{-1} \)·h\(^{-1} \) and found only 40–44% of the preexercise muscle glycogen content after 4 h of recovery (varying with the amount of glucose in the refeeding regime) (3). Therefore, we must assume that muscle glycogen was incompletely restored during the rest period between the two bouts of exercise, particularly in trial Short in which only 3 h of rest and one meal containing 4 MJ were given.

The assumption that glycogen stores were compromised during the second bout of exercise is supported by the lower mean RER found in trial Short (0.84 ± 0.02) compared with One (0.87 ± 0.02; \( P = 0.005 \)), both during and after the second bout of exercise. This indicates a shift toward decreased carbohydrate and increased fat oxidation on the second bout of exercise. Furthermore, in a recent study that used a glucose clamp technique, Galassetti et al. (9) demonstrated an increased turnover of carbohydrate fuels during a second bout of endurance exercise, even at moderate intensity. When two equal bouts of 90-min exercise are performed at 48% of \( \dot{V}O_2_{\text{max}} \) separated by 3 h of rest, a fivefold higher rate of exogenous glucose infusion was needed during the last 30 min of the second bout of exercise to maintain euglycemia. This further substantiates the argument that muscle glycogen stores in the subjects of the present study must have been minimal toward the end of the second bout of exercise, particularly because exogenous carbohydrates were not provided during exercise.

Interestingly, two recent studies have demonstrated that IL-6 production in contracting muscle is influenced by preexercise muscle glycogen content, showing a larger IL-6 production when exercise is performed in glycogen-depleted states (14, 32). Other studies have altered carbohydrate supply before (10) or during (17, 31) strenuous endurance exercise and observed an attenuating effect of increased carbohydrate availability on the IL-6 and IL-1ra responses to prolonged exercise. Furthermore, recombinant IL-6 has proved to have dose-dependent effects on glucose regulation (40) and to mediate metabolic effects during catabolic states (37). Thus we suggest that the more pronounced IL-6 at the end of the second bout of exercise observed in the present study, and the subsequently increased IL-1ra response, may be a result of muscle glycogen depletion. It could be speculated that increased IL-6 reflects an energy crisis within the working muscle, thus mediating a signal for increased substrate mobilization in other organs and tissues in the body, i.e., performing a hormonelike action (27). It has been demonstrated that IL-6 can arrest fasting-induced decline in blood glucose in a dose-dependent fashion, possibly through direct action on hepatocytes or through glucose counterregulatory hormones like glucagon, cortisol, GH, and epinephrine (40). Furthermore, infusion of IL-6 seems to mimic many of the metabolic alterations associated with a catabolic state, including increased lipolysis and fat oxidation, increased oxygen consumption and energy expenditures, and increased hepatic glucose output, although the mechanisms of action are not clear (37).

In accordance with previous studies on blood glucose regulation during strenuous endurance exercise, we observed a fall in plasma glucose during Ex-A (1, 7, 13). However, the new observation from this study is that glucose concentration appears to remain well regulated during a second bout of exercise, even in the absence of exogenous carbohydrate supply during the exercise. Moreover, a reduction from 6 to 3 h of rest and a 50% reduction in caloric intake between the two exercise sessions did not seem to further affect plasma glucose concentrations during the second bout. Also, there was no correlation between changes in plasma glucose and IL-6 concentrations from pre- to postexercise on the second bout. Thus, assuming IL-6 is involved in signaling carbohydrate substrate shortage during prolonged strenuous exercise, our observations lend support to the contention that muscle glycogen content rather than blood glucose concentration is the dominant stimulus for increased IL-6 production during high-intensity endurance exercise (27).

Epinephrine has been proposed to possess a mechanistic role in the increased levels of plasma IL-6 during
exercise (16, 24, 30, 37). In an earlier publication from the present study, we reported a fivefold higher increase in epinephrine during the second bout in trial Short compared with the first bout in trial One (28). When comparing the pre- to post-Ex-A changes in IL-6 with the corresponding changes in epinephrine, we found a significant correlation in trial Short but only a trend in trial Long. However, because peak epinephrine was 500% higher, whereas peak IL-6 was only 40% higher, during the second bout compared with the first bout, this does not indicate a strong relationship. Furthermore, Steensberg et al. (33) have shown that the exercise-induced increase in plasma IL-6 could not be mimicked by epinephrine infusion, thus suggesting only a minor role for epinephrine in stimulating IL-6 production during exercise.

All exercise sessions in the present study lasted 75 min, and the subjects performed all exercise bouts at the same relative workload, corresponding to ~75% of V̇O₂ max. However, mean oxygen uptake was slightly higher during the second bout of exercise in trial Short (3.9 ± 0.1 l/min) compared with the first bout in trial One (3.7 ± 0.1 l/min). This may be a result of increased exertion [rating of perceived exertion was higher in One (3.7 ± 0.0) than One (3.0 ± 0.7; P = 0.006), data not published], increased fat oxidation relative to carbohydrate (RER was lower in trial Short than One), altered muscle fiber recruitment, or a combination of these factors. Nevertheless, it is our opinion that this minor (4%) elevation of oxygen uptake may only account for a smaller part of the augmented IL-6 and IL-1ra responses associated with the second bout of exercise.

Three of the nine subjects showed initial resting levels of IL-6 >4 mmol/l and consistently high levels throughout the trial in one or more of the four exercise trials. IL-6 concentrations of this magnitude have not been observed at rest in previous investigations (14, 27, 34). Because all samples were analyzed in duplicates and all samples from each trial were analyzed in kits from the same batch, analytic error does not appear to be the most plausible explanation. Rather, some form of immunological activation, i.e., infection or inflammation, may have triggered a cytokine response before entering the trial (12). Interestingly, among the four subjects who needed to reduce their workload temporarily because of near exhaustion during Ex-A (see METHODS), we found all three subjects who were excluded on the basis of high resting levels of IL-6. However, none of these subjects had reported any illness symptoms within the last 5 days before or the day after the trials. In any case, it appeared reasonable to exclude these subjects from both the IL-6 and IL-1ra analysis, because changes in IL-1ra are known to be related to elevations in IL-6 (39).

Conclusion. On the basis of this study, we conclude that a second bout of high-intensity endurance exercise on the same day is associated with a more pronounced increase in IL-6 and IL-1ra compared with a single bout of similar exercise. Furthermore, a trend toward attenuation in the augmented cytokine response was observed when the rest period between the two bouts of exercise was extended from 3 to 6 h and an additional meal was served. The augmented IL-6 and IL-1ra response may be linked to glycogen depletion in the working muscle, thus representing a signal of energy shortage in the muscle and a need for increased substrate mobilization from other tissues. Further studies that use a design with repeated bouts of exercise are warranted to elucidate the mechanism(s) and biological significance behind the augmented increases in IL-6 and IL-1ra.

We are grateful for the skilful assistance of Øystein Haugen and Tone Rasmussen Ørtsland at the Norwegian University of Sport and Physical Education. This study was supported by a grant from The Norwegian Research Council and The Norwegian Olympic Committee and Confederation of Sport.

REFERENCES

16. Mazzeo RS, Donovan D, Fleschner M, Butterfield GE, Zamudio S, Wolfel EE, and Moore LG. Interleukin-6 re-

J Appl Physiol • VOL 92 • JUNE 2002 • www.jap.org


