Effect of glutamine and protein supplementation on exercise-induced decreases in salivary IgA

KAREN KRZYWKOWSKI,1,2 EMIL WOLSK PETERSEN,1,2 KENNETH OSTROWSKI,1,2 HARRIET LINK-AMSTER,4 JULIO BOZA,4 JENS HALKAER-KRISTENSEN,3 AND BENTE KLARLUND PEDERSEN1,2

1The Copenhagen Muscle Research Centre, 2Department of Infectious Diseases, and 3Department of Orthopedic Rehabilitation and Medicine, Rigshospitalet, 2200 Copenhagen, Denmark; and 4Nestlé Research Center, CH-1000 Lausanne, Switzerland

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EXERCISE HAS REPEATEDLY BEEN SHOWN to affect the immune system. Light to moderate exercise appears to have a beneficial effect on the immune system, whereas prolonged bouts of exercise and heavy training bouts cause temporary immune impairment (35). The magnitude of the alterations of the immune system reflects the intensity, duration, and chronicity of the exercise (13). Athletes appear to exhibit an increased risk of upper respiratory tract infection (URTI). Resistance to respiratory infection is provided by the mucosal immune system, with the major immunoglobulin being secretory immunoglobulin A (S-IgA). S-IgA inhibits bacterial adherence, neutralizes viruses and toxins, and prevents absorption of antigens through mucosal surfaces (23). Several reports have shown that the level of S-IgA in saliva decreases after intense prolonged and/or chronic exercise (36, 37, 39) and after shorter intense interval exercise (19, 22), whereas exercise of moderate intensity [50–80% of maximum oxygen consumption (V0₂ max)] and duration (15–45 min) has no effect on the level of S-IgA (24). The duration of the exercise-induced lowering in salivary IgA concentration has been found to last between 2 and 24 h after intense prolonged exercise (18). Although no causal relation has been established, the incidence of URTI in athletes has been related to the level of S-IgA in saliva (10, 37, 39). It is well established that prolonged exercise induces a temporary decrease in the plasma glutamine concentration (4, 5, 15, 31–33, 40). Glutamine supplementation has been demonstrated to decrease glutamine concentration (4, 5, 15, 31–33, 40). Glutamine supplementation has been demonstrated to decrease the number of URTI in athletes (6). This finding is not likely to be explained by an effect of glutamine on the function of circulating lymphocytes given that glutamine supplementation abolished exercise-induced decrease in plasma glutamine but not exercise-induced impaired lymphocyte function (31, 33).

Linking the possible association between salivary IgA and URTI (10, 37, 39) on the one hand and glutamine supplementation and URTI on the other (6) led to the hypothesis that glutamine supplementation might abolish postexercise decline in salivary IgA. Some support for this idea was found in studies performed in rats, which demonstrated that addition of glutamine to total parenteral nutrition abolished the suppressed level of biliary IgA observed in relation to standard total parenteral nutrition (3).

The purpose of this study was to examine the effect of glutamine supplementation on the exercise-induced suppression of S-IgA-mediated mucosal immunity. In addition, the effect of supplementation with protein as a source of glutamine was investigated. Protein was included as a natural source of glutamine. The placebo
possible psychological stress was therefore equal in the present, but the present study was randomized, and minor psychological stress may have been cause psychological stress decreases salivary IgA levels (14). Minor psychological stress may have been present, but the present study was randomized, and possible psychological stress was therefore equal in each trial group.

**METHODS**

**Subjects.** The experimental protocol was approved by the ethical committee of Copenhagen Community, and written, informed consent was obtained from all subjects. Eleven healthy, endurance trained sportsmen aged 23–48 yr (mean 38 yr) with $V\dot{O}_2\max$ of 47.4–68.4 ml·min$^{-1}$·kg$^{-1}$ (mean 59.9 ml·min$^{-1}$·kg$^{-1}$) and maximum heart rate of 163–198 beats/min (mean 182 beats/min) participated in the study. Descriptive characteristics of the subjects are summarized in Table 1. The study was conducted during the months of September to March.

**Experimental design.** A minimum of 3 days before the subjects’ first trial, their $V\dot{O}_2\max$ (MedGraphics CPF-S and CPX) and maximum heart rate (Polar advantage NV) were determined by using a graded exercise test on the same Krogate’s bicycle ergometer that was used in the experiments.

All subjects performed three exercise trials each separated by at least 2 wk. On each experimental day, the subjects reported to the laboratory at 8 AM in a rested and 8-h fasted condition. The subjects had been asked to avoid strenuous exercise the day before the trial and all exercise for at least 8 h. At the first appointment, the subjects’ training history and also contained 2% sucrose, 0.2% citric acid, and 0.15% lemon flavor to improve the taste. After having consumed the fifth beverage, the subject was offered a standardized meal consisting of ~200 g white bread, 65 g cheese, 150 g tomato, 150 g cucumber, 50 g lettuce, and 1 banana. Subjects were allowed to drink water ad libitum throughout the study except 10 min before sampling of saliva. At the end of each trial, the subjects were asked whether they had an impression of which product they had been given.

**Saliva collection.** Saliva samples were obtained before the exercise bout after 15 min of rest ($t = 0$ h), 20 min postexercise ($t = 2.3$ h), and 140 min postexercise ($t = 4.3$ h) as well as 4 and 22 h ($t = 6$ and $t = 24$ h) after the end of the exercise period (Table 2). The meal was consumed between the third and the fourth saliva sample. The saliva samples were ob-

| Table 1. Descriptive characteristics of subjects participating in the study |
|---|---|---|---|---|---|
| Age, yr | Weight, kg | Height, m | BMI, kg/m | $HR_{max}$, beats/min | $V\dot{O}_2\max$, ml·min$^{-1}$·kg$^{-1}$ |
| 38 (25–48) | 80.4 (73.3–90.5) | 1.82 (1.72–1.97) | 24.2 (22.7–25.7) | 182 (163–198) | 59.9 (47.4–68.4) |
| Values are means with range in parentheses ($n = 11$ subjects). BMI, body mass index; $HR_{max}$, maximal heart rate; $V\dot{O}_2\max$, maximal oxygen uptake. |
tained according to a standardized procedure: The subjects sat in an upright position with the head inclined forward. They were asked to collect saliva in the mouth for 1 min, after which they were asked to swallow. This was done to obtain a more precise flow rate. During the following 5 min, the subjects were told to expectorate into a preweighed 50-ml plastic tube (Nunc, Roskilde, Denmark) at intervals of their own choice. The subjects were told not to force salivation, and it was not (artificially) stimulated. Immediately after the 5-min period, the saliva sample was placed on ice until weighed and it was then centrifuged at 4°C for 24 min at 5,200 g to pellet mucus and cells. The supernatant was stored at −20°C until analysis.

Blood sampling. After collection of the saliva sample, blood was obtained from an antecubital vein before the beginning of the exercise bout, immediately postexercise (t = 2 h), and 2 h after the end of the exercise period (t = 4 h).

Total protein determination. A micromethod was performed in microtitrater plates (Microtec, Embrach Embraport) using the branched-chain amino acid protein assay reagent from Pierce (Pierce, Rockford, IL) and bovine serum albumin as a standard.

IgA determination. Secretory IgA (S-IgA) was determined by a sandwich ELISA. The wells of microtitrater plates (MaxiSorp, Nunc, Wohlen) were coated overnight with 100 μg per well of an α-chain-specific goat anti-human IgA (Sigma Chemical, Buchs), 20 μg/ml in coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9). All samples were tested in triplicate, and for each sample to be tested a control well was left uncoated. The wells were washed three times with PBS (0.01 M phosphate containing 0.15 M NaCl, pH 7.2) containing 0.05% Tween-20 (Bio-Rad Laboratories, Glattbrugg). Unspecific binding was blocked by incubating wells with 100 μl of Tween-20/PBS containing 0.5% caseinate K (Nestlé, batch DMU 01.12.93) for 1 h at 37°C. Plates were washed as previously described, and 100 μl of standards or saliva samples (at a suitable dilution between 1,500 and 12,000) diluted in 0.05% Tween-20/PBS were added to the wells and incubated for 1 h at 37°C. Unbound IgA was removed by washing three times, and 100 μl α-chain-specific goat anti-human IgA conjugated with alkaline phosphatase (Sigma Chemical) were added at dilution 1:5,000 in Tween-20/PBS and incubated for 1 h at 37°C. After washing, 200 μl of 1 mg/ml paranitrophenyl phosphate (Sigma Chemical) in substrate buffer were added, and plates were incubated for 30 min at 37°C. The color reaction was stopped by the addition of 100 μl 1 M NaOH, and the optical density was measured at 405 nm by using a MRX Dynex Microplate Reader (Microtec). The standard consisted of purified S-IgA (Nordic Immunological Laboratories, Tilburg) in twofold dilutions from 1.0 to 0.0625 μg/ml in Tween-20/PBS. S-IgA controls were included on each plate. To avoid interassay variability, all samples from one athlete were assayed on the same microtitrater plate.

The absolute concentration (mg/ml) of S-IgA was determined by regression analysis. The relationship between known concentrations of S-IgA (standards) and absorbance was used to interpolate S-IgA concentration in the samples. The output of IgA (mg/min) was calculated by multiplying the absolute concentration by salivary flow rate (ml/min). Salivary flow rate (ml/min) was determined by dividing the weight of saliva with the time of the sampling period and assuming that the specific gravity of saliva is 1. The relative amount of IgA to protein was determined by dividing the absolute concentration of IgA with the protein concentration.

Plasma glutamine determination. Blood was drawn into glass tubes containing EDTA and was centrifuged at 2,500 g for 15 min at 4°C. Plasma was stored at −80°C and analyzed in monoplicate by HPLC (11). Plasma glutamine concentrations were corrected for changes in plasma volume caused by dehydration according to changes in the concentrations of hemoglobin and hematocrit (8).

Clinical chemistry tests. Hematocrit and hemoglobin concentrations were determined according to standard laboratory procedures at the Department of Clinical Chemistry, the University Hospital of Copenhagen, Denmark.

Statistical analyses. All parameters in this study was analyzed by comparing each supplementation group to the placebo group. To test whether the measured parameters were influenced by time and interaction between time and treatment, a three-way ANOVA was carried out; because the measurements at different times were on the same subject and because each subject served as his own control, a paired, repeated-measures design was employed. The model is

\[ Y_{ijk} = \mu + t_i + s_j + t_k + (t_r \times t_k) + (s \times t) + E_{ijk} \]

where \( Y \) denotes the dependent variable, \( \mu \) is the overall mean, and the main effects are treatments (\( t \)), subjects (\( s \)), and time (\( t \)). Indexes \( i, j, \) and \( k \) indicate the level of the respective factors. Interactions between main effects are indicated by \( \times \). \( E \) signifies the remaining (residual) variation that is not explained by the effects in the model. When a significant interaction was found between time and treatment (indicated by \# in figures), a Student’s paired t-test was performed at each time point and the time trend was analyzed for each group separately. When a significant difference was found between two treatments (indicated by \& in figures), the time trend was also analyzed for each group separately. Conversely, when no significant difference between two treatments and between time and treatment was found, the results were considered as if arising from only one treatment. If a significant effect was found for time, the time trend in relation to the preexercise value was further analyzed with a Bonferroni-adjusted Student’s paired t-test. In this case, differences in time are indicated only for the nonplacebo group in the figures. In all cases, the untransformed data best met the requirement of normal distribution.

Note that, when evaluating parameters related to the flow rate of saliva (i.e., salivary flow rate, protein output, and IgA output), data at \( t = 24 \) h were not included in the statistical analysis because of several missing determinations of the flow rate at this time point.

Statistical calculations were performed by use of SYSTAT statistical software (SYSTAT vs. 7.0, SPSS). In all tests \( P \leq 0.05 \) was considered significant.

RESULTS

The actual oxygen consumption, heart rate, and workload determined during each trial are shown in Table 3.

Table 3. Oxygen consumption, heart rate, and workload of subjects measured during the 3 exercise trials

<table>
<thead>
<tr>
<th></th>
<th>( V_{O_2} \text{max}, % )</th>
<th>Heart Rate, %</th>
<th>Workload, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>74.8 ± 3.5</td>
<td>76.6 ± 3.2</td>
<td>68.8 ± 3.9</td>
</tr>
<tr>
<td>Glutamine</td>
<td>74.8 ± 2.7</td>
<td>76.8 ± 2.8</td>
<td>69.0 ± 4.3</td>
</tr>
<tr>
<td>Protein</td>
<td>74.2 ± 4.4</td>
<td>75.2 ± 3.3</td>
<td>69.0 ± 3.4</td>
</tr>
</tbody>
</table>

Values are means ± SD in percentage of the individuals maximum capacity as determined before the trials (\( n = 11 \) subjects). The individual value of \( V_{O_2} \text{max} \) is an average of 3 measurements obtained during the exercise period. Heart rate and workload were determined throughout the exercise period.
Table 3 as a mean of the individual measurements. No differences were found in these parameters between the trials in which subjects were supplemented with glutamine or protein compared with the placebo trial (Student’s paired t-test). There was no tendency in the subjects’ answers to the question of what product they had received during each experimental day (data not shown).

Salivary flow rate. Salivary flow rate varied over time, but the change over time did not differ among the three supplementation trials. The flow rate did not change from preexercise values to 20 min postexercise (t = 2.3 h) but was elevated at both 140 min (t = 4.3 h) and 4 h (t = 6 h) postexercise compared with preexercise values (Fig. 1).

Protein concentration. The concentration of protein in saliva (mg/ml) changed in response to exercise but was not affected by the different supplements. An increase in the concentration of protein was found in the first sample after the end of exercise (Fig. 2A). By the end of exercise, the protein concentration was on average increased 75% in all the groups. The protein concentration reverted to preexercise values after 140 min of rest (t = 4.3 h) but dropped below preexercise values at t = 6 h in the glutamine trial.

Protein output. When adjusting for changes in salivary flow rate, the protein output over time (mg/min) showed the same trends as just described for the protein concentration (Fig. 2B). Protein output varied over time, but the change over time did not differ among the three supplementation trials. The protein output was increased, in all supplemented groups, in the first postexercise sample, averaging 70% above baseline concentrations. In the placebo and the protein-supplemented groups, the protein concentration was still slightly elevated after 140 min of rest (t = 4.3 h), but 4 h after the end of exercise (t = 6 h) the protein output had returned to preexercise values in all groups.

IgA concentration. The concentration (mg/ml) of secretory IgA was influenced by exercise, but neither glutamine nor protein supplements affected the salivary IgA concentration. The concentration of IgA decreased after exercise, and the lowest concentration was observed 140 min postexercise (t = 4.3 h) (Fig. 3A). The IgA concentration was still decreased at t = 6 h in all groups. In the glutamine-supplemented group, the IgA concentration was still reduced by ~50% at t = 24 h.

IgA output. Salivary IgA output (mg/min) decreased in response to exercise with no influence of glutamine or protein supplementation (Fig. 3B). At 4.3 h after the beginning of exercise, the IgA output was decreased by 27–49%. Four hours postexercise (t = 6 h), the IgA output was still decreased in the glutamine group.

IgA relative to protein. The percentage of IgA relative to the amount of total protein was decreased by the end of exercise and remained below preexercise values throughout the study without influence of supplementation (Fig. 4). The smallest relative amount of IgA was observed at t = 140 min. At this time point, reductions were in the range 44–48% compared with preexercise values.

Plasma glutamine concentration. In the placebo group, the plasma glutamine concentration was on average decreased 15% 2 h after the end of exercise.
compared with preexercise values (Fig. 5). This decrease was abolished in the glutamine supplementation trial as well as in the protein supplementation trial.

DISCUSSION

The main finding in this study was that none of the salivary parameters evaluated showed any effect of supplementation compared with the placebo group, although both glutamine and protein supplementations abolished the exercise-induced decline in plasma glutamine concentration. It should be noticed, however, that the level of IgA the day after the exercise trial was lowest in the glutamine group. This finding, although probably not clinically significant, does not support our proposed hypothesis that glutamine supply during and after exercise is able to abolish the exercise-induced lowering in salivary IgA. Previously, Mackinnon and Hooper (21) found that changes in plasma glutamine concentration are not related to the appearance of URTI in swimmers.

In regard to the exercise effects per se, our results show that the absolute concentration of IgA, the IgA concentration relative to the total amount of protein, and the IgA output all declined after the exercise bout. In the case of the absolute concentration and the output over time, the lowest values were observed 140 min after the end of exercise independent of treatment, whereas the minimum in relative IgA concentration was observed 20 min after exercise. The latter finding is explained by the large increase in protein at this time point. The decrease in absolute concentration of IgA in response to exercise is in keeping with some previous findings (37, 39) but is not confirmed by others (36). A decrease in IgA output after exercise has previously been found (19, 20, 36), and the relative amount of IgA to protein was found to decrease in some studies (36, 39), whereas no effect of exercise was found in another (19). However, collection of saliva samples differs between studies (resting saliva, stimulated

Fig. 3. Effect of 2 h of bicycle exercise at 75% of $\dot{V}_{\text{O}_2}\text{max}$ on absolute IgA concentration (mg/ml; A) (n = 11, 10, and 11, respectively) and IgA output (mg/min; B) (n = 8, 9, and 8, respectively) in subjects supplemented with placebo (maltodextrin), glutamine, and protein. Average of each group is shown; bars are SD. Note that values of IgA output at t = 24 h were not included in the statistical analysis because of missing values. cANOVA evaluation of the output of IgA revealed a difference between the placebo and the protein group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, differences from preexercise values.

Fig. 4. Effect of 2 h of bicycle exercise at 75% of $\dot{V}_{\text{O}_2}\text{max}$ on the relative amount of IgA to protein (%) in subjects supplemented with placebo (maltodextrin), glutamine, and protein (n = 11, 10, and 11, respectively). The relative amount of IgA to protein was determined by dividing the absolute concentration of IgA with the protein concentration. Average of each group is shown; bars are SD. cANOVA revealed a difference between the placebo group and the glutamine group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, differences from preexercise values.

Fig. 5. Plasma glutamine concentration (µmol/l) in relation to 2 h of bicycle exercise at 75% of $\dot{V}_{\text{O}_2}\text{max}$. Subjects were supplemented with placebo (maltodextrin), glutamine, and protein (n = 9, 8, and 9, respectively). Average of each group is shown; bars are SD. Interaction between time and treatment as determined by ANOVA ($P < 0.05$). *P ≤ 0.05, differences from preexercise values.
whole saliva, and stimulated parotid saliva), which makes the comparison of studies difficult (2). Furthermore, the response of the mucosal immune system is affected by the duration and intensity of the exercise (20, 24).

Blannin et al. (1) suggest that the most reliable parameters of IgA measurements are IgA output and IgA-to-osmolality ratio. Our results thus indicate that the mucosal immune system is depressed at least in the 4-h postexercise period after intense, prolonged exercise with the maximum depression occurring around 140 min postexercise, when a decrease was observed in IgA output in the range of 27–49%. The results of the relative concentration of IgA are in agreement with the data on IgA output, as the observed lowering in the relative concentration ranged between 40 and 48%. Thus for several hours after the end of strenuous exercise athletes may be immunocompromised and more susceptible to infections in the upper respiratory tract. However, a normal range for the IgA level in saliva has not been established mainly because of a high interindividual variability (16). Hence, it is difficult to specify populations at risk from the actual values of the measured parameters. Furthermore, other parameters may be relevant when considering athletes’ susceptibility to URTI, such as, e.g., salivary IgM (9) and alveolar macrophages (7).

The concentration and output of total protein increased after exercise in accordance with previous findings (17, 36). Because the flow rate did not differ in the first postexercise sample compared with the preexercise value, this finding suggests either an increased protein concentration due to dehydration and/or an increased synthesis and/or secretion of several proteins. The increase in protein has previously been shown to be due to an increase in the level of most salivary proteins (36). The level of S-IgA was decreased in this study, which indicates a specific reduction in the synthesis and/or secretion of salivary IgA in response to exercise.

The salivary flow rate was found to be unchanged shortly after exercise and to increase 140 min postexercise. This is in contrast to findings by others of a reduction in the secretion rate immediately after strenuous exercise (17, 36). In one study, the largest decline in flow rate occurred after the most intense training session (19). The time point of sample collection may at least be partly responsible for the observed differences between studies given that the first postexercise salivary sample in this study was obtained ~20 min after the end of the exercise bout, whereas samples in the other studies were obtained immediately postexercise. Differences may also in part be explained by the aforementioned differences in study designs.

During the past few years, attempt has been made to identify nutritional supplements that could abolish exercise-induced immune changes. Until now, only carbohydrate loading has demonstrated significant findings (12, 25–29). Thus it has been demonstrated that carbohydrate loading diminishes the exercise effects on plasma cytokines, neutrophil, and lymphocyte traffick- ing, as well as levels of stress hormones. Supplementation with antioxidant vitamins (30) or fish oil (38) have not demonstrated any effect on circulating proinflammatory cytokine, lymphocyte, or neutrophil numbers.

The present study adds to two previous studies finding no effect of glutamine supplementation on exercise-induced immune changes (31, 33). Thus the finding of an effect of glutamine supplementation on URTI (6) is not mechanistically explained by an effect of glutamine on either salivary IgA nor lymphocyte function.

In conclusion, the exercise-induced change in salivary IgA level was not affected by glutamine or protein supplements. The present study therefore finds no support for the glutamine hypothesis, which says that exercise-induced decrease in plasma glutamine is linked with postexercise immune impairment.

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