Glutamine Supplementation Prevents the Decrease of Mitogen Response after a Treadmill Exercise in Rats

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Summary  The effect of glutamine (Gln)-supplemented diet on mitogen response decreased immediately after a treadmill exercise was examined by measuring the proliferations of peripheral blood lymphocytes (PBL) with phytohemagglutinin (PHA) and concanavalin A (ConA) in male Fisher rats. Although the plasma Gln concentration was significantly decreased in the control group immediately after a treadmill exercise (20 m/min, 60 min) compared to rested rats, plasma Gln concentration of rats fed Gln-supplemented diet for 3 weeks was significantly higher than that of control group in resting and was not significantly decreased even immediately after a treadmill exercise. In addition, proliferation of PBL with PHA or ConA and interleukin 2 (IL2) production were also significantly decreased immediately after a treadmill exercise in control group. On the contrary, their functions were almost maintained in Gln-supplemented group even immediately after a treadmill exercise. PBL from rats fed Gln-supplemented diet showed a higher response to mitogens such as PHA and ConA compared to the control group. Furthermore, their PBL showed higher incorporation of [3H]Gln compared to that of the control group irrespective of treadmill exercise. These results indicate that the preventive effect of Gln-supplemented diet on mitogen response decreased after a treadmill exercise is due to an increased response to mitogen and increased uptake of Gln as sources of fuel and nucleotides to the immune cells.

Key Words  glutamine, treadmill exercise, mitogenesis, IL2 activity, rats

Although there have been many reports on the effects of exercise training on the immune system, their results were not consistent and showed both deleterious and beneficial effects on immune functions (1,2). Furthermore, a depressed response to mitogen has been observed following short-term exercise (3), the mechanism of which was not clearly known until now. We have found and already reported that the decreased proliferation of PBL after acute exercise is associated with increased levels of plasma lactate and prostglandin E2 (PGE2) (4). In one
recent report, it was suggested that the plasma Gln concentration decreases in overtrained athletes and after long-term exercise, which may be a good predictor of overtraining (5). Gln is highly utilized by cells of the immune system and is considered to be an important fuel for immune cells (6). In fact, a decrease in plasma Gln level in vivo has shown to induce an immunosuppression (7). Furthermore, Gln is also an important amino acid for a source of purine and pyrimidine nucleotides (8). Taken together, the hypothesis is advanced that a decreased plasma Gln concentration after acute or strenous exercise causes an impairment of immune system such as mitogenesis and NK activity. Furthermore, since the major source of plasma Gln is considered to be skeletal muscle (9), overtraining or a single bout of exercise may cause a decrease of Gln release from muscle, subsequently cause a decrease in the plasma Gln level and consequently provoke immunosuppression. Since branched chain amino acids (BCAA) are considered to be an important source of nitrogen for Gln synthesis in muscle and enhance the release of Gln from muscle, Parry-Billings et al. investigated the effect of BCAA-supplemented diet on the decrease of plasma Gln concentration in overtrained athletes and after long-term exercise (marathon race) and found that BCAA supplementation prevented the decrease of plasma Gln level (10).

In this study we have investigated whether Gln-supplemented diet prevents a decrease of plasma Gln concentration after a treadmill exercise or not. We have also investigated the mechanism by which Gln-supplemented diet prevents the decrease of mitogen response after a treadmill exercise through the responsiveness of PBL with PHA and Con A and the uptake of $[\text{3H}]$Gln by PBL.

**MATERIALS AND METHODS**

*Animals.* Male F344 rats, 4 weeks old, obtained from Japan SLC (Shizuoka, Japan) were used in this experiment. They were randomly divided into two groups and fed control or 3% Gln-supplemented diet for 3 weeks (Table 1). Each group consisted of 24 rats. Food and water were given ad libitum. This experiment was repeated 3 times and representative data are presented in this paper.

| Table 1. Composition of experimental diets. |
|--------------------------------------------|
| Control (%) | Gln-suppl (%) |
|-------------|---------------|
| Vitamin-free casein | 20 | 20 |
| Sucrose      | 10 | 10 |
| Cornstarch   | 57 | 54 |
| Stripped corn oil | 8 | 8 |
| Mineral mixture | 4 | 4 |
| Vitamin mixture | 1 | 1 |
| L-Glutamine  | —  | 3 |
| Energy       |    | 4.2 kcal/g |

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Exercise regimen. After 3 weeks, rats from both the control and Gln-supplemented groups underwent a treadmill exercise at 20 m/min for 60 min. To avoid the effect of circadian rhythms, treadmill exercise was performed at night (8:00 to 10:00 P.M.). Then, their blood samples were collected by heparinized syringe from the inferior vena cava under anesthesia with Nembutal (0.1 ml/100 g BW, Abbott Laboratories, North Chicago, IL) immediately and 60 min after a treadmill exercise. The blood samples from sedentary rats were also collected simultaneously.

Analysis of blood samples. Blood samples were stored on ice and their plasmas were collected by centrifugation within 60 min after collection. Plasma was stored at −20°C or below. Neutralized and perchloric acid-treated plasma was prepared for the assay of Gln concentration in plasma as described previously (11).

Peripheral blood lymphocytes (PBL). PBL were isolated from heparinized peripheral blood by using Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation and counted microscopically as described previously (12).

Splenocytes. Spleen was removed and the weight was measured. Then, they were minced with scissors and passed through a stainless steel mesh. The numbers of their lymphocytes were counted microscopically. Viabilities of their lymphocytes were also measured by trypan blue dye exclusion.

Mitogenesis assay. Splenocytes and PBL isolated from each animal were adjusted to 1 × 10⁶ cells/ml in RPMI 1640 culture medium supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethansulfonic acid (HEPES, Sigma Chemical Co., St. Louis, MO), 5 μM 2-mercaptoethanol (2-ME, Sigma), and 5% heat-inactivated fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY), respectively. Splenocytes and PBL with or without mitogens such as phytohemagglutinin (PHA, 10 μg/ml, Sigma) and concanavalin A (ConA, 5 μg/ml, Sigma), were plated in 96-well microtiter plates, incubated at 37°C in humidified incubator with 5% CO₂ and 95% air for 72 h, and then pulsed with [³H]thymidine (specific activity 25 μCi/mmol, New England Nuclear, Boston, MA). After 18 h, they were harvested by an automated sample harvester (Flow Laboratories, Rockville, MD). The radioactivity was determined by a liquid scintillation counter (LSC-703, Aloka Corp., Tokyo). The data are indicated as counts per minute (cpm). In a separate experiment, data are presented as the percent proliferation which was calculated by assigning 100% to the stimulation index (SI) of PBL with PHA and ConA in resting (pre-exercise) and comparing it to the SI of PBL immediately and 60 min after acute treadmill exercise. These values express the net responsiveness of PBL to PHA and ConA except for the effect of treadmill exercise on PBL proliferation incubated in vitro with medium only.

Interleukin 2 (IL2) assay. Splenocytes or PBL (1 × 10⁷ cells/ml) isolated from each animal were cultured with ConA (5.0 μg/ml) in RPMI 1640 culture medium supplemented with sodium pyruvate (Sigma), 10% heat-inactivated FBS, and 5 μM 2-ME for 48 h. Then, IL2 activity in the culture medium was measured by the proliferation of IL2-dependent CTLL-2 cells (13). Five thousand CTLL
cells in a volume of 100 μl were added to each well and the plates were incubated at 37°C for 24 h. Then, 1 μCi of [3H]thymidine was added to each well and further incubated for 24 h. The incorporation of [3H]thymidine into DNA of CTLL cells was measured by a liquid scintillation counter (LSC-703, Aloka Corp.). IL2 activity in the culture medium was expressed as U/ml in comparison with the activity of murine recombinant IL2 (Genzyme, Boston, MA).

Flow cytometry analysis. PBL isolated from each animal were stained with both fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4 monoclonal antibody (mAb) (W3/25, Serotec Ltd., Oxford, U.K.) and phycoerythrin (PE)-conjugated anti-rat CD8 mAb (OX8, Serotec Ltd.) for two-color staining. Stained cells were fixed in 0.1% paraformaldehyde in saline and analyzed with a FACScan flow cytometer and Consort 30 software program (Becton Dickinson, Co., Mountain View, CA) after excluding dead cells by using forward and side light scatters as described previously (14).

Incorporation of [3H]Gln by PBL. PBL (1 × 10⁶ cells/ml) were incubated with 1 μCi of L-[3H]Gln (specific activity 30–60 Ci/mmol, New England Nuclear) at 37°C. After 4, 20, 48, and 72 h, they were harvested by an automated sample harvester. The radioactivity was determined by a liquid scintillation counter (LSC-703, Aloka Corp.). The data are indicated as cpm.

Statistical analysis. The results are presented as means ± SD (n = 7). The data were evaluated statistically by analysis of variance (ANOVA) with separation of treatment means by Duncan’s Multiple Range Test using a statistical analysis program (Systat, Inc., Evanston, IL). P value < 0.05 was regarded as significant.

RESULTS

Changes in food intake and body weight in rats fed Gln-supplemented diet

Rats were given control or 3% Gln-supplemented diet for 3 weeks. During experimental period both food intake and body weight gain were not significantly different between the dietary groups. The final body weight of rats from both groups was 127 ± 11.8 g in the control group and 128 ± 11.2 g in the Gln-supplemented group.

Changes in plasma Gln concentration after a treadmill exercise

In the control rats, the concentration of plasma Gln was significantly decreased immediately after treadmill exercise (p < 0.05) and recovered to the sedentary level at 60 min after the termination of exercise. On the contrary, plasma Gln concentration in the Gln-supplemented group was significantly higher than that of the control group at resting (p < 0.05). In addition, plasma Gln concentration in Gln-supplemented group was scarcely influenced by treadmill exercise and was maintained at the same level as resting even after treadmill exercise (Fig. 1).
Mitogenic responses of PBL to PHA and ConA in rats fed control or Gln-supplemented diet after a treadmill exercise

When rats had undergone a single exhaustive treadmill exercise, PBL from the control group showed a significant decrease of proliferation with PHA immediately after exercise (Fig. 2). Furthermore, PBL proliferation, which decreased with PHA and ConA in the control group following a treadmill exercise, returned to the pre-exercise level at 60 min after the termination of exercise. In the Gln-supplemented group PBL had constant responses to mitogens such as PHA and ConA and showed similar proliferation to that of pre-exercise even after a treadmill exercise. In pre-exercise, the proliferation of PBL with ConA was higher in rats fed Gln-supplemented diet than in control rats.

Interleukin 2 (IL2) production by PBL from rats fed control or Gln-supplemented diet after a treadmill exercise

IL2 production from PBL was also significantly decreased in the control group, whereas PBL from the Gln-supplemented group maintained the same level as IL2 production in the rested rats (Fig. 3).

Responsiveness of PBL to mitogens such as PHA and ConA in rats fed control or Gln-supplemented diet after a treadmill exercise

The responsiveness of PBL to PHA or ConA was significantly decreased in the control group immediately and 60 min after a treadmill exercise (Fig. 4). On the contrary, PBL from the Gln-supplemented group did not show any decreased responses to mitogens even after a treadmill exercise and maintained the same responsiveness as shown in pre-exercise (Pre). In pre-exercise, the stimulation...
Fig. 2. Proliferation of peripheral blood lymphocytes (PBL) with PHA (A) or ConA (B) in rats fed control (○) or Gln-supplemented (●) diet before (Pre), and immediately and 60 min after a treadmill exercise. PBL were isolated from rats before and after a treadmill exercise. Data are means±SD from 7 rats. *Significantly different (p<0.05) value from rested rats of the control group.

Fig. 3. Interleukin 2 (IL2) production from PBL of rats fed control (○) or Gln-supplemented (●) diet after a treadmill exercise. PBL were isolated from rats before (Pre), and immediately and 60 min after a treadmill exercise. Data are means±SD from 7 rats. *Significantly different (p<0.05) value from rested rats of control group.
Fig. 4. The responsiveness of PBL to mitogens such as PHA (A) or ConA (B) in rats fed control (○) or Gln-supplemented (●) diet after a treadmill exercise. PBL were isolated before (Pre), and immediately and 60 min after a treadmill exercise. In pre-exercise, the stimulation index (SI) of PBL with PHA or ConA was 89.4 ± 7.4, 94 ± 10.1 in the control group and 121 ± 11.5, 156 ± 18.7 in the Gln-supplemented group. Data are means ± SD from 7 rats. *Significantly different (p < 0.05) from rested rats of control group.

Index (SI) of PBL with PHA or ConA was significantly higher in the Gln-supplemented group than that of control group (p < 0.01).

Proportions of T cell subsets in PBL from control or Gln-supplemented groups after a treadmill exercise

The proportion of CD4⁺CD8⁻ T cells in PBL was significantly increased in both the control (Pre: 40.2 ± 4.2%, Post: 58.5 ± 3.5%) and Gln-supplemented groups (Pre: 45.6 ± 2.8%, Post: 56.4 ± 3.2%) immediately after a treadmill exercise, whereas the proportion of CD4⁻CD8⁺ T cells in PBL was scarcely affected by a treadmill exercise (Fig. 5).

Changes in [³H]Gln incorporation by PBL after a treadmill exercise

PBL from the control group showed similar incorporation of [³H]Gln before
Fig. 5. Changes of the proportions of CD4+CD8− (A) and CD4−CD8+ (B) T cells in PBL of rats fed control (○) or Gln-supplemented (●) diet after a treadmill exercise. PBL were isolated from rats before (Pre), and immediately and 60 min after a treadmill exercise. Data are means±SD from 7 rats. Significantly different from rested rats: **p<0.01, ***p<0.001.

Fig. 6. The uptake of [3H]Gln by PBL of rats fed control or Gln-supplemented diet after a treadmill exercise. PBL were isolated from rats before and after a treadmill exercise. Rested (○) and exercised (△) rats in the control group. Rested (●) and exercised (▲) rats in the Gln-supplemented group. Data are means±SD from 7 rats. Significantly different from rested rats of the control group: #p<0.05, ##p<0.01. Significantly different from rested rats of the Gln-supplemented group: *p<0.05, **p<0.01.
and after a treadmill exercise, whereas PBL from the Gln-supplemented group showed a higher incorporation of \[^3\text{H}\]Gln after a treadmill exercise than that of rested rats (Fig. 6). In addition, the incorporation of \[^3\text{H}\]Gln by PBL of rested rats from Gln-supplemented group was significantly higher compared to those of both rested and exercised rats in the control group.

**DISCUSSION**

In the present study, we found a marked decrease of plasma Gln after a treadmill exercise. It has already been reported that the plasma Gln concentration decreases in overtrained athletes and after long-term exercise, which is associated with a decrease in Gln released by skeletal muscle as the major source of Gln (6). In addition, it has been suggested that the decrease of plasma Gln concentration is associated with immunosuppression after a single bout of exercise or overtraining (10). As shown in Figs. 2 and 3, the present study has also indicated that the proliferation of PBL with PHA and ConA, and the production of IL2 from PBL are greatly reduced immediately after a treadmill exercise. Furthermore, these immunosuppressions, inducing immediately after a treadmill exercise, could be recovered by Gln-supplemented diet, as shown in Figs. 2 and 3. These results suggest that the immunosuppressions induced immediately after a treadmill exercise are associated with the decrease of plasma Gln concentration after a treadmill exercise.

The mechanisms by which Gln supplementation prevents the decrease of mitogen response induced after a treadmill exercise are considered as follows: a) the responsiveness of PBL to mitogens such as PHA or ConA may be enhanced by taking Gln-supplemented diet, b) the proportions of T-cell subsets in PBL could be changed and the proportion of helper T cells may be increased by Gln supplementation, and c) Gln supplementation may induce a higher uptake of Gln as an important fuel for immune cells, which may induce higher proliferation of PBL in response to PHA or ConA. As shown in Fig. 4, the responsiveness of PBL to PHA or ConA was remarkably depressed in the control group immediately and at 60 min after a treadmill exercise, whereas PBL from the Gln-supplemented group maintained the same responsiveness of PBL to PHA or ConA as that of rested rats even after a treadmill exercise. This phenomenon could be explained by our previous report showing that Gln-containing dipeptides and free Gln enhanced phagocytic activity of alveolar macrophages and blastogenic response of splenic lymphocytes \textit{in vitro} (15). Since Gln can directly stimulate immune functions, the increased Gln level in plasma of rats fed Gln-supplemented diet as shown in Fig. 1 may induce a higher responsiveness of PBL to PHA or ConA. In fact, the stimulation index (SI) of PBL with PHA or ConA was significantly higher in the Gln-supplemented group compared to the control group.

As the second explanation for the preventive effect of Gln supplementation on mitogen response decreased immediately after a treadmill exercise, the qualitative changes of T cells are considered. Since the proportion of CD4\(^+\)CD8\(^-\) T cells in
PBL was significantly increased in both the control and Gln-supplemented groups after a treadmill exercise (Fig. 5), the decrease of mitogen response immediately after a treadmill exercise is difficult to explain in terms of changes in T cell subsets of PBL. There are some reports on the effect of exercise on T cell subsets (16–18), most of which have indicated an increase in the proportion of CD4+CD8+ T cells in PBL after exercise associated with a decrease in cellular immune functions following acute exercise. In the present study, we did not find a decrease of CD4+CD8+ T cells but the increase of CD4+CD8− T cells after a treadmill exercise, as shown in Fig. 5. Although this result seems to conflict with the decreased proliferation of PBL after a treadmill exercise, this discrepancy could be explained by different proportions of T cell subsets such as TH1 and TH2 in PBL, namely, an increase in the proportion of TH2 cells in the control group and an increase in the proportion of TH1 cells in the Gln-supplemented group.

Since Gln is an important amino acid as a source of both fuel and nucleotides for immune cells (6,8), a Gln-supplemented diet may increase uptake of Gln by PBL due to the adaptation to the higher Gln concentration and result in higher proliferation of PBL. In fact, the uptake of [3H]Gln by PBL was significantly higher in the Gln-supplemented group than in the control group (Fig. 6). The uptake of [3H]Gln by PBL was further increased in the Gln-supplemented group following a treadmill exercise. Furthermore, the proportion of splenic lymphocytes in S-phase, DNA synthetic phase, was increased in the Gln-supplemented group compared with the control group, irrespective of treadmill exercise (19). These results suggest that PBL from the Gln-supplemented group are activated by increased Gln uptake.

In conclusion, the present study demonstrates that (a) the decrease in the Gln plasma level after a treadmill exercise is associated with a decrease in the mitogen response, and (b) the Gln-supplemented diet prevents the decrease of mitogen response immediately after a treadmill exercise, which is, in part, due to the increased responsiveness of PBL and the higher uptake of Gln as a source of fuel and nucleotides for the immune cells.

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