Effect of Dietary Amino Acids on Behavior and Serum Levels of Amino Acids in Stress Loaded Rats

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Summary Interrelationships between behavior and concentrations of serum amino acids in stressed rats with immobilization and water immersion were investigated. Rats were subjected to 7 h of immobilization with water-immersion stress (IWS) in each sequential day, and serum amino acids were then determined. On the first day, serum taurine, threonine, valine, leucine, isoleucine, phenylalanine, lysine, and histidine (increased-type of amino acids) were significantly increased, but alanine (decreased-type) was significantly decreased. On days 3 and 7, the increase was retained, except for threonine, histidine, and lysine. Spontaneous activities (locomotion, rearing behavior, hole-poking) under loading water-immersion stress were significantly decreased, but a supplementation of branched-chain amino acids (BCAA) led to recovery. We suggest that pretreatment with some kind of increased type of amino acid, such as BCAA, might effectively prevent decline in spontaneous activities evoked by water-immersion stress.

Key Words branched-chain amino acids, taurine, behavior, immobilization, water-immersion stress

Present-day humans are environmentally subjected to physical and/or mental stress, and homeostasis is disturbed (1–5). Chronic fatigue (6, 7), gastric ulcers (8, 9), hypophagia (10), and insomnia (11) can be evoked by stress. In previous studies on humans (12) and rats (1, 13), it was found that acute stress can increase catecholamines, serotonin, and steroid hormones in the brain and the adrenal gland. Thus increases in blood pressure and heart rates, the activation of gluconeogenesis, and protein catabolism are evoked by stress (2).

For several reasons we have reached the hypothesis that stressors (including IWS: immobilization with water-immersion stress) elicit amino acids from amino acid pools in a stressor-specific manner. The concentration of humoral-free amino acids, which account for only 0.5% of all amino acids in the body, readily change in a stress specific manner (14), and other small changes are also evident (1, 15–17). It is known that there are intercellular and/or extracellular amino acid pools in liver (18), muscle (19), and brain (20). Moreover, it is conceivable that these amino acid pools play an important part as a supplier of free amino acids into blood. Phenylalanine, tyrosine, and tryptophan are precursors of neurotransmitters, namely, adrenaline, noradrenaline, dopamine, and serotonin; thus a correlation exists between the metabolism of these neurotransmitters and amino acid concentrations (21). Amino acids are not only unique materials of many kinds of proteins in our body, hormones, but also nitrogen donors to nucleic acids and important energy sources.

We reported that serum amino acid concentrations were changed in iron-deficient anemic rats (22). Furthermore, an oral coadministration of proline and iron led to a prominent increase of the counts of red blood cells and hemoglobin in iron-deficient anemic rats and significantly overcame the behavioral disorders evoked by iron-deficient anemia. We asked whether the other types of stress also lead to specific amino acid increases in serum and whether an oral administration of this amino acid would also improve the behavioral activity in stressed rats.

In this study, we first investigated changes in the reaction kinetics of internal amino acids under conditions of IWS. We then examined whether the supplementation of branched-chain amino acids (BCAA), increased under IWS, and proline (as negative control), increased with iron deficiency, would prevent stress reactions by using the biochemical and behavioral methods.

MATERIALS AND METHODS

Animals and experimental design. Male Wistar rats (age 6 wk; Japan SLC, Hamamatsu, Japan) were maintained at 25±2°C under a 12 h light (7:00–19:00 h) and dark cycle and fed a commercial nonpurified diet (CE-2: Japan Clea Inc., Tokyo, Japan) and tap water for 3 d. To accustom the rats to the experimental condition, they were initially given free access to an experimental diet (Table 1) for 1 d before being divided into groups.

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Table 1. Composition of basal diet.

| Component        | Amount (%) |
|------------------|------------|
| Casein*          | 20.00      |
| α-Cornstarch*    | 43.25      |
| Sucrose*         | 21.60      |
| Cellulose*       | 5.00       |
| Corn oil†        | 5.00       |
| Mineral mix*     | 4.00       |
| Vitamin mix‡     | 1.00       |
| Choline-Cl**     | 0.15       |
| **Total**        | 100.00     |

* Purchased from Oriental Yeast Co., Tokyo, Japan.
† Purchased from Honen Co., Tokyo, Japan.
‡ Vitamin (AIN-93G) and mineral (AIN-93G) mixtures were purchased from Nihon Nosan K.K., Yokohama, Japan.
** Purchased from Wako Pure Chemical Co., Osaka, Japan.

Thirty-six rats were grouped into six groups, namely, 1, 3, and 7 experimental days with and without IWS (n = 6).

For immobilization, the rats were individually housed in stainless-steel cages in a room with controlled temperature (25 ± 2°C) and humidity (55%) and given free access to the diet and distilled water. IWS rats were maintained in a restricted posture by the use of metal mesh and immersed to the neck in water at 27 ± 1°C for 7 h/d for 1, 3, and 7 d. Thereafter the rats were decapitated on 1, 3, and 7 d, and blood was collected from the cervical area. Tissues were immediately removed, frozen on dry ice, and stored at -80°C until assay.

For 3 d, other rats (controls) were fed the control diet (12% casein diet), a diet supplemented with valine + leucine + isoleucine, or one supplemented with proline. The compositions of the diets are given in Table 2. These rats were decapitated on the last day of the experiment, and blood was collected from the cervical area.

Behavioral analysis. The rats were carefully placed in the center of the hole board on the hole-poking apparatus, and active motor behavior was measured for 10 min (13). The apparatus consisted of an opaque plastic area of 70×70 cm divided into 16 square fields of 17.5×17.5 cm and surrounded by walls 50 cm high. A hole 2 cm in diameter was drilled into the squares adjacent to the walls, and the apparatus was elevated by about 2 cm to allow for hole-poking. Four types of behavior were assessed: locomotion (entering another field by at least 3/4 of the body); rearing on the hind legs; hole poking; and grooming. Each activity was monitored and recorded with a video system; the tapes were subsequently displayed to an observer blinded to the experimental conditions.

The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of the University of Shizuoka.

Biochemical analyses. Serum amino acid concentrations were determined with an automatic amino acid analyzer (L-8500; Hitachi Co. Ltd., Tokyo, Japan). The serum corticosterone concentration was measured with DPC Rat Corticosterone Kits (Diagnostic Products Co., Los Angeles, USA).

Statistics. The statistical significance of the differences between values was determined by an analysis of variance followed by a post hoc l.s.d. test, when appropriate, or a Student’s t-test.

RESULTS

Effects of IWS to body weight, food intake, and organ weight

The results of mean daily growth, food intake, and organ weights are shown in Table 3. Body weight and food intake of the stressed rats was significantly lower than in the control rats on days 3 and 7. The weights of body organs (except brain) generally changed with IWS loading. Significant weight loss evoked by IWS occurred in liver, thymus, and spleen, whereas the weight of adrenal glands increased (days 3 and 7).

Effects of IWS on serum amino acid concentrations

In 1 d of IWS, an increase in serum taurine, threonine, valine, isoleucine, leucine, phenylalanine, lysine, and histidine concentrations or a decrease of alanine concentration (p < 0.01) were noted (Table 4-1). In regard to day 3 of IWS, the concentrations of serum taurine, valine, isoleucine, leucine, and arginine increased, and threonine, serine, glutamic acid, alanine, tyrosine, and tryptophan concentrations decreased (Table 4-2). On day 7, serum taurine, valine, isoleucine, leucine, and phenylalanine significantly increased (p < 0.01), and glutamic acid, alanine, tryptophan, aspartagine (p < 0.01), and aspartic acid, threonine, serine, methio-
Table 3. Effect of IWS on body weight, food intake, and organ weight of rats on days 1, 3, and 7.

| Measurement                  | 1 d                      | 3 d                      | 7 d                      |
|------------------------------|--------------------------|--------------------------|--------------------------|
|                              | Control                  | Stress                   | Control                  | Stress                   | Control                  | Stress                   |
| Body weight (g)              | 134.6±3.8                | 141.4±3.1                | 144.2±3.2                | 127.6±2.8*               | 155.6±5.8*               | 135.6±5.9*               |
| Body weight gain (g)         | 1.4±0.4                  | 6.3±0.8*                 | 11.9±0.9                 | -5.0±1.5*                | 16.9±2.0                 | -16.4±1.3**              |
| Food intake (g/d)            | 11.8±0.5                 | 12.7±0.8                 | 11.4±0.6                 | 6.6±0.5**                | 14.9±0.8                 | 9.3±1.4*                 |

Gastrocnemius

| Adrenal glands were measured on both sides. Gastrocnemius was measured on the left side. Values are means±SE for 6 rats. Data were analyzed by Student's t-test (*p<0.05, **p<0.01).|

Table 4-1. Serum amino acid concentrations in IWS-loaded rats on day 1.

| Amino acids (nmol/mL) | Control | Stress |
|-----------------------|---------|--------|
| Taurine               | 356.1±29.0 | 1623.0±104.0** |
| Aspartic acid         | 31.5±2.6   | 31.7±1.7 |
| Threonine             | 309.1±21.0 | 477.6±34.0** |
| Serine                | 239.0±9.7  | 222.3±13.0 |
| Glutamic acid         | 191.8±7.3  | 172.1±9.1 |
| Glycine               | 228.8±9.1  | 203.6±13.0 |
| Alanine               | 560.6±35.0 | 353.7±24.0** |
| Valine                | 222.2±7.4  | 826.5±45.0** |
| Cystine               | 42.4±3.6   | 46.5±6.0 |
| Methionine            | 62.4±3.2   | 57.3±3.0 |
| Isoleucine            | 102.8±2.4  | 305.6±13.0** |
| Leucine               | 171.7±4.2  | 662.2±34.0** |
| Tyrosine              | 124.2±14.0 | 145.1±6.3 |
| Phenylalanine         | 73.3±2.8   | 111.7±3.7** |
| Tryptophan            | 149.7±4.7  | 151.7±6.3 |
| Lysine                | 578.0±33.0 | 1250.0±63.0** |
| Histidine             | 108.9±40.4 | 233.3±15.0** |
| Arginine              | 180.5±6.3  | 150.3±16.0 |
| Asparagine            | 93.9±5.9   | 91.1±4.3 |
| Proline               | 218.9±9.2  | 201.3±14.0 |

Each value is expressed as the mean±SE of 6 rats per group. Data were analyzed by Student's t-test (**p<0.01).

nine, and arginine (p<0.05) significantly decreased (Table 4-3). In those of amino acids, taurine, valine, isoleucine, leucine, and phenylalanine were significantly increased in the IWS group, through in the experimental periods, than control group. Especially BCAA, namely, valine, isoleucine, and leucine, increased most prominently in all the experimental days (Table 4-1–3).

Table 4-2. Serum amino acid concentrations in IWS-loaded rats on day 3.

| Amino acids (nmol/mL) | Control | Stress |
|-----------------------|---------|--------|
| Taurine               | 365.7±22.0 | 665.6±36.0** |
| Aspartic acid         | 32.6±1.7 | 34.1±2.4 |
| Threonine             | 340.5±22.0 | 247.6±10.0** |
| Serine                | 297.2±11.0 | 239.5±5.6** |
| Glutamic acid         | 207.6±3.6 | 107.2±5.0** |
| Glycine               | 259.3±3.7 | 282.8±11.0 |
| Alanine               | 725.8±40.0 | 309.4±20.0** |
| Valine                | 230.3±7.8 | 1033.0±83.0** |
| Cystine               | 52.0±3.0 | 53.4±4.4 |
| Methionine            | 72.5±3.0 | 70.2±4.6 |
| Isoleucine            | 115.0±4.8 | 490.4±44.0** |
| Leucine               | 184.9±6.7 | 891.9±79.0** |
| Tyrosine              | 148.2±4.5 | 121.7±3.4** |
| Phenylalanine         | 85.4±2.4 | 135.4±2.8** |
| Tryptophan            | 176.0±4.1 | 980.0±4.9** |
| Lysine                | 686.2±23.0 | 728.5±45.0 |
| Histidine             | 109.9±2.5 | 153.3±4.0 |
| Arginine              | 197.3±5.9 | 250.4±12.0** |
| Asparagine            | 89.4±10.4 | 55.9±5.0 |
| Proline               | 249.4±9.7 | 278.8±14.0 |

Each value is expressed as the mean±SE of 6 rats per group. Data were analyzed by Student's t-test (**p<0.01).

Effects of dietary BCAA and proline on body weight, food intake, and organ weight in IWS-loaded rats

Although the food intake by the proline-supplemented group was significantly lower than in the basal- and BCAA-supplemented group (p<0.01), there were no differences in body weight changes in the groups. The liver and thymus weights of the BCAA-supplemented diet group were significantly higher than those on the control diet (p<0.05), suggesting that oral ad-
ministrative BCAA can ameliorate the weight loss evoked by IWS at least in these organs. However, BCAA did not overcome hypertrophy of the adrenal gland (Table 5).

### Biochemical effects of dietary BCAA and proline on IWS-loaded rats

In stressed rats fed the BCAA-supplemented diet, serum valine, isoleucine, and proline concentrations were increased, and taurine, glutamic acid, and glycine were decreased. In regard to IWS, the proline-supplemented diet increased serum cystine and proline concentrations. No significant differences were found in serum taurine levels between the basal diet group and the proline supplemented group (Table 6).

#### Behavioral effects of dietary BCAA and proline on IWS-loaded rats

Behavioral activities such as locomotion, rearing, grooming, and hole-poking of stressed rats fed BCAA-supplemented diets were determined with the hole-board apparatus. All parameters were significantly diminished on IWS-loaded rats (p<0.01; Fig. 1). Rearing was significantly ameliorated by rats on the BCAA-supplemented diet. Hole-poking behavior tended to increase in the BCAA-supplemented group, but not significantly. Moreover, grooming behavior improved, but locomotion did not (Fig. 2).

### DISCUSSION

#### Changes in serum amino acid concentrations with IWS

From the data of mean daily growth, food intake, and organ weights, we first ensured that IWS provokes a severe status of stress in experimental rats. Furthermore, serum amino acids respond markedly and in a highly individual way to IWS. From our hypothesis mentioned in the introduction, the increased types of amino acids are of great interest.

In a study on humans, oral medication with BCAA (valine, isoleucine, and leucine) significantly ameliorated the consciousness level of hepatic encephalopathy patients (23). Moreover, an athlete can enhance ability by ingesting BCAA taken to mitigate fatigue; the prevention of failure (24, 25) or controlling the disassembly of muscular protein is known again (26, 27).

From these previous reports and the present data, it is conceivable that BCAA is a presumable candidate of amino acids to improve behavioral disorder evoked by IWS stress.

In the next paragraph, we tried to clarify whether an oral administration of BCAA can improve behavioral and/or biochemical disorder by IWS loading.

#### Improvement of behavioral disorders of IWS rats given dietary BCAA

The effects of orally administered BCAA on growth, food intake, and organ weight in stressed rats suggest...
that BCAA can ameliorate the weight loss evoked by IWS in at least these organs. However, BCAA administration did not improve the hypertrophy of the adrenal gland. Furthermore, the concentrations of serum corticosterone in the stressed rat fed the BCAA- or proline-supplemented diet are of no significant difference among these test groups (data not shown), suggesting that BCAA improved the behavioral parameter (see below paragraph) without affecting the adrenal gland function disturbed by IWS. The hypertrophy of the adrenal gland and an increase in serum corticosterone levels are increased with stress.

Table 6. Effect of dietary BCAA and proline on serum amino acid concentrations in IWS-loaded rats.

| Amino acids      | (nmol/mL) | Basal diet | + Proline | + BCAA | p value |
|------------------|-----------|------------|-----------|--------|---------|
| Taurine          |           | 936.7±135.0 | 636.6±65.0 | 497.6±45.0 | 0.01    |
| Aspartic acid    | 25.4±1.9  | 25.6±5.5   | 22.8±2.0  | NS     |
| Threonine        | 399.1±40.0 | 406.9±44.0 | 305.7±18.0 | NS     |
| Serine           | 245.8±26.0 | 291.6±34.0 | 218.9±15.0 | NS     |
| Glutamic acid    | 83.2±7.3b | 91.5±12.0b | 51.2±3.3b | 0.01    |
| Glutamine        | 856.2±52.0 | 863.1±60.0 | 878.9±45.0 | NS     |
| Glycine          | 139.4±19.0a | 132.1±12.0b | 91.9±9.3a | 0.05    |
| Alanine          | 213.5±35.0 | 268.3±45.0 | 208.0±14.0 | NS     |
| Valine           | 699.7±108.0 | 608.8±70.0 | 1590.0±240.0 | 0.01   |
| Cystine          | 51.2±6.1a | 73.2±8.6b | 63.4±2.5ab | 0.05    |
| Methionine       | 47.5±5.1  | 57.1±6.7   | 50.9±3.3  | NS     |
| Isoleucine       | 288.1±37.0a | 244.9±26.0b | 471.6±71.0b | 0.05   |
| Leucine          | 575.7±103.0 | 526.9±72.0 | 764.6±83.0 | NS     |
| Tyrosine         | 100.2±14.0 | 115.6±13.0 | 90.5±3.6  | NS     |
| Phenylalanine    | 85.7±5.0  | 86.7±3.1   | 75.6±2.6  | NS     |
| Tryptophan       | 83.8±12.0 | 92.7±10.0  | 99.6±3.6  | NS     |
| Lysine           | 1008.0±138.0 | 933.1±96.0 | 839.5±47.0 | NS     |
| Histidine        | 173.3±17.6 | 188.8±15.0 | 157.0±5.6 | NS     |
| Arginine         | 114.3±12.0 | 110.9±8.7  | 118.0±7.7 | NS     |
| Asparagine       | 42.0±4.1  | 47.3±11.0  | 35.9±5.1  | NS     |
| Proline          | 133.5±18.0a | 1766.0±548.0b | 162.5±14.0a | 0.01   |

Each value is expressed as the mean±SE of 6 rats per group. Data were analyzed by one-way ANOVA with l.s.d. (p<0.05, p<0.01).

Fig. 1. Hole-board behavior by IWS-loaded rats. Each value is the mean for 6 rats per group. The data were analyzed by Student's t-test. Vertical bars indicate SE (a, p<0.05).
Table 6 shows that serum amino acids respond markedly and in a highly individual way to IWS and diet. Perhaps one of the most intriguing pieces of data was that the high level of serum taurine evoked by IWS was ameliorated by BCAA application. Other workers found that plasma taurine levels exhibit the sharp rise during restraint stress (14), as we also noted (Table 4-1-3). Cooper and Lombardini (28) showed that a 2 mg/kg injection, s.c., of adrenaline, which increases in the plasma during stress, caused a significant fall of taurine in cardiac tissue and an increase in the concentration of blood taurine in rats. Thus dietary BCAA can ameliorate stress reactions mediated by adrenaline.

Rearing behavior, which may also constitute a form of exploration (13), was significantly ameliorated by a BCAA-supplemented diet in comparison with a basal diet and a proline-supplemented diet. The hole-poking behavior, also an exploratory behavior (13), tended to increase in the BCAA-supplemented group but not significantly. Moreover, grooming, a nonexploratory body reference behavior, was also improved, but locomotion was not.

To our knowledge, these data constitute the first description of a parallel, BCAA-induced reversal of the behavioral and biochemical deficits caused by IWS. Especially, grooming behavior was almost completely improved. The rate of improvement of rearing behavior, however, was significant but poor, suggesting that the BCAA administration wasn’t enough to improve the disorder of exploratory behavior provoked by IWS.

We recently observed that oral proline administration can enhance the significant increase in the number of red blood cells and behavioral parameters in iron-deficient anemic rat (22). In this study, however, we didn’t observe significant behavioral and biochemical amelioration in rat by proline administration, indicating that the improvement effect of dietary amino acid is stressor specific.

We noticed a big difference of weight gain between the 20% casein diet+IWS (3 d: Table 3) and the 12% casein diet+IWS (3 d: Table 5). In the previous study, Yokogoshi et al. (29) demonstrated that the body weights of rats fed the 10% casein diet were greater than 25% of the casein diet group for the first 3-4 d. Protein synthesis in liver, however, was gradually decreased with the increase of dietary protein (0-25%). This earlier study may provide a nutritional basis for the present observation.

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