Effects of Neurotrophin® on Immunodeficiency and Ulcer-Development of Rats Exposed to Activity-Stress

Chiaki HARA and Nobuya OGAWA

Department of Pharmacology, Ehime University School of Medicine,
Shigenobu-cho, Onsen-gun, Ehime 791-02, Japan

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Abstract—Young adult rats exposed to a 1 hr feeding schedule in activity-wheel cages daily, show immunological incompetence and stomach ulcer. This stress procedure is called "activity-stress (AS)". The present study examined the effects of neurotrophin (NSP), which is an extract from rabbit skin tissues inflamed with vaccinia virus, on the immunosuppression and stomach ulcer induced by AS. The i.p. treated NSP showed a significant immunopotential effect (50 and 100 mg/kg), but did not significantly prevent the ulceration, although i.p. treated NSP decreased the incidence of ulcer. The p.o. treated NSP revealed both immunopotential (50 and 100 mg/kg) and antiulcerogenic effects (100 mg/kg). However, the i.p. treatment of NSP seems to prevent atrophy of the thymus and spleen, but did not improve hypertrophy of the adrenals induced by AS. The p.o. treated NSP improved atrophy of the spleen and hypertrophy of the adrenals, but did not improve atrophy of the thymus. Since immunodeficiency and ulcer-production by AS are considered to be phenomena appearing in the exhaustion stage of the organism, the present study suggests that NSP can heal immunological incompetence and stomach-ulcer induced by stress. In addition, the present study discussed the different pharmacological activities of NSP based on differences between administration routes.

Young adult rats that are housed in running wheel cages and fed 1 hr daily develop large lesions in the glandular stomach and die within a few days. Rats that develop stomach lesions and die significantly run more during the experimental period as compared with rats which survive and are lesion-free (1). Therefore, a high level of running activity is a necessary component of the ulceration. Accordingly, the ulcer was designated as "activity-stress (AS) ulcer" by Pare and Houser (2).

On the other hand, rats which were exposed to AS revealed atrophy of the thymus and spleen which are regarded as belonging to the lymphatic system (3). In addition, antibody titers in their sera significantly diminished (4, 5). This indicates that the AS-exposed rats suffer from immunological incompetence as well as stomach ulcer. Therefore, the AS-exposed rats seem to be a

beneficial model for a parallel comparison of influences of stress on immunocompetence and ulcer-development in the same individuals.

Neurotrophin (NSP) is an extract from rabbit skin tissues inflamed with vaccinia virus. Accordingly, NSP itself is a crude substance containing some biologically active ingredients. For the past two decades NSP has been used clinically in allergic diseases (6). In addition, its effectiveness on pain of the orthopedic field has been reported (7, 8). In animal studies, NSP was confirmed to have an analgesic effect (9, 10). Moreover, NSP is indicated to heal pathophysiological symptoms of animals exposed to stress in spite of having no influence on healthy animals (11, 12). Especially, NSP exerted preventative effects on stomach ulcer induced by restraint and water immersion stress and on histamine-induced duodenal

ulcer (13). Moreover, NSP has an immunopotential effect on various immunodeficiency models such as immunodepression in spontaneously hypertensive rats (14) and immunosuppression induced by SART (specific stress caused by alternation of rhythm in temperature) stress (15). However, the mechanisms of action and the biologically active ingredient of NSP are unknown.

Therefore, the present study examined effects of NSP on immunosuppression and stomach ulcer induced by AS in order to study the mode of action of NSP.

Materials and Methods

Animals: Male Wistar strain rats supplied from Japan Clea Laboratory Co., Ltd. were used as subjects. These animals were 7 weeks old at the beginning of the 1 hr feeding schedule. For at least 2 weeks before the 1 hr feeding schedule was started, they were housed in a temperature-controlled room ($23 \pm 1^\circ\text{C}$) with reversed 12:12 light-dark (LD) cycle (lights on 21:00–9:00).

Apparatus: Twenty running-wheel cages (Yayoi Medical Instrument Co., Ltd., Tokyo, Japan) were used. Each running-wheel was equipped with an adjoining cage ($40 \times 15 \times 15$ cm). A sliding door separated the cage from its adjoining wheel. The wheel consisted of a wire mesh drum, 10 cm wide and 83 cm in diameter. Wheel revolutions were automatically recorded at intervals of 30 min by a micro-computer system.

Procedure: The animals were divided into 4 groups as follows: 1) free-feeding group with no drug treatment in the activity-wheel cage, 2) 1 hr feeding group with intraperitoneal treatment of drugs in the activity-wheel cage, 3) 1 hr feeding group with oral treatment of drugs in the activity-wheel cage, and 4) 1 hr feeding group with no drug treatment in the activity-wheel cage. The experiment was performed under the reversed LD cycle condition, since rats which were exposed to activity-stress in the dark revealed a higher incidence of ulcer (1, 3). The animals were allowed to adapt to the reversed LD cycle for at least 2 weeks under ad lib. feeding conditions. After the adaptation to the running wheel cage for at least 3 days, the 1 hr feeding schedule (10:00–11:00

A.M.) was started. The animals were immunized by duplicate treatment of antigen on Day-7 and Day-1 before the 1 hr feeding schedule was started. NSP was daily administered once a day just before the feeding time. The 1 hr feeding schedule was ceased when the control animals died. Then, the surviving animals were sacrificed by ether. The stomach, thymus, spleen and adrenals were dissected out. One hr after 1% formalin injection into the stomach, the stomach was opened by cutting along the greater curvature, washed thoroughly with saline, and inspected for ulcer. The number of ulcers was counted, and the extent of ulceration was determined by measuring the length of each ulcer. Wet weights were obtained for the thymus, spleen and adrenals, and they were expressed as mg/g of body weight.

Immunization and titration of antibody:

Rats were injected into the tail vein with 0.5 ml of solution containing 10^8 sheep erythrocytes as antigen and hemophilus pertusis vaccine (10^9 organisms) as adjuvant. Blood samples were obtained by retroorbital puncture. Titration of hemagglutination (HA) was carried out on the diluted individual sera. Two-fold serial dilutions of serum of 0.025 ml volume were added to 0.025 ml of a sheep erythrocyte solution in a concentration of 10^8 organisms/ml. Antibody titer was expressed as the dilution of serum which served to produce HA. The titration procedure was performed according to our previous study (4, 5).

Drugs: NSP was generously donated by Nippon Zoki Pharmaceutical Co., Ltd. In the present study, both the injectable solution of NSP (5 mg/ml) and its concentrated solution of NSP (50 mg/ml, 100 mg/ml) were used. The former was administered intraperitoneally with the injection volume of 1 ml/100 g body weight. Saline was used as the control. The latter was administered orally with the injection volume of 0.2 ml/100 g body weight. Vehicle containing extract from healthy rabbit skin was used as the control. Doses of NSP used in the present study were decided in reference to the published papers (13–15).

Statistics: Only the data from animals showing a significant increase of the wheel-

activity as compared to the activity level at the beginning of 1 hr feeding were adopted, since a high level of running wheel-activity is essential for getting the activity-stress symptoms. Severities of ulcer and immunity were assessed by the Mann-Whitney *U*-test. Changes of organ weights were evaluated by Student's *t*-test. Evaluation of running activity was carried out by analysis of variance followed by Scheffe's test.

Results

Figure 1 shows body weight changes and running-activity of rats administered NSP intraperitoneally during the 1 hr feeding period. In the intraperitoneal administration, NSP-treated rats revealed a low running-activity compared to saline treated ones ($F(2,24)=4.773$, $P<0.05$). On the other hand, there was no significant difference between NSP and saline in the body weight changes. In the oral administration, there were no differences between vehicle and NSP in either body weight changes or running-activity (data not shown).

Figure 2 represents the effects of NSP on

development of activity-stress ulcer. The free-feeding group had no ulcers. The 1-hr feeding group without administration of any drug solutions revealed stomach ulcer. Both the saline-treated group and vehicle-treated group also showed stomach ulcer. Intraperitoneal administration of NSP did not show a significant ulcer-preventing effect compared to saline, although the incidence of ulcer seems to be reduced. On the other hand, oral administration of 100 mg NSP significantly prevented the ulceration and decreased the incidence of ulcer compared to the vehicle treated group ($P<0.05$).

Figure 3 represents the effect of NSP on immunosuppression induced by AS. The antibody titer of the 1 hr feeding group with no drug treatment was lower than that of the free-feeding group ($P<0.01$). Antibody titers of groups with either saline or vehicle also were lower compared to that of the free-feeding group ($P<0.01$). In the intraperitoneal administration of NSP, either 50 mg ($P<0.05$) or 100 mg ($P<0.01$) of NSP significantly prevented reduction of antibody titer by AS compared to saline. In

Fig. 1. Influences of neurotrophin (NSP) administered i.p. on body weight and running activity changes induced by activity-stress. * $P<0.05$: statistically different from saline (Scheffe's test). Saline, 50 and 100 mg groups consist of 7, 10 and 10 animals, respectively.

Fig. 2. Effect of neurotropin (NSP) on development of activity-stress ulcer. * $P < 0.05$: statistically different from vehicle or saline. † $P < 0.05$, †† $P < 0.01$: statistically different from free-feeding group (Mann-Whitney U -test, one-tailed). () represents incidence of rats with ulcer (%). Free-feeding and 1 hr feeding groups consist of 5 and 9 animals, respectively. In the i.p. treatment of NSP, saline, 50 and 100 mg groups consist of 7, 10 and 10 animals, respectively. In the p.o. treatment, vehicle, 25, 50 and 100 mg groups consist of 5, 5, 4 and 4 animals, respectively.

the oral administration, antibody titers of rats with 50 mg ($P < 0.05$) and 100 mg ($P < 0.05$) of NSP showed a higher value compared to those of rats with vehicle.

Figure 4 shows the effects of NSP on relative organ weights of the thymus, spleen and adrenals of rats exposed to AS. The organ weights of the thymus and spleen of the 1 hr feeding group with no drug treatment, saline-treated group and vehicle-treated group significantly decreased compared to those of the free feeding group ($P < 0.01$). In the intraperitoneal treatment of NSP, the effects on the atrophy of the thymus and spleen were not statistically significant compared to saline. However, the thymus and spleen weights of rats with 100 mg of NSP did not markedly reduce compared to those of the free-feeding rats. Therefore, intraperitoneally treated NSP seems to prevent dose-dependently atrophy of the thymus and

spleen of rats exposed to AS. The oral treatment did not prevent the atrophy of the thymus. The atrophy of the spleen also was not significantly improved by the oral treatment, although it seems to be improved dose-dependently. The adrenal weights in the 1 hr feeding, saline-treated and vehicle-treated groups indicated hypertrophy compared to that of the free-feeding group. The oral treatment of NSP dose-dependently improved the hypertrophy. Especially, 100 mg NSP significantly reduced the hypertrophy ($P < 0.05$). On the other hand, the intraperitoneal treatment of NSP had no effect on the hypertrophy of the adrenals induced by AS.

Discussion

The purpose of the present study was to examine the effects of NSP on immunological incompetence and stomach ulcer found in the same subjects exposed to the activity-

Fig. 3. Effect of neurotrophin (NSP) on immunosuppression induced by activity-stress. * $P < 0.05$, ** $P < 0.001$: statistically different from vehicle or saline. †† $P < 0.01$: statistically different from free-feeding group (Mann-Whitney U -test, one-tailed). Antibody titer is expressed as the dilution of serum which served to produce hemagglutination. See Fig. 2 legend for animal number of each group.

stress paradigm. In addition, the effects of NSP were compared between the intraperitoneal and oral administrations.

The results of the present study indicated that rats exposed to AS revealed immunosuppression, ulcer-production, atrophy of the thymus and spleen, and hypertrophy of the adrenals compared to the free-feeding group, as we have previously reported (3–5).

Intraperitoneally administered NSP dose-dependently alleviated immunodeficiency, but did not prevent the ulceration. In addition, the intraperitoneal administration of NSP seems to prevent atrophy of the thymus and spleen, but not hypertrophy of the adrenals. In contrast to the intraperitoneal administration, the oral administration of NSP prevented both immunodeficiency and ulceration. However, although the oral administration had no effect on atrophy of the thymus, it alleviated atrophy of the spleen

and hypertrophy of the adrenals.

Thus, these results suggest that there are some differences between the intraperitoneal and oral administrations of NSP in its pharmacological actions. The first discrepancy between the two administration routes was found in the ulcer-preventing effect. The intraperitoneal administration of NSP has been reported to have an ulcer-preventing effect in various stress ulcer models (13). However, the results of the present study indicated that the intraperitoneal treatment did not exert a significant ulcer-preventing effect. The ulceration of AS is known to correlate with hyper-running activity. Intraperitoneal treatment with a 100 mg of NSP depressed running-activity compared to saline. This makes us expect a suppression of ulceration. However, marked suppression of ulceration was not found, although NSP treatment reduced the ulcer-

Fig. 4. Influence of neurotrophin (NSP) on relative organ weight changes of thymus, spleen and adrenals induced by activity-stress. * $P < 0.05$, ** $P < 0.001$: statistically different from vehicle or saline. † $P < 0.05$, †† $P < 0.01$: statistically different from free-feeding group (Student's *t*-test, one-tailed). See Fig. 2 legend for animal number of each group.

index and incidence of rats with ulcer compared to saline. The ulcer-index of the saline group was larger than those of the 1 hr feeding group with no drug treatment and the vehicle group. In addition, rats which were administered NSP intraperitoneally exerted hypertrophy of the adrenals in spite of attenuation of running-activity. Accordingly, the daily repeated intraperitoneal treatment itself might adversely act on exhausted rats. Moreover, the reduction of running activity seems to be based on the physical adverse effect due to the intraperitoneal treatment of a high dose of NSP. Therefore, NSP seems to have an ulcer-preventing effect basically because of a decrease in the incidence of ulcer.

The second discrepancy between the two administration routes is found in the thymus weights. The intraperitoneal administration of NSP dose-dependently alleviated immunodeficiency as well as atrophy of the

thymus and spleen which are regarded as belonging to the lymphatic system. The fact is interesting in comparison with the results of the oral administration of NSP. The oral treatment of NSP dose-dependently prevented ulceration and alleviated immunodeficiency. In addition, atrophy of the spleen and hypertrophy of the adrenals were improved, but atrophy of the thymus was not alleviated. Since the oral administration of NSP improved hypertrophy of the adrenals, the oral administration seems to reduce stress-level. NSP is known to enhance immune function. Especially, it has been reported that NSP activated the T-cell functions in the spontaneously hypertensive rats (14) or in an *in vitro* study (16). Therefore, the immunopotentiality found in the intraperitoneal treatment of NSP seems to be based on the direct action on the thymus, since the immunopotentiality effect was found despite a stressful situation accom-

panied with ulcer-production and hypertrophy of the adrenals. On the other hand, in the oral administration of NSP, the effect on the immunodeficiency seems to be based on an indirect action due to attenuation of stress-level, since ulceration and hypertrophy of the adrenals were improved without alleviation of atrophy of the thymus. The indirect action seems to be the central action. This is supported by our previous study which indicated that psychotropic drugs, especially antidepressants, prevented the ulceration without suppression of running activity (17). Moreover, the fact that frequency analysis of the EEG of normal rats with NSP (100 and 200 mg/kg, p.o.) exerted an increase of the theta wave component in the hippocampal EEG (C. Hara et al., unpublished data) and the previous papers that NSP has an analgesic effect conceivably based on its central action (9, 10) seems to support the hypothesis of the central action.

Thus, the results of the present study indicated that there were differences between the intraperitoneal and oral administrations in the pharmacological profile of NSP. Such a difference is likely to rise, since NSP itself is a crude substance extracted from the rabbit skin tissues inflamed with vaccinia virus, and its active ingredients are not identified yet. Therefore, we hypothesize that NSP contains at least the following two active substances: one is a substance which directly potentiates the thymus function and is ineffective in the oral administration, and the other is a substance which attenuates stress-level via the central nervous system and is effective in the oral administration.

In conclusion, the results of the present study indicate that NSP is effective on immunodeficiency and ulceration in the exhaustion stage like that induced by exposure of AS. In addition, a difference between the administration routes in the pharmacological profile of NSP was found in the present study. This suggests that NSP contains several biologically active ingredients, since NSP itself is a crude substance. At present, however, such assumptions are under discussion. Therefore, further studies are needed.

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