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The Galectin-1 level in serum as a novel marker for stress

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Abstract Galectin-1(Gal-1), a carbohydrate-binding protein with an affinity for β-galactoside, is widely expressed in various normal and pathological tissues and it also plays an important role in regulating immune cell homeostasis and tumorigenesis. This study investigated the effects of restraint stress on serum Gal-1 by Western blot analyses and enzyme-linked immunosorbent assays. The Gal-1 levels of the restraint-stress group were significantly higher than those of the control group. However, this increase by stress was not obvious in adolescent rats. The pattern of these changes was similar to that of corticosterone. Furthermore, this Gal-1 increase in the serum was prevented by pre-treatment with a neurotoxin 6-hydroxydopamine (6-OHDA), which destroys the noradrenergic nerve terminals. However, a bilateral adrenalectomy (ADX) had no effect on the Gal-1 increase. These results suggest that Gal-1 is a candidate stress marker protein and that the stress-induced increase of Gal-1 in serum is regulated by the sympathetic nervous system under stress conditions.

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Introduction

Stress is inherent in modern society, and it has the potential to disturb the normal functions of many systems of the human body, including the nervous system, the endocrine system, and the immune system [1]. Therefore, it is important to resolve stress problems. Markers that show stress at an early stage will therefore be useful for diagnostic purposes. Researchers have attempted to establish methods to evaluate both pathological and physiological stress using biochemical markers, such as the concentrations of corticosterone, ACTH, catecholamine, prolactin, leptin, and chromogranin A in the peripheral blood, saliva, or urine [2–11].

Gal-1, the first protein in the galectin family to be discovered, is present in various normal and pathological tissues and organs, including smooth muscles, the liver, and the thymus. Gal-1 is a multifunctional protein that is implicated in a variety of biological activities [12, 13],

such as immune-cell homeostasis, tumorigenesis, nerve regeneration after injury, and the inflammatory response [14–19]. However, the effect of various types of stress on the serum Gal-1 level is unclear. The current study demonstrated that Gal-1 quickly responds to stress, and Gal-1 may therefore be a good candidate as a stress marker.

Material & methods

Animals

Ninety male Sprague–Dawley rats (SLC Japan, Shizuoka, Japan), ranging in age from 4 weeks to 6 months, were maintained in a temperature-controlled room (22±3°C) with a 12-h light/dark cycle (light sun at 7:00 a.m.). Three to five rats per cage were housed with free access to water and food. All experiments were carried out according to the Guidelines for Animal Experimentation of Kanagawa Dental College in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Stress procedures

The animals were divided into two groups, namely, the restraint-stress group and the control group. Animals were tied to a wooden board for only 30 min for short restraint stress (Experiments 1 and 3 in Fig. 1). The rats were tied to a board for 2, 4, or 6 h for long restraint stress (Experiment 2 in Fig. 1). Leg fasteners held the rats in a spread-eagle supine position.

Adrenalectomy

Adrenalectomized rats (14-week old) were purchased from SLC Japan. Brief surgical procedures were the following. Under deep anesthesia, animals were made about 1.5 cm incision on the right dorsal skin above the 12th costa. Then, the right abdominal muscle was also incised. The right adrenal gland was removed with forceps after blood vessels were occluded. After extraction, the incision was sutured. The left adrenal gland was similarly removed. Two weeks later, the adrenalectomized rats were served for short restraint-stress experiments.

Chemical sympathectomy

A chemical sympathectomy was performed on 10-week old rats by the intravenous injection of 6-hydroxydopamine (6-OHDA; Sigma-Aldrich Chemical Co., St. Louis, MO) according to the previous papers [20–22]. The 6-OHDA was diluted to 100 mg/ml in physiological saline containing 0.1% of ascorbic acid (Experiment 3 in Fig. 1) and 100 mg/kg was dosed into the tail vein.

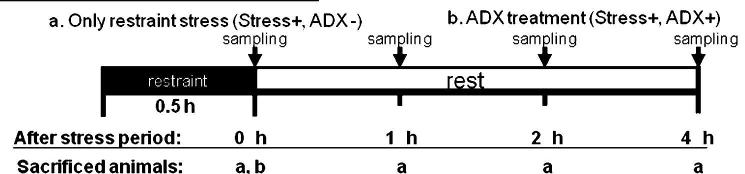
Sample preparations from rats exposed to stress

Trunk blood was collected from animals by decapitation under anesthesia with 2-bromo-2-chloro-1,1,1-trifluoroethane (2 ml/kg; Takeda Chemical Industries, Osaka, Japan). The blood was collected in plastic tubes, centrifuged at 25,000 rpm for 25 min at room temperature, and stored at −80°C until it was analyzed.

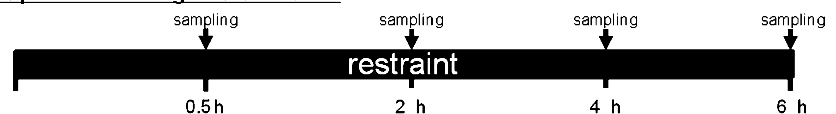
Experimental procedure

Experiment 1 : short restraint stress

a. Only restraint stress (Stress+, ADX-) b. ADX treatment (Stress+, ADX+)



Experiment 2 : long restraint stress



Experiment 3 : 6-OHDA

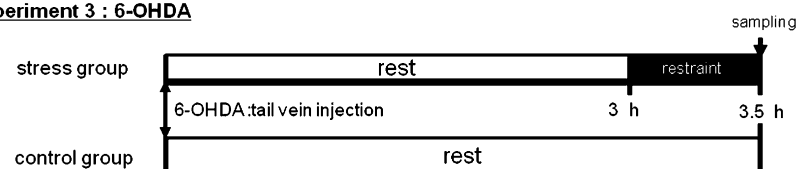


Fig. 1 Experimental protocols and time schedule. Experiment 1 indicates the time schedule for short restraint stress. The black bar indicates the time of restraint stress and arrows indicate the times of blood sampling. The adrenalectomized rats (b) were sacrificed at the end of stress and the rats (a) that did not undergo a surgical operation were sacrificed at the end of stress, and 1 h, 2 h, and 4 h after stress. Experiment 2 indicates the time schedule of long restraint stress. Arrows indicate sampling time during restraint. Experiment 3 indicates the time schedule for 6-OHDA treated rats

Rats that did not undergo a surgical procedure underwent a 30-min stress period, and then trunk blood was collected at 0, 1, 2, or 4 h after the end of the stress period. The rats in the latter three groups were returned to their cages at the end of the restraint period, where they had free access to food and water. Adrenalectomized rats were separated into two groups, consisting of those with the short 30-min restraint stress and those without. Trunk blood was collected as described above. In experiment 2, the rats were exposed to stress for 0.5, 2, 4, and 6 h, and the blood was collected immediately after each stress period. Trunk blood was collected from the rats treated with 6-OHDA either after 30-min restraint stress or without stress. The stress experiments were all carried out between 10:00 a.m. and 12:00 noon to avoid the influence of the pre-delivery activation of the hypothalamic-pituitary-adrenal axis (HPA axis). All of the control rats were kept in individual cages.

Quantitative Western analysis

The total protein level in the serum samples was measured using the Bradford assay. Equal amounts of protein from each group were separated by SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). The transferred membranes were blocked with 5% non-fat skim milk in a solution of sodium phosphate buffer containing 0.01% Tween 20 and 0.85% NaCl (PBS-T) for 1 h at room temperature. The nitrocellulose membranes were incubated for 1 h in rabbit anti-Gal-1 polyclonal antibody (2.7 μg/ml) diluted in PBS-T containing 5% non-fat dried milk. The anti-Gal-1 polyclonal antibodies were produced in a rabbit by the injection of recombinant human Gal-1 and purified by protein-A-affinity chromatography. The antibody specificity was already reported elsewhere [18]. The membranes were rinsed with PBS-T and incubated with goat peroxides-conjugated anti-rabbit antibody (Dako Cytomation, Glostrup, Denmark) for 1 h at room temperature and then rinsed with PBS-T. The immunocomplexes on the membrane were visualized by chemiluminescence using an enhanced Lumi-Light Plus Western Blotting Substrate (Roche Diagnostics, Basel, Switzerland) and detected with the LAS-3000 imaging system (Fuji Film, Tokyo, Japan). The appropriate signal bands were identified using Multi-gauge X (Fuji Film, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Blood samples stored at −80°C were thawed on ice and the levels of Gal-1 were then quantified by ELISA using the rabbit anti-Gal-1 antibody and the mouse Gal-1 Duo Set ELISA Development Kit (R&D Systems, Minneapolis, Minnesota). Preliminary experiments compared various combinations of anti-Gal-1 antibodies and Gal-1 (recombinant human Gal-1 and recombinant mouse Gal-1) for the ELISA because of the absence of commercially available ELISA kit for rat. Finally, the following combination was selected based on the reproducibility and sensitivity, namely, the rabbit anti-Gal-1 antibody that was same one used in the Western blot analysis as the capture antibody, biotinylated goat anti-mouse-Gal-1 antibody in the ELISA kit as the detection antibody and human Gal-1 as a standard antigen. Furthermore, each corticosterone level was quantified using the appropriate Coat-A-Count Rat Corticosterone Kit (DPC; Diagnostic Products Corporation, Los Angeles, CA, USA).

Data analysis

All data were analyzed using a one-way analysis of variance (ANOVA) to compare the Gal-1 levels within the groups undergoing short-duration stress, and Student's *t*-test with the post hoc Tukey's multiple comparison was used to compare the groups.

Results

Effect of short-duration restraint stress on the serum Gal-1 level

The serum Gal-1 levels at the end of the short (30 min) restraint stress were analyzed by Western blotting at various ages (Fig. 2). The intensity of the Gal-1 band in the stressed group was stronger than that in the control group for rats aged 10 weeks, 14 weeks, and 6 months. The intensity of the Gal-1 band in samples from the 4-week-old rats varied depending on the sample. However, there seemed to be no differences between the control and the short restraint-stress groups (Fig. 2). Quantification using ELISA showed results that were similar to those obtained for the Western blot analysis. The average value of the Gal-1 level in each age group is shown in Fig. 3. The increase in the serum Gal-1 levels in the rats subjected to short restraint stress was statistically significant in the 10-week-old group ($p<0.01$), 14-week-old group ($p<0.05$), and 6-month-old rat group ($p<0.01$). However, the short-duration restraint stress had no significant effect on the serum Gal-1 level in the 4-week-old rats (Fig. 3a).

The serum Gal-1 level in the 10-week-old rats was measured at 0 h, 1 h, 2 h, and 4 h after the short restraint stress by ELISA. The average values at each time are shown in Fig. 4A. The average value (mean±standard deviation) of the serum Gal-1 level before stress exposure (control) was 8.85±0.22 ng/ml. The average values were 17.11±1.57, 20.23±2.14, 12.66±0.65, and 9.95±0.88 ng/ml at 0, 1, 2, and 4 h, respectively, after short restraint stress. An

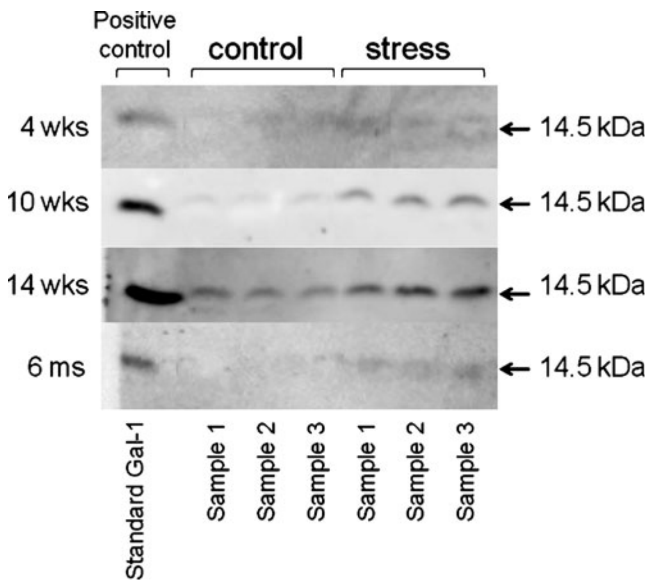


Fig. 2 A Western blotting analysis of 4-week (4 wks), 10-week (10 wks), 14-week (14 wks), and 6-month-old (6 ms) rats. Recombinant human Gal-1 was used as a positive control. Note 14.5 kDa bands (arrows) in stressed rats are more intense than those of control rats except for 4-week-old rats

ANOVA analysis showed that the increase from the control level was statistically significant at 0 h ($p<0.01$) and 1 h ($p<0.01$). However, after 1 h, the Gal-1 level gradually decreased and returned to almost the same level as the control at 4 h. This decrease at 2 and 4 h was statistically significant ($p<0.01$ at 2 h and $p<0.01$ at 4 h) compared to the level of 1 h. These changes were compared to those of the serum corticosterone level, which is one of the standard stress markers. The corticosterone level had already reached the maximum at the end of the short restraint stress (at 0 h) and then gradually decreased (Fig. 4B). The higher initial value was statistically significant compared to the control, and the decreases at 1 h ($p<0.01$), 2 h ($p<0.01$) and 4 h ($p<0.01$) were also statistically significant compared to the initial level of corticosterone by ANOVA.

Gal-1 levels during long restraint stress

A long restraint-stress experiment (Experiment 2 in Fig. 1) was performed to evaluate the changes in the serum Gal-1 levels under continuous stress (Fig. 5). The serum Gal-1 level significantly increased at 30 min after stress exposure (15.68±2.50 ng/ml; $p<0.01$, ANOVA) compared to the control level (8.85±0.50 ng/ml) and then rapidly decreased to the control level at 2 h (9.08±0.86 ng/ml; $p<0.01$ compared to the level of 30 min, ANOVA). Thereafter, the level was relatively constant (7.11±0.57 ng/ml at 4 h; 7.17± 0.45 ng/ml at 6 h). The corticosterone level rapidly increased (Fig. 5B) from a control level of 264±57.68 ng/ml to 1132.25±119.08 ng/ml at 30 min ($p<0.01$ compared to the control level; ANOVA), and then decreased to 535.5± 52.53 ng/ml at 2 h ($p<0.01$ compared to the level of 30 min). The levels after 2 h were relatively constant (488.5±49.64 ng/ml at 4 h and 607.6±11.2 ng/ml at 6 h).

Effects of adrenalectomy and 6-OHDA injection on the stress-induced Gal-1 increase

An adrenalectomy was used to exclude the possible influence of corticosterone. Only a trace of corticosterone was detected in the serum of control rats and in that of adrenalectomized rats subjected to short stress (Fig. 6A). On the other hand, the short restraint stress significantly increased Gal-1 levels in adrenalectomized rats, from 6.66±1.01 ng/ml (unstressed) to 16. 61±2.07 (stressed) (Fig. 6B). The Gal-1 levels in the adrenalectomized and non-stressed rats were lower than those in non-adrenalectomized and non-stressed rats (Fig. 6B) suggesting the effects of adrenalectomy itself on serum Gal-1 levels. However, these differences were not statistically significant. In addition, the serum Gal-1 levels in the control rats (non-adrenalectomized and non-stressed) of the adrenalectomy experiment (Fig. 6B) tended to be higher than those of control rats of other experiments (Figs. 3b, d, 4A and 5A), however, these differences were

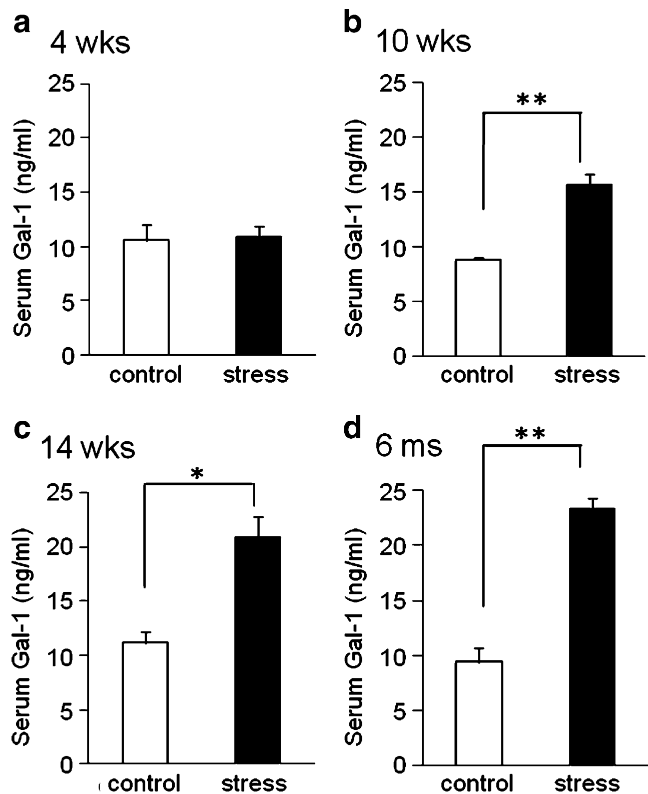


Fig. 3 The levels of Gal-1 in serum from rats of different ages were analyzed with ELISA after a 30-min restraint-stress period. Values represent the means±SD in each group. *indicates a significant difference at $p<0.05$ and **a significant difference at $p<0.01$

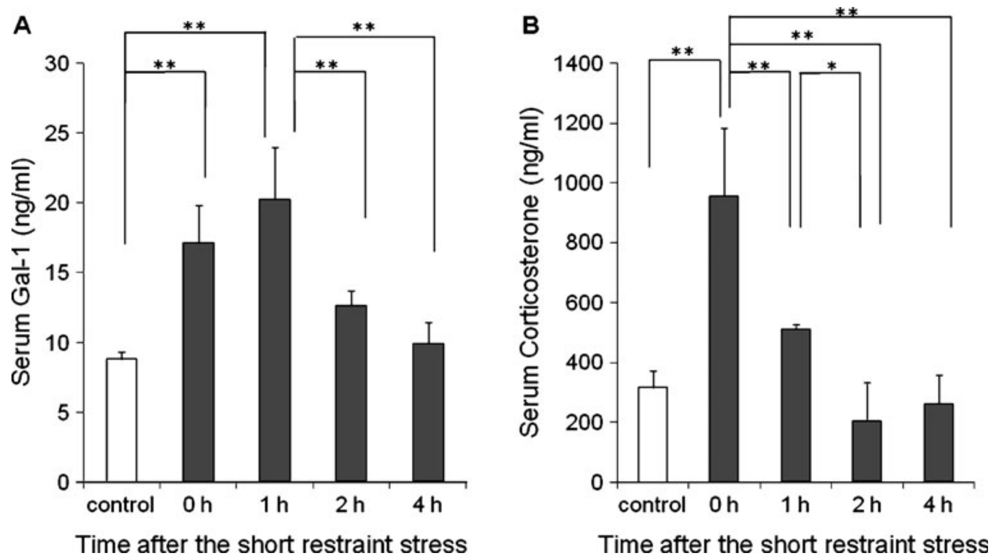


Fig. 4 The serum Gal-1 level (**A**) and corticosterone level (**B**) at 30 min of restraint stress (n=3–5) at the end of stress (0 h), 1 h, 2 h, and 4 h after stress. The values represent the means±SD in each group. *A significant difference at $p<0.05$ and **indicates a significant difference at $p<0.01$

not statistically significant based on $p<0.05$. In contrast to the adrenalectomy, chemical sympathectomy with 6-OHDA did prevent the rise in Gal-1 in conjunction with stress (11.37±1.54 ng/ml in unstressed rats compared to 13.22± 2.03 ng/ml in stressed rats) (Fig. 7). There is no statistically significant difference between the Gal-1 levels of unstressed rats in the sympathectomy (Fig. 7) and those of other control rats (Figs. 3, 4A and 5A), though the levels of unstressed rats in the sympathectomy tended to be slightly higher than those of the control rats.

Discussion

The present study demonstrated a rapid (within 1 h) and marked increase in the serum Gal-1 level after short restraint stress in rats older than 10 weeks old, but not in adolescent rats. This suggests that maturity is the critical determinant of the serum Gal-1 level changes caused by stress. In contrast, a significant increase in the plasma corticosterone levels was observed at all ages, including the adolescent rats as observed in previous papers [7, 23]. The Gal-1 increase in response to stress was a transient phenomenon similar to the increase of corticosterone in serum suggesting that Gal-1 is a novel marker for the early stage of stress after exposure to stress. This serum Gal-1 response was confirmed by the long-term stress experiment to be similar to that of corticosterone.

Corticosterone acts on activated macrophages to reduce the expression of IL-1β, IL-6, and others in response to stress [17, 24]. Similarly, Gal-1 suppresses the secretion of IL-2, a proinflammatory cytokine from T-cells [25]. Furthermore,

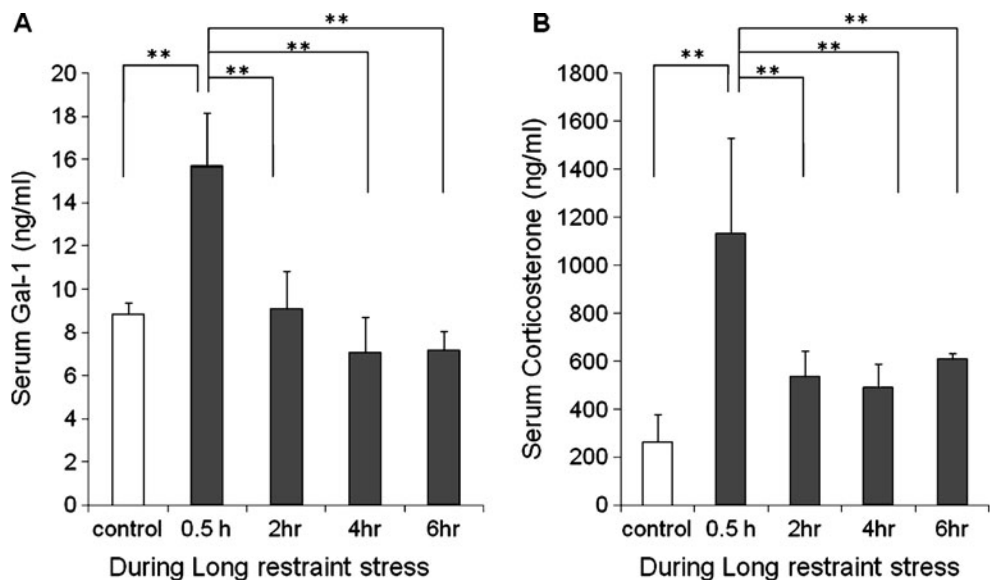


Fig. 5 The serum levels of Gal-1 (**A**) and corticosterone (**B**) of continuous restraint stress for 0.5, 2, 4, and 6 h (n=4–7). Values shown represent the means±SD in each group. **indicates a significant difference at $p<0.01$

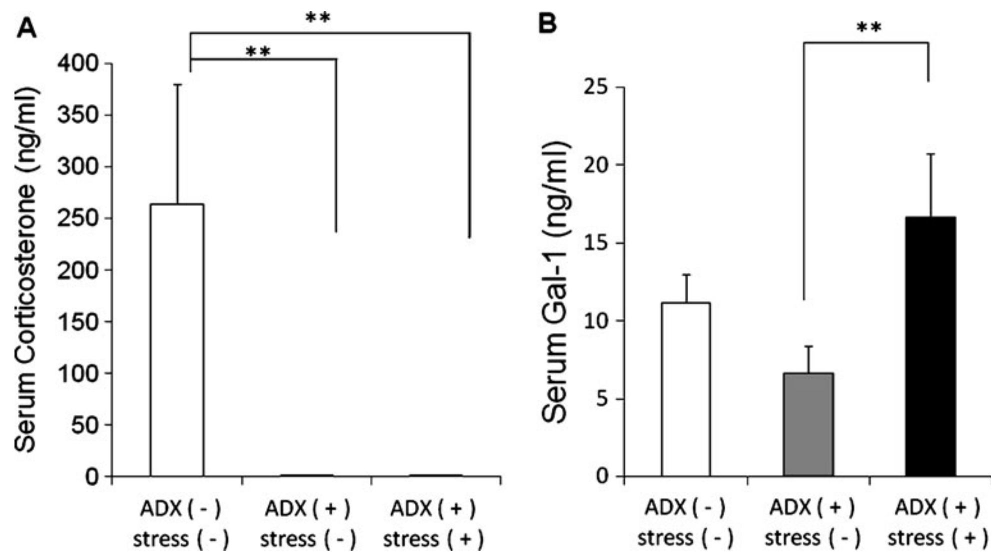


Fig. 6 The serum levels of corticosterone (**A**) and Gal-1 (**B**) were analyzed with ELISA of adrenalectomized (ADX) rats after short restraint stress (n=3–4). The values represent the means±SD in each group. **indicates a significant difference at p<0.01

Gal-1 promotes the secretion of IL-10 and an anti-inflammatory cytokine from T-cells [26]. In addition, oxidized Gal-1 regulates the macrophages to improve injured peripheral nerves [27]. Therefore, the oxidized form of Gal-1 may work on macrophages to reduce up-regulated cytokines similar to corticosterone in the early stage of stress.

The significant elevation of serum Gal-1 in response to restraint stress was quenched by the noradrenalinergic nerve terminal destruction induced by injecting 6-OHDA, but it was not influenced by a bilateral ADX. This suggests that the stress-induced serum Gal-1 is mediated by the activation of the sympathetic nervous system. Various reactions of the autonomic nervous system against stress have already been reported [2].

The corticosterone levels increased in the adolescent rats in contrast to the serum Gal-1 level in adolescent rats. Similar results were obtained under other types of restraint stress [28]. Hence, the HPA axis against restraint stress functions in adolescence rats, but the sympathetic nervous system does not yet function in the adolescent rats to induce Gal-1 increases as a response against stress. Recent studies support this hypothesis by showing that restraint stress markedly reduces the depolarized release of noradrenalin in 4-week-old rats, while increasing the release in 10-week-old rats [23].

Gal-1 protein is widely expressed in various normal and pathological tissues and organs such as the liver, brain and lung [29]. Among these organs, the liver is a candidate of the organ sources contributing the increase of serum Gal-1 levels caused by the restraint stress. Hepatic stellate cells contain Gal-1 and are situated in the perisinusoidal space. Furthermore, the stellate cells secreted Gal-1 in culture medium in a response against carbon tetrachloride stimulation [30]. Lymphatic organs, such as spleen and thymus, are also the candidates. Gal-1 is produced in the organs and is known to be involved in the immune and inflammatory responses, as mentioned above. However, further study is required to clarify the organ sources of serum Gal-1, underlying mechanisms to increase the serum Gal-1 levels, and target organs of the Gal-1.

Conclusion

The present set of *in vivo* experiments demonstrated, for the first time, that restraint stress markedly increases the serum Gal-1 level in adult rats probably through the sympathetic nervous system, but not through the HPA axis. The serum Gal-1 level therefore plays an important role in preventing physiological and/or psychological stress. The Gal-1 level in the peripheral blood can thus be used to clinically monitor stress as a novel marker protein for stress.

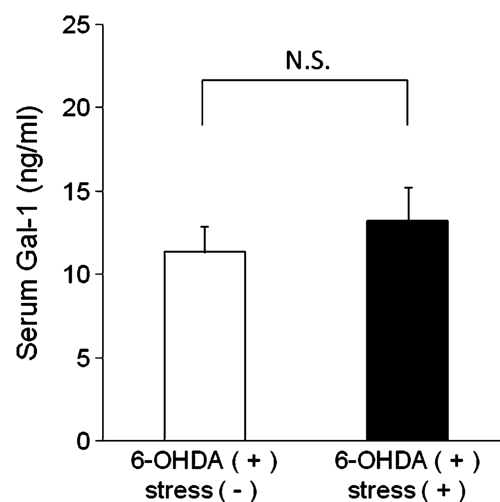


Fig. 7 The serum Gal-1 levels of rats treated with 6-OHDA after short restraint stress (black bar) and without the stress (open bar). Note no statistical significance was observed between them (N.S.)

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