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ORIGINAL PAPER

Estimation of restraint stress in rats using salivary amylase activity

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Abstract The rat is an ideal model animal for studying physical and psychological stresses. Recent human studies have shown that salivary amylase activity is a useful biomarker of stress in our social life. To estimate the usefulness of amylase activity as a biomarker of stress in rats, we analyzed changes in physiological parameters including amylase activity and anatomical variables, which were induced by a mild restraint of paws (10 min, 3 times/week, 9 weeks). The quantities of food and water intake and excretion amount of the stress rats were smaller than those of the control rats during the experimental period (5–13 weeks). The body weight of the stress rats decreased compared with that of the control rats. Moreover, the enlargement of the adrenal gland was confirmed in the stress rats, indicating that the mild restraint caused a chronic stress response. The amylase activities of the stress rats were significantly greater than those of the control rats at 5 weeks of age. However, the amylase activity of the stress rats decreased compared with that of the control rats after 6 weeks of age. These results indicate that amylase activity is increased by acute stress and reduced by chronic stress, which is caused by repeated restraint stress. In conclusion, amylase activity is a useful biomarker of acute and chronic stresses in rats.

Keywords Amylase · Biomarker · Irritable bowel syndrome · Rat · Restraint stress

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Introduction

In our social life, we receive many types of stress stimulus (stressor) and show various responses to stressors such as avoidance behavior and clinical conditions characterized by widespread pain. The mechanisms by which stressors adversely affect patients have generally been considered to be at the level of the central nervous system. Stressors are initially detected by the sensory system, and their information is transmitted to the brain. Then, the release of corticosteroid and noradrenaline, which are caused by the activity of hypophysis and sympathetic nerves, respectively, changes the physiological state, e.g., blood pressure and heart rate. Physical measurements such as electroencephalogram [1], blood pressure [2], and heart rate [3] measurements are conventionally used to evaluate the stress response.

Recently, in human and animal studies, salivary amylase has been used as a biomarker associated with certain physical and psychological stresses [4–8] and proposed as a marker for activity of the sympathetic nervous system [9, 10]. Salivary amylase was first found to be induced by surgical stress [11], and the close association between salivary amylase activity and blood noradrenaline level has been demonstrated in a physiological study [12]. The secretion of salivary amylase by the salivary glands is regulated by the sympathetic nervous–adrenomedullary system, which controls blood noradrenaline and adrenaline levels [9, 12–14]. Two regulatory pathways for increasing salivary amylase activity, i.e., hormonal regulation by noradrenaline and direct innervation, are known in this system [15]. Hormonal regulation is delayed by loading stress, whereas direct innervation directly stimulates the secretion of salivary amylase and elicits a quicker response than hormonal regulation [16, 17]. It has also been reported

that salivary amylase elicits a more sensitive response to psychological stress than cortisol produced in the hypothalamic–adrenocortical pathway [17]. Thus, salivary amylase activity may be utilized as an excellent index of psychological stress in animal experiments.

The rat is an ideal model animal for studying the many mechanisms underlying behavioral and physiological characteristics. When rats are used in learning tests, they receive experimental stress such as a punishment stimulus as an anxiety/dread experience. They also receive stress in elevated plus-maze and water maze tests, which does not give invasive stimuli. To evaluate stress level in rats during experiments, we determined the changes in blood pressure, heart rate, and body weight [e.g., 18, 19]. Anatomical variables of the adrenal glands and thymus have also been used as indices of stress level after experiments [e.g., 20]. However, it is difficult to measure the above-mentioned indices of stress level under noninvasive conditions. To the best of our knowledge, there is as yet insufficient study of useful and noninvasive biomarkers as indicators of stress during rat experiments [9], although secretion of salivary amylase by autonomic nervous stimulation in rats has been reported [21, 22].

Irritable bowel syndrome (IBS) is exacerbated by stressful life events [e.g., 23, 24]. The symptoms of IBS related to gastrointestinal dysfunction include abdominal cramping with pain, and concurrent abnormal bowel habits such as episodes of diarrhoea, constipation, or both. IBS is most likely a multifactorial biopsychosocial disorder in which physiological, psychological, behavioral, and environmental factors all contribute to the clinical expression of the disorder. In the present study, we determined whether salivary amylase activity, which is used as a stress biomarker in human studies, is a useful index of stress using the IBS model rats, when subject to restraint stress [25].

Materials and methods

Animals

Fischer 344 (F344) male rats ($n = 24$) were used in the experiments. Animals at 4 weeks of age were purchased from CLEA Japan, and a pair of rats was maintained in a standard rodent cage until 14 weeks of age. Twelve rats were given restraint stress as described below and called the stress rats. Twelve rats that were not given the stress were called the control rats. They were housed with ad libitum food and water in their cages at 25 °C in a light–dark controlled room (lights on 0700–1900 hours). All experiments were performed in accordance with the guidelines of the Physiological Society of Japan and approved by the Animal Care and Use Committee of Iwate University.

Restraint stress

Respective forepaws and hindpaws of the stress rats were lightly bound with rubber bands 3 times a week (at 5–13 weeks of age) to develop IBS. At 1700 hours on Monday, Wednesday, and Friday, the rats whose paws were bound were placed in an acrylic cage (30 cm × 55 cm × 10 cm high) with spread Kimtowel for 10 min (restraint period). Their saliva was collected immediately after release from the stress by cutting the rubber band. The control rats were placed in an acrylic cage without the restraint in the same time schedule as that for the stress rats.

Measurements of changes in physiological variables

To estimate the changes in physiological variables, the quantities of food and water intakes, and excretion amount of the rats in each cage were measured on Monday, Wednesday, and Friday. The values of 3 measurements were summed up to measure the weekly values. The stress rats excreted soft feces during the restraint period. The excretion amount included the amount of soft feces during this period. Body weight was measured every Friday.

Saliva was collected using KISO-wet tester (KISO Science, Yokohama, Japan), which was placed under the tongue, approximately 20 s after the restraint period on Monday, Wednesday, and Friday. Saliva weight was immediately measured using a precision balance (HR-202i; A&D, Tokyo, Japan) and diluted with saline 100-fold in the sampling tube. In the present measurement, 1 g of saliva was regarded as 1 ml of liquid [7]. The saliva was stored in a freezer at −20 °C until analysis. The average concentration of salivary amylase was calculated every week using 3 measurements per week.

The salivary amylase activity of the collected saliva samples was measured using a liquid enzymatic method reagent (Espa AMY liquid 2; Nipro, Japan) and a clinical automatic analyzer (Miracle Ace 919; Nipro). The enzymatic method reagent contained nitrophenylated oligosaccharides as substrates, and changes in the absorbance were analyzed by a clinical automatic analyzer at 37 °C [26]. Amylase activity was expressed in international units/ml of saliva (U/ml).

Anatomical analysis

When the rats reached at 14 weeks of age, they were euthanized with an overdose of pentobarbital sodium (1–2 ml/animal i.p.) (Kyoritsu Seiyaku, Tokyo, Japan).

Then, the adrenal glands, thymus and gastrointestinal tract were removed carefully. The weights of the adrenal glands and thymus were measured with a precision balance (HR-202i; A&D), and the intestine was meticulously observed as to its condition.

Statistical analyses

The differences in the time courses of the quantities of food and water intake, excretion amount, body weight, and amylase activity between the stress and control rats were statistically examined using two-way ANOVA. Individual comparisons of physiological parameters at each week between the stress and control rats were analyzed by Student's *t* test.

The fractions of the weights of the adrenal glands and thymus relative to the body weight were used for statistical analyses. To calculate the fractions, the weights of the adrenal glands and thymus of each rat were divided by the body weight at 13 weeks of age. The significance of the differences in the weights of the adrenal glands and thymus between the stress and control rats was also analyzed using Student's *t* test. Values are presented as means ± SD.

Results

Mild restraint caused chronic stress

The stress rats showed a significant decrease in the quantity of food intake after 6 weeks of age compared with the control rats ($P < 0.05$ at 6 weeks of age, $P < 0.01$ after 7 weeks of age) (Fig. 1a). The quantity of water intake of the stress rats was smaller than that of the control rats during the experimental period ($F_{1,8} = 8.31$, $P < 0.05$), although there was no significant difference in the quantity of water intake for each week between the stress and control rats (Fig. 1b). The stress rats showed decreased excretion amount after 6 weeks of age compared with the control rats ($P < 0.01$) (Fig. 1c). The body weight of the stress rats was less than that of the control rats ($P < 0.05$ at 6 weeks of age, $P < 0.01$ after 7 weeks of age) (Fig. 1d). These results suggest that the mild restraint of paws caused stress response in physiological processes.

The adrenal glands of the stress rats were larger than those of the control rats (Fig. 2a), and thus the weight of the adrenal glands of the stress rats was greater than that of the control rats (0.10 ± 0.02 g in the stress rats,

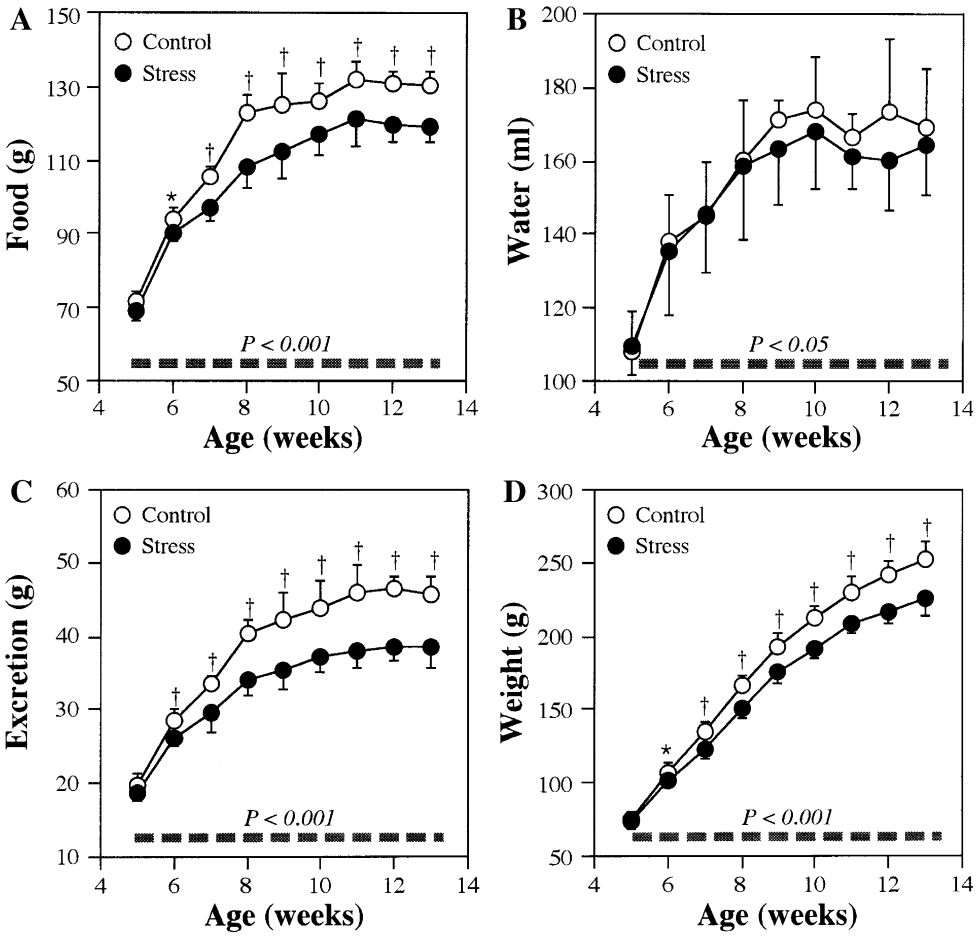

Fig. 1 Changes in quantities of food intake (**a**) and water intake (**b**), excretion amount (**c**), and body weight (**d**) during experimental period. The quantities of food intake, excretion amount, and body weight of the stress rats were less than those of the control rats during the experiment (*horizontal dot bars*, food $F_{1,8} = 49.68$, $P < 0.001$; excretion $F_{1,8} = 45.00$, $P < 0.001$; weight $F_{1,8} = 28.63$, $P < 0.001$). The quantity of water intake of the stress rats was also less than that of the control rats during the experiment (*horizontal dot bar*, $F_{1,8} = 8.31$, $P < 0.05$), although there was no significant difference for each week. *Open* and *closed circles* indicate the values of the control and stress rats, respectively. *Error bars* SD, ($n = 12$). $^{*}P < 0.05$ and $^{\dagger}P < 0.01$ compared with the stress rats for each week

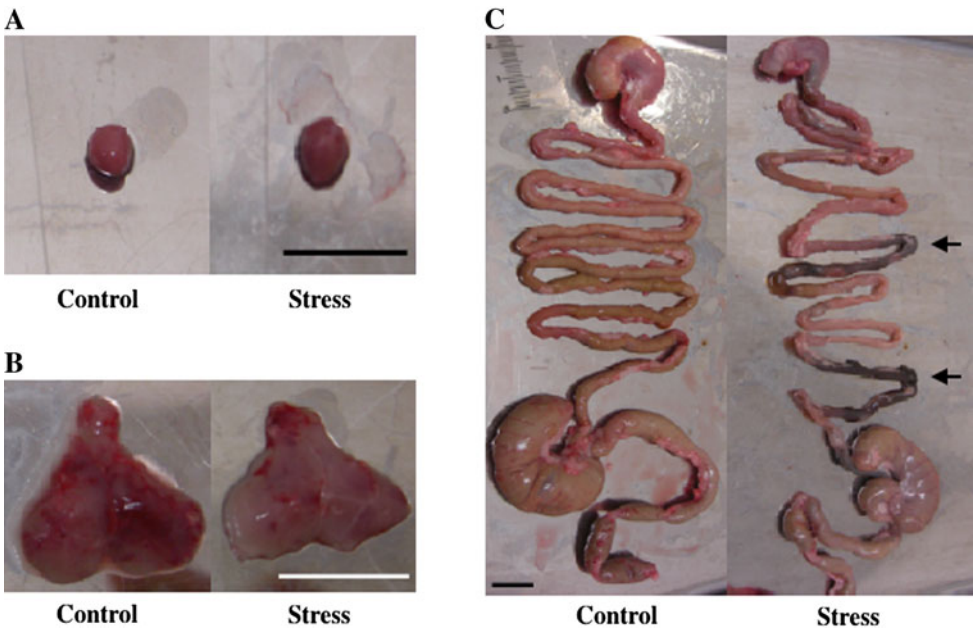

Fig. 2 Representative photographs of adrenal glands (**a**), thymus (**b**), and gastrointestinal tract (**c**) from control and stress rats (14 weeks of age). The adrenal glands were larger and the thymus was smaller in the stress rats than in the control rats. Intestinal bleeding was observed in the stress rats (*arrowhead*). *Scale bars* 1 cm

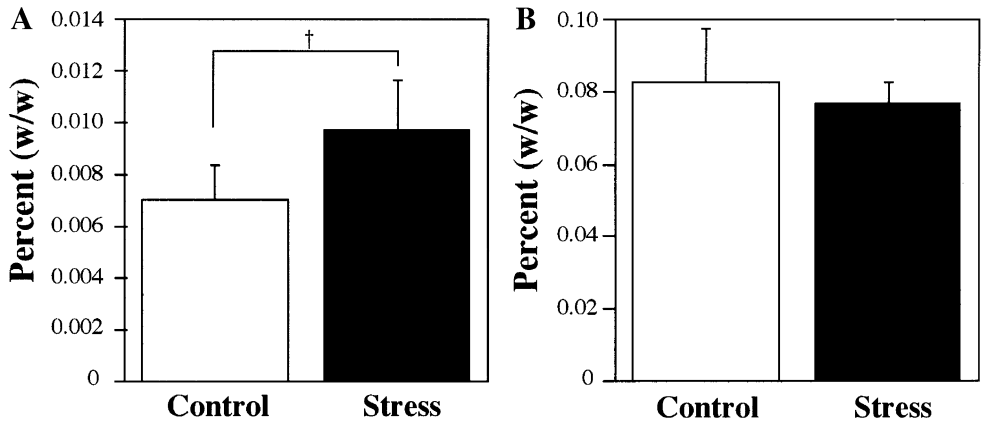

Fig. 3 Fractions of weights of the adrenal glands (**a**) and thymus (**b**) relative to body weight of control and stress rats (14 weeks of age). Data show percent values. The fraction of the weight of the adrenal glands of the stress rats was greater than that of the control rats ($^{\dagger}P < 0.01$). There was no significant difference in the fraction of the weight of the thymus between the stress and control rats. *White* and *black columns* indicate the percent values of the control and stress rats, respectively. *Error bars* SD ($n = 12$)

0.07 ± 0.01 g in the control rats; $P < 0.05$). The fraction of the weight of the adrenal glands relative to the body weight of the stress rats was also greater than that of the control rats ($P < 0.01$) (Fig. 3a). On the other hand, the thymus of the stress rats was smaller than that of the control rats (Fig. 2b). Although the weight of the thymus of the stress rats was less than that of the control rats (0.77 ± 0.15 g in the stress rats, 0.82 ± 0.15 g in the control rats; $P < 0.05$), there was no significant difference in the fraction of the weight of the thymus between the stress and control rats (Fig. 3b). Intestinal bleeding was observed in eight of the stress rats (i.e., two-thirds of all stress rats) (Fig. 2c).

Salivary amylase activity varied during stress period

At 5 weeks of age, the salivary amylase activity of the stress rats significantly increased compared with that of the control rats (Fig. 4). After 6 weeks of age, however, the amylase activity of the stress rats (6–13 weeks of age) was significantly lower than that of the control rats ($F_{1,7} = 23.22$, $P < 0.01$). At 9, 11, and 13 weeks of age, we observed a significant decrease in the amylase activity of the stress rats compared with that of the control rats. The observations indicate that the activity of salivary amylase is increased by acute stress and reduced by chronic stress in rats.

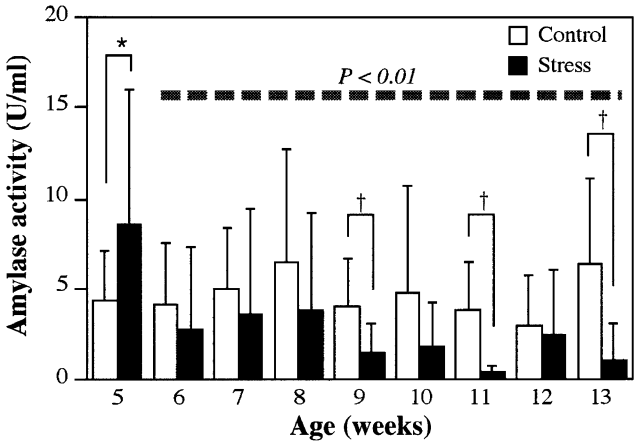

Fig. 4 Amylase activities of control (*white columns*) and stress (*black columns*) rats during experiment. The amylase activity of the stress rats at 5 weeks of age was significantly greater than that of the control rats ($^{*}P < 0.05$). The activity of the stress rats after 6 weeks of age decreased compared with that of the control rats (*horizontal dot bar*, $F_{1,7} = 23.22$, $P < 0.01$). A significant decrease in the amylase activity of the stress rats at 9, 11 and 13 week of age was observed compared with that of the control rats ($^{\dagger}P < 0.01$). *White* and *black columns* indicate the amylase activity of the control and stress rats, respectively. Amylase activity was expressed in international units/ml of saliva (U/ml). *Error bars* SD ($n = 12$)

Discussion

Chronic stress causes a decrease in body weight and changes in physiological variables because of the unbalanced homeostasis between the sympathetic nervous system and the parasympathetic nervous system. The unbalanced homeostasis during a stress period causes a decrease in the rate of secretion of mucus and induces intestinal bleeding [27]. When animals receive stimuli from stressors, the pituitary gland secretes the adrenocorticotrophic hormone (ACTH), which causes hyperplasia of the adrenal glands and atrophy of the thymus [27, 28]. In this study, the physiological variables, such as quantities of food and water intake, excretion amount, and body weight, of the stress rats were less than those of the control rats (Fig. 1). The weights of the adrenal glands and thymus also changed during the stress period (Figs. 2, 3) indicating that the mild restraint of paws caused a chronic change in hormonal secretion. We usually observed the excretion of soft feces during the stress period. It is known that the model rats with IBS demonstrated an increase in soft fecal excretion during restraint stimuli [25]. These observations suggest that the symptoms of IBS in stress rats are caused by applying light-binding stimuli to their paws and show a chronic stress response to the repeated applications of stimuli in our experimental protocol.

The psychological stress response such as IBS could produce physiological effects similar to those produced by physical changes in various physiological systems. The activation of the hypothalamus–pituitary–adrenal system, which is activated by stressors, results in the enhancement of blood cortisol secretion [28]. It has been revealed in human studies that salivary cortisol level correlates with serum cortisol level [29], indicating that salivary cortisol has an advantage for the analysis of stress response [7, 8, 16]. The sympatho-adrenomedullary system is also particularly involved in eliciting stress response. Salivary amylase, which is secreted by the salivary glands in response to sympathetic stimuli, is one of the major salivary enzymes associated with blood noradrenaline level, which is a useful index of sympatho-adrenomedullary system activity [12, 30]. The changes in salivary amylase activity can be quickly detected using a small amount of saliva compared with that in salivary cortisol activity [15], and salivary amylase level changes more significantly than salivary cortisol level [31]. Therefore, we conclude that salivary amylase is a useful biomarker for evaluating acute stress response in both humans [6, 7, 15, 32] and rats [9].

We revealed that the amylase activity of stress rats at 5 weeks of age significantly increased compared with that of control rats (Fig. 4). On the other hand, after 6 weeks of age, the activity of the stress rats declined compared with that of the control rats, whereas that of the control rats was almost the same as that during the experimental period (Fig. 4). The salivary amylase activity of healthy humans is constant at a younger age because the function of the salivary gland is relatively unchanged [33, 34]. These results indicate that salivary amylase activity is increased by acute stress and reduced by chronic stress in rats. It has been reported in rat experiments that acute stress, such as restraint and immobilization, elevated heart rate and arterial blood pressure [e.g., 35], and increased plasma noradrenalin level [e.g., 36] due to the activation of the sympathetic nervous system. Indeed, high sympathetic and low parasympathetic tone has been observed in IBS patients [37]. The sympathetic activity promoted the secretion of salivary amylase [9, 10]. A strong correlation between stress-induced adrenocorticotropic hormone release and stress-induced intestinal dysfunction was also revealed in rats under conditions of mild restraint stress [25]. These reports support the notion in the present study that salivary amylase activity is increased by acute stress. On the other hand, the parasympathetic nervous system plays a significant role in salivary amylase release [21, 22, 38], suggesting that a change in amylase activity was caused by a change in the sympathetic/parasympathetic balance. Wolf et al. [8] have reported that the high chronic stress is associated with a lower salivary amylase output in the study of human children with asthma, and suggested that salivary amylase is more sensitive to social characteristics of chronic diseases involving an altered autonomic nervous system activity. The repeated applications of

binding stimuli to the rat paws in our experiment caused digestive dysfunction and may have led a change in the balance of the sympathetic and parasympathetic nervous systems, leading to lower amylase activity.

In many studies using rats, the animals receive boundless acute and chronic stresses during experiments. For example, the step-through and water maze tests are usually used to determine the level of learning and memory. Both the punishment stimulus in the step-through test and the water stimulus in the water maze test are strong stressors for rats [39]. The results of these studies include not only the effects of learning and memory but also the effects of stress during examination. To obtain an accurate evaluation in rat studies, we may need to determine the stress level of rats during experiments. Salivary amylase activity should be a useful biomarker for the objective assessment of acute and chronic stresses in rat experiments.

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