Effects of hyperoxic inhalation on psychological stress-induced salivary biomarkers

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Abstract
This study examined the effects of hyperoxic inhalation on psychological stress-induced salivary biomarkers. To induce psychological stress, eight males (22–24 year old) were performed a simple mathematical calculation. After the task, the subjects inspired either normal air or 100% O$_2$ for 30 min. The control subjects (control trial) did not perform the calculation task and inspired normal air. These three trials were randomly performed at an interval of at least one week, and the two calculation trials with and without 100% O$_2$ inhalation were performed using a single-blinded design. A tendency for increase in salivary cortisol (s-cortisol) and chromogranin A (s-CgA) concentrations, and a significant increase in salivary α-amylase (s-amylase) activity were observed following the task. Hyperoxic inhalation did not affect s-cortisol and s-CgA secretion, but decreased the s-amylase activity. Changes in the increased rate of s-amylase activity and s-CgA concentration showed a significant negative correlation with each other, after the task. These results imply that hyperoxic inhalation attenuates a part of autonomic excitability resulting from psychological stress. Although both s-amylase and s-CgA are employed as biomarkers of autonomic excitability, the s-amylase and s-CgA do not appear to be regulated by the same autonomic nervous system.

Psychological stress activates the hypothalamic-pituitary-adrenal (HPA) axis and the sympathoadrenal medullary (SAM) system (6, 15, 25). Salivary cortisol (s-cortisol), a biomarker of the HPA axis, and salivary α-amylase (s-amylase) and chromogranin A (s-CgA), biomarkers of the SAM system, have been examined over the past decade in psychological studies (3, 10, 19). Cortisol is secreted from the adrenal gland into the serum and saliva under psychological and physical stress, and that the tendencies of secretion in both serum and saliva are nearly consistent with each other (4, 11). Alpha-amylase is secreted from the salivary gland, which is innervated by both sympathetic and parasympathetic nerves (5, 24). Both psychological and physical stress stimulates the salivary gland to increase the secretion of s-amylase (3, 5). CgA exists in various endocrine organs and its secretion is regulated by the sympathetic nervous system (7, 29). Furthermore, CgA in the submandibular gland responds to psychological stress but not to physical stress of moderate intensity (1, 16). Although both s-amylase and s-CgA are employed as biomarkers of autonomic excitability, they may not be regulated completely by the same autonomic nervous system.

Hyperoxic inhalation reduces heart rate and causes hyperoxic bradycardia, which suggests that hyperoxia modulates the autonomic nervous system (2, 12, 14). Some spectral analysis studies on heart rate variability under hyperoxic conditions showed that hyperoxia activates the parasympathetic nervous system (13, 14). A previous study conducted by us...
showed that pre-exposure to hyperbaric hyperoxia attenuates high-intensity exercise performance associated with the lower integrated electromyography with respect to time, implying that hyperoxic inhalation suppresses sympathetic excitability (9). These results suggest that hyperoxia modulates the SAM system. Therefore, it is hypothesized that hyperoxic inhalation regulates the SAM system, which leads to suppression of the sympathetic activity, including a decrease in s-amylase activity and s-CgA concentration but does not influence the HPA axis. This study investigates the effects of hyperoxic inhalation on the psychological stress-induced secretion of biomarkers (s-cortisol, s-amylase, and s-CgA).

MATERIALS AND METHODS

Subjects. Eight healthy, non-smoking males (mean age ± SD, 22.9 ± 1.0 years) participated in this study. All experimental procedures were conducted in accordance with the Declaration of Helsinki. The subjects were informed of the experimental risks and signed an informed consent document approved by the Human Subject Research Committee of the University of Tokyo and submitted the document prior to the investigation. This investigation was approved by the Human Subject Research Committee of the University of Tokyo.

Procedure. A simple mathematical calculation was employed to induce psychological stress, which is a common method used in clinical psychology and occupational mental health studies (8, 28). The subjects were instructed to perform a serial calculation of random numbers printed on paper for a period of 15 min, following which they were again instructed to complete an additional 15-min task after a 5-minute interval. After completion of the task, the subjects inspired either normal air (NA trial) or 100% O₂ (hyperoxic; HO trial) for 30 min through a Douglas bag with a face mask. The control subjects (control trial) did not perform the calculation task and inspired normal air for 65 min. These three trials were randomly performed at an interval of at least one week. Additionally, both the NA and HO trials used a single-blinded design. The individual trials were conducted at the same time of the day to reduce the effects of any diurnal variation on the bioassay results. Although oxidative damage to humans should also be considered, our previous study showed that the exposure to hyperbaric hyperoxia (100% O₂ at 1.3 atmospheres absolute for 50 min) does not cause any oxidative damage (9).

Salivary measurements. S-cortisol and s-CgA concentrations, and s-amylase activity were measured to evaluate the psychological stress before (baseline) and immediately after (post-point) the task, and after the inspiration of normal air or 100% O₂ for 30 min (post-30). Salivary samples of the control subjects were collected at the same points as that of the experimental subjects. All the salivary samples were collected using salivary collection tubes (Salivette; Assist Co., Ltd., Tokyo, Japan), wherein the subjects were required to rinse their mouth with distilled water to remove any potential contaminant that might affect the s-cortisol, s-CgA and s-amylase levels in the samples. All the salivary samples were centrifuged at 5000 × g for 5 min at 20°C and stored at −80°C until analysis. S-cortisol was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Salimetrics LLC, State College, PA, USA) according to the manufacturer’s protocol. S-amylase activity was measured using a kinetic measurement of s-α-amylase kit (Salimetrics LLC) according to the manufacturer’s protocol. S-CgA was measured using an ELISA kit (Yanaihara Institute Inc., Shizuoka, Japan) according to the manufacturer’s protocol, which was standardized by total protein concentration that was determined using a protein determination kit (Bio-Rad, Richmond, CA, USA). The values were normalized as percentages of the baseline. As s-amylase activity and s-CgA concentration were reported to be regulated by the SAM system, the correlation of their individual levels at post-point was analyzed. All analyses conducted in duplicates.

Statistical analyses. All values are expressed as mean ± SD. A two-way analysis of variance (ANOVA) with repeated measures for groups (control, NA and HO trials) and time (baseline, post-point and post-30) was used to test the interactions and main effects. When the interactions or main effects reached significant levels, Fischer’s PLSD post hoc test was used to identify the significant differences. An alpha of P ≤ 0.05 was considered statistically significant for all comparisons. Correlation analyses were used to compare the changes in s-CgA concentration and s-amylase activity.

RESULTS

The levels of s-cortisol in both NA and HO trials showed a tendency to increase at post-point (NA trial, 122.2 ± 70.7%; HO trial, 151.1 ± 75.7%; control, 91.5 ± 19.1%) compared to baseline, but the changes were not significant (Fig. 1a). The increase levels
tended to remain high with or without hyperoxic inhalation at post-30 (NA trial, 124.7 ± 64.2%; HO trial, 155.8 ± 126.0%; control, 84.5 ± 37.6%). The levels of s-CgA in both NA and HO trials also showed a tendency to increase at post-point (NA trial, 129.8 ± 14.3%; HO trial, 122.7 ± 35.4%; control, 94.5 ± 10.6%) compared to baseline, but the changes were not significant (Fig. 1b). Additionally, the s-CgA level in the NA trial was significantly (*P* ≤ 0.05) higher than that in the HO and control trials (Fig. 1c). A significant negative correlation (*P* ≤ 0.05) between the s-CgA activity and s-CgA concentration at post-point was observed (Fig. 2).

**DISCUSSION**

The HPA axis and the SAM system are activated when humans are exposed to psychological stressors (6, 15, 25). To reduce psychological stress, previous studies investigated the effects of various procedures such as spa bathing and coffee drinking (26, 27). Present study employed hyperoxic (100% O₂) inhalation as an attempt to reduce stress. Secretion of cortisol, which is a biomarker of the HPA axis, is a major component of the psychological stress response. It has been reported that academic examinations and trier social stress test (TSST) enhance the release of s-cortisol (6, 25). Present study has indicated that hyperoxia after a simple mathematical calculation task does not suppress the increased s-cortisol levels on completion of the task. In agreement with these results, it is hypothesized that hyperoxic inhalation does not influence s-cortisol concentration after the task because hyperoxia influences only the autonomic nervous system and not the HPA axis. On the other hand, present study also hypothesized that hyperoxic inhalation after the task

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**Fig. 1** Changes in salivary cortisol (a) and CgA (b) concentrations, and amylase (c) activity respond to a simple mathematical calculation task and hypoxia inhalation. Mean ± SD. N = 8 for each trial. *Significant difference (*P* ≤ 0.05) between the NA and the other two trials, and compared to baseline in the NA trial. †Significant difference (*P* ≤ 0.05) compared to baseline in the NA and HO trials.

**Fig. 2** Correlation between s-CgA concentration and s-amylase activity at post-point. Although the biomarkers increased at post-point, the individual increased rate showed a negative correlation (*P* ≤ 0.05) with each other.
suppresses the increased s-amyrase activity and s-CgA levels on completion of the task. Results of the present study have indicated that hyperoxic inhalation suppresses the increased s-amyrase activity after the task, but not the increased s-CgA concentration. Thus, it is in disagreement with the proposed hypothesis.

S-amyrase activity, which is a biomarker of the SAM system, is known to be enhanced under psychological and physical stressors, such as the TSST and bicycle exercise (5, 18). CgA is a major soluble protein in adrenal chromaffin cells and adrenergic neurons (7, 29), which is co-released with catecholamines into plasma and saliva when the SAM system is stimulated (22, 23, 29). Interestingly, it is observed that CgA responds to psychological stress but not to a moderately intense exercise-induced physical stress (1, 16, 17). These results imply that neither the s-amyrase nor the s-CgA is completely regulated by the same SAM system. In the present study, a simple mathematical calculation task causes a significant increase in the s-amyrase activity and a tendency for the CgA concentration to increase immediately after the task. This result is almost in agreement with previous studies that used various tests to induce psychological stress (16, 17, 21). However, a significant negative correlation ($P \leq 0.05$) was found between the increase rate of s-amyrase activity and s-CgA concentration after the task (Fig. 2). It has been reported that psychological stress induced by academic examination does not increase the s-CgA concentration (19). Noto et al. reported that a mental arithmetic task increases the s-amyrase activity but not the s-CgA concentration (20). Nakane et al. reported that a word processing task significantly increases the s-CgA concentration (17). Although the underlying mechanisms for this phenomenon remains unclear, differences in s-amyrase and s-CgA secretion in any individual may need to reach a threshold that is dependent on the quantity and quality of the psychological stressor. Additionally, these results suggest that evaluating either s-amyrase or s-CgA alone is insufficient to properly assess the activity of SAM system.

In conclusions, hyperoxic inhalation did not affect the s-cortisol levels, but decreased the s-amyrase activity. Although s-CgA is also known as a biomarker of the SAM system, hyperoxic inhalation did not affect its secretion. Both s-amyrase and s-CgA levels increased due to psychological stressors (calculation-task stressor), and the individual changes in the increase rate showed a negative correlation with each other. These results suggest that neither s-amyrase nor s-CgA is completely regulated by the same autonomic nervous system, and that both biomarkers should be measured simultaneously in salivary samples when psychological stressors are evaluated. Although hyperoxic inhalation decreased the s-amyrase activity, further investigations are required to investigate whether hyperoxia attenuates the psychological stress.

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