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Effects of chronic academic stress on mental state and expression of *glucocorticoid receptor α* and *β* isoforms in healthy Japanese medical students

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Abstract
Chronic academic stress responses were assessed by measuring mental state, salivary cortisol levels, and the *glucocorticoid receptor* (*GR*) gene expression in healthy Japanese medical students challenging the national medical license examination. Mental states of 17 male and 9 female medical undergraduates, aged 25.0 ± 1.2 years (mean ± SD), were assessed by the State and Trait Anxiety Inventory (STAI) and the Self-Rating Depression Scale (SDS) 2 months before, 2 days before, and 1 month after the examination. At the same time points, saliva and blood were collected. STAI-state scores peaked 2 days before the examination. Scores on STAI-trait and SDS, and salivary cortisol levels were consistently higher during the pre-examination period. One month after the examination, all these measures had significantly decreased to baseline levels. Real-time reverse transcription PCR showed that this chronic anxious state did not change the expression of the functional *GRα* mRNA isoform in peripheral leukocytes, while it resulted in reduced expression of the *GRβ* isoform 2 days before the examination. Our results replicate and extend a significant impact of chronic academic stressors on the mental state of healthy Japanese medical students and suggest a possible association of *GRβ* gene in response to psychological stress.

Keywords: *Academic stress, glucocorticoid receptor, medical student, salivary cortisol, Self-Rating Depression Scale (SDS), State and Trait Anxiety Inventory (STAI)*

Introduction

It is well known that both acute and chronic stress can affect a range of psychological and physiological outcomes. During both acute and sustained psychological stress exposure, the activation of the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system can play a major role in immune response (Vedhara et al. 2000), increased vulnerability to disease (Reiche et al. 2004, 2005), and a more rapid progression of existing health conditions (Leserman et al. 2000). In addition, overactivation of the HPA axis resulting in enhanced release of glucocorticoids is primarily responsible for the dysregulation of cellular and humoral immune responses after chronic psychological stress (Glaser and Kiecolt-Glaser 2005). These findings have been observed in several highly stressed groups, such as students undergoing academic assessments (Lacey et al. 2000; Ng et al. 2003; Loft et al. 2007) or spousal caregivers (Segerstrom and Miller 2004). However, it remains largely unknown as to how chronic stressors have an impact on the neuroendocrine responses.

Academic stress during an examination period in medical students has been used as a model for the study of neuroendocrine responses to stress (see Michaud et al. 2008). Using this model, a number of studies have indicated that academic stress activates the HPA axis and increases cortisol release (Malarkey et al. 1995; Lacey et al. 2000; Lucini et al. 2002; Ng et al. 2003; Weekes et al. 2006; Preuss et al. 2010). Some studies showed either no change in cortisol

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secretion or even decreased release of cortisol before examination (Vedhara et al. 2000; Loft et al. 2007). The inconsistent findings may be partially due to the differences in the individual variations in the cortisol reactivity (McEwen 2007).

One candidate factor related to cortisol reactivity is the *glucocorticoid receptor* (*GR*) gene, which plays an important role in the adaptation to stress and in the regulation of the negative feedback of the HPA axis (Holsboer 2001; Pariante et al. 2001). *GR*, a member of the nuclear receptor superfamily, binds glucocorticoids in the cytoplasm and then translocates into the nucleus to act as a transcription factor, resulting in the inhibition of synthesis and secretion of both corticotropin-releasing hormone and adrenocorticotropic hormone. In response to stress, *GR* mediates various glucocorticoid actions on the brain, including shrinkage of neural dendrites, suppressed neurogenesis, and reduced serotonin metabolism (Lopez et al. 1998; Sapolsky et al. 2000; Lupien et al. 2009).

Differences in *GR* responsiveness can be partially attributed to the existence of two *GR* mRNA isoforms (*GRα* and *GRβ*), resulting from alternative splicing of *GR* pre-mRNA (Lu and Cidlowski 2005). *GRα* mediates glucocorticoid effects, but *GRβ* does not bind glucocorticoids and exerts a dominant-negative effect on *GRα*-mediated transcription (Oakley et al. 1999; Vottero and Chrousos 1999). The expression of *GRα* mRNA was significantly reduced in patients with bipolar disorder or major depression, but *GRβ* expression was not altered (Matsubara et al. 2006). A significant association between life stress and diminished expression of *GRα* in leukocytes was also documented in children with asthma (Miller and Chen 2006). However, the biological function of *GRβ* remains largely unknown and controversial (Carlstedt-Duke 1999; Vottero and Chrousos 1999). Furthermore, it is not fully understood how these *GR* isoforms have an impact on the stress response.

To address this issue, we examined psychological measures, salivary cortisol levels, and *GRα* and *GRβ* mRNA expressions in peripheral blood leukocytes of 26 medical students exposed to the academic stress of the Japanese national medical license examination.

Methods

Subjects and study design

A total of 26 Japanese subjects were recruited from a group of sixth-year undergraduate medical students of the University of Tokushima. The study was approved by the Human Study Committee of the University of Tokushima, Japan. Written informed consent was obtained from all participants.

The students were aged between 23 and 29 years with a mean age of 25.0 years (SD = 1.2) and consisted of 17 males and 9 females. Subjects were all non-smokers and did not take any medication for 3 months prior to and during the experimental period. We also confirmed by an interview that all subjects had no serious somatic or mental disorder in the past or present.

All medical students are required to pass the national examination for the Japanese medical license to become clinical physicians. The national examination consists of a 3-day paper test and is the most stressful event for medical students. Samplings were done at three time points: 2 months and 2 days before the examination (the pre-examination period), and 1 month after the examination (the post-examination period). All subjects were different from those used in our previous study (Kawai et al. 2007). One male student was excluded from the psychological analysis due to missing values in State and Trait Anxiety Inventory (STAI) state, STAI trait, and Self-Rating Depression Scale (SDS) scores taken 2 months before the examination.

Salivary cortisol

Saliva was collected from participants using Salivette sampling devices (Sarstadt, Inc., Rommelsdorf, Germany; Tsubouchi et al. 2006). The Salivette devices stimulate the flow of saliva, enough of which are collected within 1 min. Saliva was collected on the same sampling days between 16:00 and 17:00 to avoid diurnal fluctuations. After centrifugation, saliva was stored at −80°C until analysis. Salivary cortisol was assayed with a commercial enzyme immunoassay kit (Ciron, Tokyo, Japan).

Psychological measures

We evaluated psychological status with two questionnaires: the STAI (Spielberger et al. 1983) and the SDS invented by Zung (Zung 1965). Two parts of STAI serve to assess the general tendency to be anxious as a personality trait (STAI trait) and the degree of anxiety at a particular moment as a situation-dependent state (STAI state; Eppley et al. 1989; Eckhardt-Henn et al. 2003). Both scales of STAI trait and STAI state consist of 20 self-report items with each item scoring from 1 to 4 (for a full score of 80) with a lower score reflecting a better psychological status. Depressive status was measured by SDS consisting of 20 items on a four-point scale with a lower score representing a more favorable mood. The maximum score of the SDS is 80. The reliability of the Japanese versions of SDS and STAI has been previously established (Fukuda and Kobayashi 1973; Nakazato and Mizuguchi 1982).

Peripheral blood lymphocytes, RNA, and cDNA preparation

Blood samples were obtained from the subjects on the same sampling days after the collection of saliva. Venous blood was immediately poured into PAXgene Blood RNA tubes (Becton Dickinson, Franklin Lakes,

NJ, USA). After the contents were sufficiently mixed, tubes were placed for 2 h at room temperature and then stored at −80°C. Although the stability of RNA in PAXgene tubes is guaranteed up to 2.5 years (Kruhoffer et al. 2007), total RNA was extracted within 1 month using a PAXgene Blood RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The total RNA yield was measured by NanoDrop-1000 (Nanodrop, Wilmington, DE, USA). After treatment of samples with a DNase kit (Qiagen), 1 μg of total RNA was used for cDNA synthesis using oligo-dT primers and a PrimeScript® First Strand cDNA Synthesis kit (Takara, Shiga, Japan). The cDNA was stored at −80°C until analysis.

Real-time quantitative PCR

Real-time quantitative PCR was performed with an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. PCR conditions were 95°C for 15 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Amplification of the single PCR product was confirmed by monitoring the dissociation curve. Amplification curve was automatically applied to be a suitable baseline and threshold cycle. To generate standard curves, different concentrations of cDNA were generated from premium total RNA (Clontech, Palo Alto, CA, USA) isolated from human peripheral leukocytes.

The relative quantification method was employed for quantification of *GR* mRNA expression (NM_001018077). *Glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) mRNA expression was used as an endogenous control within the same sample, and quantity values were normalized to *GAPDH* mRNA expressions (NM_002046). All measurements were independently performed in triplicate. Expression of two mRNA isoforms of *GR*, *GRα* and *GRβ*, was analyzed. Primer sequences were as follows: 5′-GAA CTG GCA GCG GTT TTA TC-3′ and 5′-TCT CGG GGA ATT CAA TAC TCA-3′ for *GRα*, 5′-CCA TTG TCA AGA GGG AAG GA-3′ and 5′-TGT GTG AGA TGT GCT TTC TGG-3′ for *GRβ*, and 5′-AGC CAC ATC GCT CAG ACA C-3′ and 5′-GCC CAA TAC GAC CAA ATC C-3′ for *GAPDH* (see Figure S1). Primer sequences for *GRα* and *GRβ* were the same as described previously (Matsubara et al. 2006).

Data analysis

Results were analyzed using SPSS version 15.0J. Comparisons of psychological measures and salivary cortisol levels were analyzed with repeated measures one-way analysis of variance (ANOVA) and *post hoc* testing (Bonferroni test). A possible effect of gender was analyzed with repeated measures two-way ANOVA. Data for *GRα* and *GRβ* mRNA are presented as the value of relative quantification. The comparisons were analyzed with repeated measures ANOVA and *post hoc* testing (Bonferroni test). Pearson's correlations were calculated to assess correlations between *GRα* and *GRβ* mRNA expressions and salivary cortisol levels. For all statistical analyses, $P < 0.05$ was considered to be significant.

Results

Changes in psychological measures and salivary cortisol levels

In the Japanese version of STAI, the threshold value for anxiety was determined to be a score of >40 (Nakazato and Mizuguchi 1982). Two months before the license examination, STAI-state scores (43.8 ± 10.0; mean ± SD) were already higher than the threshold, further increased to 53.8 ± 9.7 2 days before, and returned back to a normal range within 1 month after the examination (32.6 ± 8.0; Figure 1). A repeated one-way ANOVA found a significant effect of time point ($F_{2,48} = 56.89$, $P < 0.001$), and Bonferroni *post hoc* test showed significant differences between all time points evaluated ($P < 0.01$).

The STAI-trait score, by definition, is a trait-like measure. Two months and 2 days before the license examination, STAI-trait scores (42.9 ± 10.1 and 43.0 ± 10.4, respectively) were higher than the threshold, and this measure decreased 1 month after the examination (37.1 ± 9.0). We found a significant effect of time on this measure with repeated one-way ANOVA ($F_{2,48} = 6.49$, $P = 0.003$; Figure 1). According to Bonferroni test, STAI-trait scores were significantly elevated both 2 months ($P < 0.01$) and 2 days ($P < 0.05$) before the examination, compared with scores recorded 1 month after the examination.

In the Japanese version of SDS, scores above 40 are considered to indicate a state of depression (Fukuda and Kobayashi 1973). Although all measurements were within the normal range (37.8 ± 7.6, 39.4 ± 7.7, and 34.2 ± 6.4, respectively), a significant effect of time was found with one-way ANOVA ($F_{2,48} = 12.97$, $P < 0.001$; Figure 1). Bonferroni *post hoc* test revealed significant elevation of SDS scores measured 2 months ($P < 0.05$) and 2 days ($P < 0.01$) before the examination, compared with those measured 1 month after the examination (Figure 1). Thus, consistently high STAI and SDS scores were observed over the pre-examination period with a peak score observed 2 days before the examination.

During the pre-examination period, salivary cortisol concentrations were 0.35 ± 0.11 and 0.32 ± 0.12 μg/dl (mean ± SD), 2 months and 2 days before the examination, respectively (Figure 1). After the examination, the levels of salivary cortisol were reduced to 0.25 ± 0.06 μg/dl. Repeated one-way ANOVA revealed significant differences in salivary cortisol among the time points ($F_{2,50} = 8.54$, $P < 0.001$).

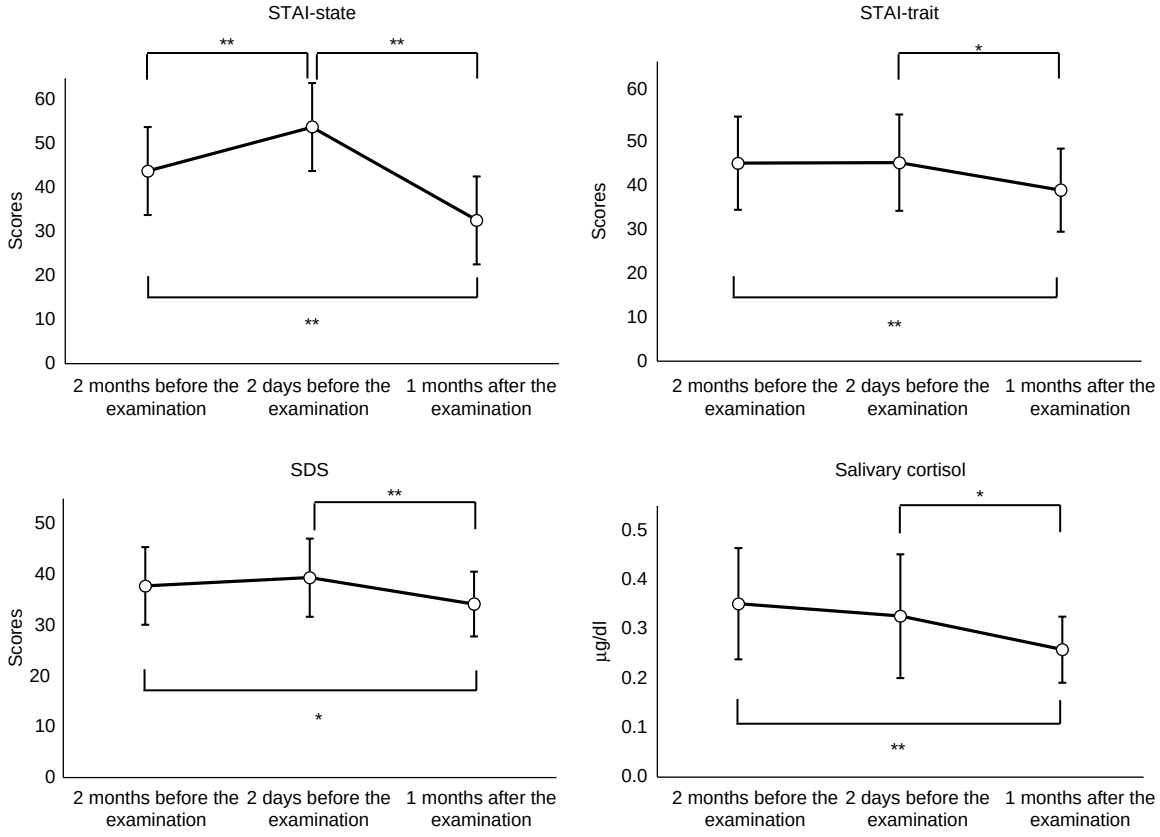

Figure 1. Changes in psychological measures and salivary cortisol levels in 26 medical students. STAI state, STAI trait, SDS, and salivary cortisol levels were assessed 2 months and 2 days before the examination (the pre-examination period), and 1 month after the examination (the post-examination period). Values are presented as mean ± SD. $\star P < 0.05$ and $\star\star P < 0.01$ by repeated measures one-way ANOVA and *post hoc* test.

According to the Bonferroni test, salivary cortisol levels were significantly high both 2 months ($P < 0.01$) and 2 days ($P < 0.05$) before the examination, compared with those 1 month after the examination.

These data indicate that a stress response was likely to have initiated several months prior to the examination, while measures recorded 1 month after the examination may better represent true baseline values. There was no significant correlation between psychological measures and salivary cortisol levels at each time point (data not shown).

Effect of gender

As the number of subjects was relatively small (17 males and 9 females), subjects were not separated for the analysis of each gender. To evaluate a possible effect of gender (male and female), repeated two-way ANOVA was performed with gender as the between-subject factor and time (three time points) as the within-subject factor (Table I). ANOVA revealed no significant time-by-gender interaction on STAI and SDS scores, or salivary cortisol levels. Thus, although academic-related stress caused significantly higher psychological test scores and elevated salivary cortisol concentrations during the pre-examination period, there was no significant effect of gender in our subjects.

Expressions of two GR gene isoforms, GRα and GRβ mRNA

We checked the slope in a standard curve for each PCR with the same RNA from the control leukocytes as a standard and examined whether each PCR had similar reaction efficiency (Figure S2). Regarding mRNA expressions of *GAPDH* and *GRα*, real-time quantitative PCR showed high correlations between RNA quantity and cycle threshold ($r^2 = 0.998$ and 0.993). In the case

Table I. Summary of repeated measures two-way ANOVA results.

Variable	*F* value	*P* value
STAI-state		
Time	54.526	<0.001
Time-by-gender	1.611	0.211
STAI-trait		
Time	5.790	0.006
Time-by-gender	0.599	0.550
SDS		
Time	14.247	<0.001
Time-by-gender	1.644	0.204
Salivary cortisol		
Time	10.937	<0.001
Time-by-gender	2.378	0.104

Note: The factors of time (three time points) and time-by-gender (male and female) were analyzed.

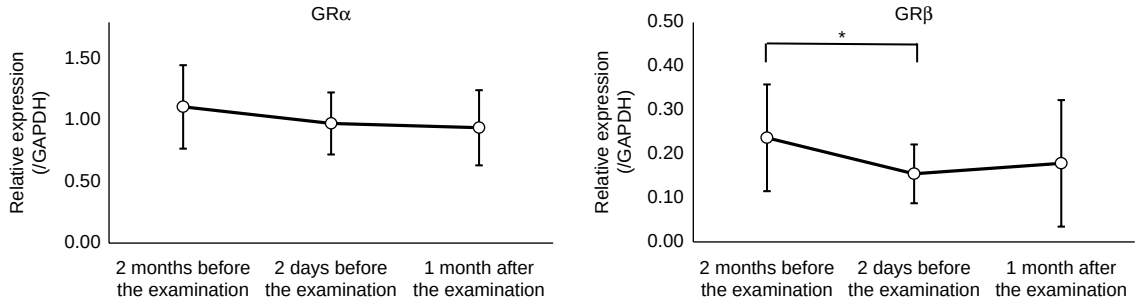

Figure 2. Changes in *GRα* and *GRβ* mRNA expression in 26 medical students. RNA was prepared 2 months and 2 days before the examination (the pre-examination period), and 1 month after the examination (the post-examination period). The amounts of *GRα* and *GRβ* mRNAs were normalized to *GAPDH* mRNA. Values are presented as mean ± SD. $*P < 0.05$ by repeated measures one-way ANOVA and *post hoc* test.

of *GRβ* mRNA measurement, the correlation value ($r^2 = 0.921$) was slightly reduced. Moreover, there was an increase in cycle threshold (ranging from 29.7 to 33.5), suggesting a low expression of *GRβ* mRNA in the peripheral blood leukocytes.

Regarding *GRα* mRNA expressions, no significant difference was found during the pre- and post-examination period ($F_{2,50} = 2.48$, $P = 0.094$; Figure 2). In contrast, there was a significant difference in *GRβ* mRNA expression ($F_{2,50} = 3.69$, $P < 0.05$). Specifically, a significant reduction of *GRβ* expression was observed 2 days before the examination with Bonferroni test ($P < 0.05$), but a significant recovery of *GRβ* expression was not observed after the examination.

Correlation between expressions of GRα and GRβ mRNA

To examine whether the reduced expression of *GRβ* mRNA was associated with differential *GRα* mRNA expression, we analyzed the correlation between *GRα* and *GRβ* mRNA expressions (Figure 3). A positive correlation was found 2 days before the examination ($r^2 = 0.226$ and $P < 0.05$), suggesting that an aberrant alternative splicing of the *GR* gene did not occur in our subjects at this time point.

Correlation between two GR gene isoforms and salivary cortisol

To test whether the expression of *GRα* and *GRβ* mRNAs was linked with physiological status, we analyzed the correlation between *GRα* and *GRβ* mRNA expressions and salivary cortisol concentrations (Figure 4). Regarding *GRα* mRNA, there were no significant correlations during the analyzed period. In contrast, a positive correlation was observed 2 months before the examination between *GRβ* mRNA and salivary cortisol levels ($r^2 = 0.260$ and $P < 0.05$). However, there was no significant correlation at any other time point.

Discussion

In this study, the national medical license examination was used to investigate psychological and physiological responses in 26 Japanese medical students. The study design was effective in measuring increases in the levels of both psychological variables and salivary cortisol, while there was no gender difference. Additionally, we assessed the expressions of two *GR* gene isoforms to evaluate a possible effect on cortisol reactivity and found a decreased expression of *GRβ* mRNA 2 days before the examination, the most stressful time point evaluated.

Stress resulting from medical training and academic examination has been of great interest in recent studies (Stewart et al. 1995, 1997; Dahlin et al. 2005). Stress during education could lead to mental distress and have a negative impact on learning (Dahlin et al. 2005). In particular, the national medical license examination is probably the most stressful event for all medical students. If the students fail to pass the examination, they lose their positions in hospitals and

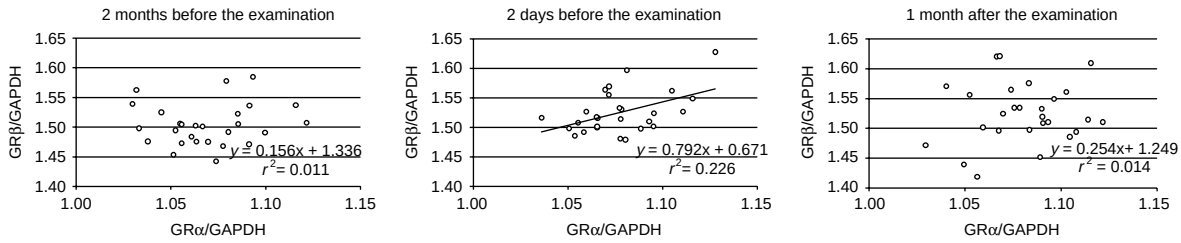

Figure 3. Correlation between *GRα* and *GRβ* mRNA expression. The amounts of *GRα* and *GRβ* mRNAs were normalized to *GAPDH* mRNA. A significant correlation was found 2 days before the examination ($r^2 = 0.226$ and $P < 0.05$), but not 2 months before the examination ($r^2 = 0.011$) or 2 months after the examination ($r^2 = 0.014$).

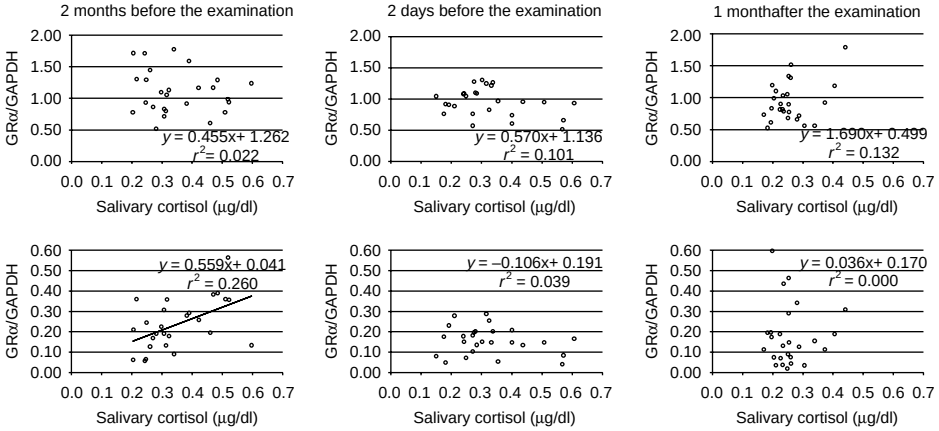

Figure 4. Correlation between *GR* gene isoforms and salivary cortisol levels. The amounts of *GRα* and *GRβ* mRNAs were normalized to *GAPDH* mRNA. Regarding *GRα* expression, there were no significant correlations during the analyzed period. Two months before the examination, a significant correlation was found between salivary cortisol levels and *GRβ* expression ($r^2 = 0.260$ and $P < 0.05$). No significant correlations were observed at the other time points.

have to retake the examination in the next year. The preparation period for the national examination has a significant impact on the psychological state of medical students. Consistent with our previous finding (Kawai et al. 2007), in the final stage of medical education (sixth grade), Japanese medical students were under pressure during the pre-examination period but were more relaxed after the examination. The design of this study seems to represent a suitable model for the analysis of chronic psychological stress.

There are several suggestive biomarkers for the measurement of psychological stress levels. In particular, salivary cortisol is frequently used as a biomarker of psychological stress and related mental or physical diseases (Hellhammer et al. 2009). Most studies consider salivary cortisol to be a reliable measure for monitoring the HPA axis response to adapt to stress. Our findings replicate and extend previous observations of elevated salivary cortisol in response to academic examination stress (Malarkey et al. 1995; Lacey et al. 2000; Lucini et al. 2002; Ng et al. 2003; Weekes et al. 2006; Preuss et al. 2010). In contrast, there are some studies reporting no significant influence of examinations on cortisol release (Vedhara et al. 2000; Loft et al. 2007). Although salivary cortisol is a potentially valuable tool to measure the psychological status of students, the stress response to salivary cortisol is rather complex and modulated by numerous factors (Hellhammer et al. 2009).

It is well documented that there is considerable variability in the sensitivity to glucocorticoids within the general population (Charmandari et al. 2004). Of note, one important determinant of glucocorticoid actions is the characteristics of the *GR* gene. The *GR* gene produces two alternative splice isoforms, *GRα* and *GRβ*, which differentially modulate glucocorticoid effects on different target tissues. *GRα* mediates glucocorticoid effects, but *GRβ* does not bind glucocorticoids and exerts a dominant-negative effect on *GRα*-mediated transcription (Oakley et al. 1999; Vottero and Chrousos 1999). Although *GRβ* mRNA is expressed at lower levels in lymphocytes (DeRijk et al. 2003; Pedersen and Vedeckis 2003; Matsubara et al. 2006), a possible function of *GRβ* has been proposed as an antagonist for *GRα*-mediated glucocorticoid signal. However, the biological function of *GRβ* remains largely unknown and controversial (Carlstedt-Duke 1999; Vottero and Chrousos 1999).

Preparation for the national medical license examination caused prolonged anxiety and depressive mood in the medical students, but functional *GRα* expression was not changed even when measured at the most stressful time (2 days before the national examination). This finding is inconsistent with a previous observation that bipolar disorder or major depression patients showed an aberrant alternative splicing of the *GR* gene (i.e. a selective downregulation of *GRα* isoform; Matsubara et al. 2006). In our healthy students, *GRβ* mRNA expression was significantly reduced 2 days before the examination, which seems to represent a beneficial effect in terms of potentially increasing the *GRα*/*GRβ* expression ratio. The expected effect of the increased ratio would be the production of a sufficient glucocorticoid signal which would help the students adapt to the coming stressful event. In addition, *GRβ* mRNA expressions were positively correlated with salivary cortisol levels 2 months before the examination despite no significant correlation among the *GRα* mRNA expressions. *GR*-mediated glucocorticoid signals are modified by many complex factors including *GR* phosphorylation, nuclear localization, and interaction with other molecules (Lu and Cidlowski 2005). In addition, the present findings implicate the involvement of *GRβ* isoform in the regulation of stress responses in healthy subjects. However, our findings do not imply a causal relationship. Future studies are required to further test this hypothesis.

There are several limitations of this study. First, the study subjects were restricted to Japanese medical students of the sixth year and not representative of the entire student group. Second, the total number of subjects was relatively small ($n = 26$), although our study was designed as a longitudinal one in which subjects served as their own control, thus reducing variability. This potentially resulted in non-significant findings due to a lack of statistical power. With respect to gender differences in cortisol levels, previous studies have revealed mixed results (Kajantie and Phillips 2006). A stronger cortisol response was observed in male students (Weekes et al. 2006), but another report did not find any gender differences (Schoofs et al. 2008). In conjunction with a potential effect of gender, future studies are needed that employ large samples with valid physiological markers and well-designed stress models (Stowell 2003).

In conclusion, 26 healthy Japanese medical students of sixth grade were exposed to the mental stress of an academic examination and displayed increase in salivary cortisol levels, although no gender effects were detected. We also measured the mRNA expressions of two *GR* gene isoforms during the stressful situations, and *GRβ* expression was significantly reduced at the most stressful time point. These data suggest that *GRβ* isoform may be involved in the regulation of psychological stress response in healthy subjects.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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