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Relationship between Salivary Chromogranin-A and Stress Induced by Simulated Monotonous Driving

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Abstract: The purpose of the present study was to evaluate the use of salivary Chromogranin-A (CgA), which is already used in general as a mental stress marker, for studying the stressful situation created by simulated monotonous driving. After informed consent 25 healthy male & female subjects were studied, under constant environment-controlled conditions. We measured the following physiological variables: blood pressure (BP), cardiac output (CO), total peripheral resistance (TPR), normalized pulse volume (NPV) as an index of alpha-adrenergic sympathetic activity to the finger arteriolar vessels, levels of cortisol and CgA during monotonous driving. The induced stress led to the expected decreases in NPV and increases in TPR and BP caused by peripherally-related sympathetic acceleration. However, CgA levels were found to fall gradually in accordance with the gradual increase of subjective rating of stress (SRS) and significantly ($p<0.01$) decreased over the period of the simulated monotonous driving.

Our hypothesis for the gradual decrease of CgA levels during the simulated monotonous driving is as follows. CgA, catestatin and Catecholamines are co-released into the extra-cellular environment. Peripheral sympathetic activity was accelerated by stress resulting from the simulated monotonous driving. Upon peripheral vessel constriction, an increase in total peripheral resistance (TPR) then increased BP which, in turn, activated catestatin. Consequently, secretion of CgA was blocked by the co-secreted catestatin from chromaffin granules. The results obtained strongly indicate that, although CgA has been reported as a possible marker of stress, CgA levels are not increased in the stressful situation of simulated monotonous driving.

Keywords: *Cardiovascular monitoring; Monotonous driving; Salivary Chromogranin-A; Stress*

CgA : Chromogranin-A

BP : blood pressure

CO : cardiac output

TPR : total peripheral resistance

NPV : normalized pulse volume

SRS : subjective rating of stress

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1. INTRODUCTION

There are many stressors in our modern day society which can threaten our health and life. The automobile is regarded in most societies as an essential commodity, however there have been considerable increases in traffic accidents every year in most industrialized nations. According to the WHO, traffic accidents cause two deaths every minute and 1.5 injuries every second [1]. Drivers may frequently be faced with too many driving stresses, such as right or left turns at busy intersections, traveling at too high a speed, or combinations of tasks requiring decisions and actions. Surprisingly, stress can also occur when drivers are trying to shake off their drowsiness in situations when they are under considerably less pressure to make decisions despite needing to perform ongoing monotonous driving tasks [2]. Decreased vigilance and fatigue are major factors accounting for driver error when under this form of increased stress [3, 4], and these are among the most common causes of traffic accidents. In order to understand these complex issues, and thereby hopefully reduce the number of traffic accidents, it is important to perform objective measurements of physiological phenomena thought to be associated with driver stress. Such measurements should include hormones and physiological signals in subjects whilst they are performing driving tasks.

Possible biochemical implications of stress have been evaluated by measuring cortisol and catecholamine as a neuroendocrine stress markers [5, 6]. However, the measurement of these hormones for this purpose has several limitations, such as the need for the invasive sampling of blood, although cortisol can be measured in saliva, and the fact that cortisol takes 20-30 mins to be secreted after stress [7] and is also influenced by circadian rhythm [8, 9]. The levels reached a peak value at 1 hour after awakening, then decreased gradually during the day. Nevertheless, since hormone levels in saliva are known to reflect well the levels in plasma, measurement of salivary stress hormones offers the prospect of a very convenient and useful approach for research into stress. Examples of possible salivary secreted hormones are immunoglobulin-A (sIgA) [10, 11], salivary amylase [12], and salivary Chromogranin-A (CgA) [13]. Salivary CgA was recently reported to be a useful biochemical marker of stress. The major advantage of the use of salivary CgA is, therefore, that its sampling is non-invasive and it can be performed in non-stressful conditions. Furthermore it is secreted immediately after stress [14] without the influence of circadian rhythm [9].

CgA, a 48-kDa acidic polypeptide, is the major soluble protein in the core of catecholamine (CA) storage vesicles. This protein is believed to play an intracellular role as a key regulator of secretory granule biogenesis and an extracellular function as a precursor of several regulatory peptides for the endocrine and nuroendocrine cells. CgA is also a pro-hormone, subject to proteolytic cleavage during and after secretion, yielding several biologically active peptides such as pancreastatin, vasostatin, prochromacin, chromacin, and catestatin. Pancreastatin inhibits glucose stimulated insulin release from pancreatic islet β-cells and parathyroid hormone release from chief cells, and vasostatin relaxes vascular smooth muscle and inhibits parathyroid hormone release. The novel fragment of CgA termed catestatin is a potent inhibitor of CA release, acting as a non-competitive antagonist on the nicotinic cholinergic receptor. CgA, catestatin and CA are co-released into the extra-cellular environment [15]. CA mediates sympathoadrenal activity on cardiovascular target cells to increase blood pressure. The release of CA is then subsequently blocked by the co-secreted

catestatin from chromaffin granules [16-19].

The purpose of the present study was to evaluate the use of salivary CgA, which is already used in general as a mental stress marker, in response to the stressful situation created by simulated monotonous driving. Under laboratory conditions, in healthy subjects during simulated monotonous driving, we have measured physiological variables together with salivary CgA levels.

2. MATERIALS AND METHOD

A. Experimental Setup

Fig. 1 shows a schematic diagram of our experimental system. This can measure physiological signals such as blood pressure (BP), cardiac output (CO), total peripheral resistance (TPR), normalized pulse volume (NPV) and levels of CgA during simulated monotonous driving. Saliva samples were collected every 10 minutes (Fig.1-(a)) and levels of CgA and cortisol were immediately measured, using enzyme linked immunosorbent assay (ELISA), by Yanaihara Institute Inc. The BP & CO monitoring systems were developed as experimental instruments and have been described fully elsewhere [20-22]. The BP system, utilizing the volume-compensation principle, is capable of measuring instantaneous BP in the finger (Fig.1-(b)), and the admittance cardiograph provides an instantaneous indication of CO (Fig.1-(c)). The finger photo-plethysmograph (FPG) (MPN1001, Medisens Co. Ltd., Japan) consists of a near-infrared light source and a photosensor which were placed on opposite sides of the distal part of the basal phalanx of the left middle finger (Fig.1-(d)). Normalized pulse volume (NPV) was obtained from the DC and AC (pulsatile) components of the FPG signal. This measure has been proposed as a useful index of α-adrenergic sympathetic activity in the finger arteriolar vessels [23].

The main part of the experimental system comprises of a video projector (LV-5210, Canon Co., Ltd., Japan) and an 80-inch screen for displaying an image to the subject, a driver's seat and car steering wheel, two CCD cameras to monitor the subject, and two conventional PCs. To conduct the experiment the subject was asked to sit down quietly on the driver's seat with their left hand held horizontally on an armrest at heart level and to operate a car simulator steering wheel with their right-hand. All of the output signals from these devices were stored in one of the two desktop PCs *via* a 16-bit A/D converter with 1-ms sampling interval for the purpose of real-time display using LabVIEW 7 Express (National Instruments Co., Ltd., USA).

B. Measurement Quantities

We acquired the following data during the experiment: beat-by-beat systolic (SBP), mean (MBP) and diastolic blood pressure (DBP) in the subject's left forefinger at the proximal phalanx; beat-by-beat cardiac output (CO) with the RR interval of the ECG (RR); beat-by-beat total peripheral resistance (TPR (=MBP/CO)); beat-by-beat normalized pulse volume (NPV) as an indication of local peripheral resistance; and levels of salivary CgA and cortisol. In this experiment the level of stress as estimated from the answers given in a questionnaire every 10-min, was used as a reference, termed the "Subjective rating of stress" (SRS). Although the sensation of stress is difficult to report, because it is purely subjective, we used the reporting by the subject of their perceived stress on a 9-level scale by selecting any number between Level-1 (not stressful at all) and Level-9 (extremely stressful); this is referred to as *Kakizaki's* method [24].

C. Subjects & Procedures

After giving informed consent 25 healthy male & female subjects [26.8±8.0 SD yrs] without known cardiovascular disorders participated in the present preliminary experiment. The subjects were asked to maintain their regular daily routines and sleeping/waking hours from at least 2 days before the experiment took place. They were studied in a temperature-controlled quiet room held at 25 °C, the study beginning at 9:00 am. The subjects were requested to sit down on the driver's seat where they could drive on a simulated oval test-

course. After resting for 10-min (baseline session) the subjects drove for a maximum of 120-min (simulated driving session). The session was terminated earlier than the 120-min maximum if the simulated vehicle was deemed to have moved out of the specified lane. After the simulated driving session the subject rested for 5-min (end session). In order to simulate a real monotonous driving situation, each subject was previously informed that they had to continue driving safely as though they were actually driving, and that they should maintain a speed of 80~120 km/h, and to drive within a specified lane. Saliva was collected every 10 minutes during experiment.

3. RESULTS

Fig. 2 shows an example of 135-min trend-charts of physiological variables together with those of CgA level and reference of stress level (subjective rating of stress : SRS) obtained in one subject. As shown on the graph, NPV, as a possible marker of the peripheral sympathetic activity, indicates that vasomotor constriction gradually accelerates during the simulated driving session as compared to that in the baseline session. RR and CO are essentially constant. TPR clearly demonstrates that the vascular resistance is gradually increased. BP gradually increases during the driving session. The levels of CgA and cortisol were found to fall gradually as the SRS gradually increased. Salivary cortisol has generally been used as a biological maker of stress and, to compare this with CgA levels, measurements using enzyme linked immunosorbent assay (ELISA) from just 3 subjects in this present experiment were used.

We found that monotonous driving stress led to the expected decreases in NPV and increases in TPR and BP due to peripherally-related sympathetic acceleration. This finding has already been demonstrated in our earlier work [25]. It could be inferred from these results that the driver felt considerably stressed during the simulated driving.

Fig. 3 shows the time course of the means±SDs (n=25) of the percentage change of the CgA level, MBP, RR, CO, TPR and NPV reactions in the simulated driving session period as compared to the baseline session. The right-hand panel indicates the means±SDs of the average values of the change of the physiological measurements during whole driving sessions with respect to the baseline values obtained for all subjects. Also, the asterisks indicate the level of statistical significance for the changes, according to the *Wilcoxon* test (*$p<0.05$, **$p<0.01$). Since the duration of the simulated driving session was different for each subject, the horizontal axis is normalized and shown as the %-age of each driving session period (%-time of driving session).

As is seen in Fig 3 it is clearly demonstrated that the MBP gradually increases and NPV decreases during the driving session, and these changes are statistically significant ($p<0.01$). These results were caused by constrictive peripheral vasomotor regulation. Furthermore, CgA levels were statistically significantly decreased ($p<0.01$) over the period of the simulated monotonous driving. These findings lead us to suggest that CgA levels are not increased in a stress-induced monotonous driving situation.

4. DISCUSSION

The study described here attempted to investigate the possible use of changes in salivary Chromogranin-A (CgA) levels that occur during simulated monotonous driving for assessing an automobile driver's stress levels which, in turn, is hypothesized to reflect their physiological potential to drive safely. In fact, there appears to be an optimal level of stress educing high performance during driving; this may be described by The Yerkes-Dodson law, which is an empirical relationship between performance and stress [26]. In order for a driver to be able to control their vehicle safely, making appropriate decisions when confronted with demanding or hazardous circumstances, and remaining vigilant during monotonous driving, it is generally agreed that they need to be alert. The purpose of our work, and that of others, is to develop some kind of in-car technology, based on the measurement of physiological status, to help ensure a driver is appropriately alert. In order to realize such a system in the future, it is important to investigate thoroughly the physiological reactions, including neuroendocrine markers, during driving.

CgA is a glycoprotein stored in the dense core granules of the adrenal medulla and of many

neuroendocrine cells and neurons. This protein is believed to play an intracellular role as a key regulator of secretory granule biogenesis and an extracellular function as a precursor of several regulatory peptides for the endocrine, cardiovascular, and nervous systems [27-31]. In the cytoplasm, CA is synthesized and transported into the dense core secretory granules (DSG). CA then binds CgA for storage in the core of the granules. Upon stimulation by acetylcholine (Ach), nicotinic cholinergic receptor permitting influx of Na+, which depolarizes the cell membrane, permitting influx of Ca2+ though voltage gated calcium channels. CgA is an acidic protein co-released with catecholamines (CA) and catestatin during exocytosis from chromaffin cells. Secreted CA triggers cardiovascular target cells to augment blood flow. A novel fragment of CgA, known as catestatin, inhibits CA release from chromaffin cells and noradrenergic neurons by acting as a non-competitive nicotinic cholinergic antagonist, and may therefore constitute an endogenous autocrine feedback regulator of sympathoadrenal activity (Fig. 4). When BP is increased by CA, the catestatin fragment of CgA inhibits CA and CgA release [32-35].

Fig. 5 shows a schematic drawing of a circulation regulatory model proposed by Deboer et al. [36] and a secretion regulatory mechanism of CgA. BP is feedback-controlled by the heart and/or the peripheral vessels through the reciprocal action of sympathetic activity and vagal activity so as to maintain the BP at a desired level. In this model, the heart and peripheral vessels modify cardiac output (CO) and total peripheral resistance of the whole body (TPR), respectively. The controlled BP is therefore calculated as "*BP=CO*TPR*". BP is detected by the baroreceptor pressure sensor. Catestatin acts to enhance baroreceptor activity, increasing vagal tone whilst decreasing sympathetic activity [37]. As a nicotinic cholinergic antagonist, the best documented effect of catestatin is to inhibit catecholamine release. Catecholamines have important central effects to influence efferent vagal and sympathetic outflow. Catestatin may play a protective role against the future development of hypertension [37].

In this study, peripherally-related sympathetic activity, vasomotor constriction, was accelerated by stress stimulation during the simulated monotonous driving. Upon peripheral vessel constriction, an increase ($p<0.01$) in total peripheral resistance (TPR) as well as a decrease ($p<0.01$) in NPV then increased BP ($p<0.01$) in correspondence with the increase ($p<0.01$) of subjective rating of stress (SRS). These results imply that, despite being in monotonous situations, drivers must still face demands, such as 'keeping an eye on surroundings', 'performing on-going monotonous tasks under constrained situations', 'shaking off their drowsiness' and so on. The results obtained here strongly indicate that long hours of driving under such monotonous situations can actually make a driver considerably stressful, resulting in a gradual rise in BP caused by an increase in vasoconstriction through acceleration of sympathetic activity. Furthermore, the results also indicate that the rise in BP during monotonous driving would be influenced by a regulation of peripheral vasoconstriction, TPR, rather than by cardiac function, CO. Thus, sympathoadrenal activity, acting mainly on the heart through circulatory blood, is not activated in this monotonous situation. It is known that catestatin is associated with augmented baroreflex sensitivity [37], wherein activation of catestatin is caused by the increase of BP, and secretion of CgA is blocked by the co-secreted catestatin from chromaffin granules. This would explain why in our study CgA levels gradually decreased during the simulated monotonous driving, despite CgA having been reported as a possible marker of stress. Although we have not measured Catecholamine levels in this experiment, because this requires invasive blood sampling, it seems likely that they might also be decreased in the monotonous driving situation.

5. CONCLUSIONS

In healthy volunteers during simulated monotonous driving, we have successfully measured physiological variables together with salivary CgA levels. The level of stress, as indicated by the subjective rating of stress and other physiological stress markers, was significantly increased, but the CgA level gradually decreased over the period of the simulated monotonous driving. The results obtained strongly indicate that, although CgA has been reported as a possible marker of stress, CgA levels are not increased in the stressful situation of simulated monotonous driving. Further experiments are needed to test this finding under actual in-car surroundings.

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Fig. 1 Schematic of experimental setup for physiological measurement during simulated monotonous driving. See text for explanation.

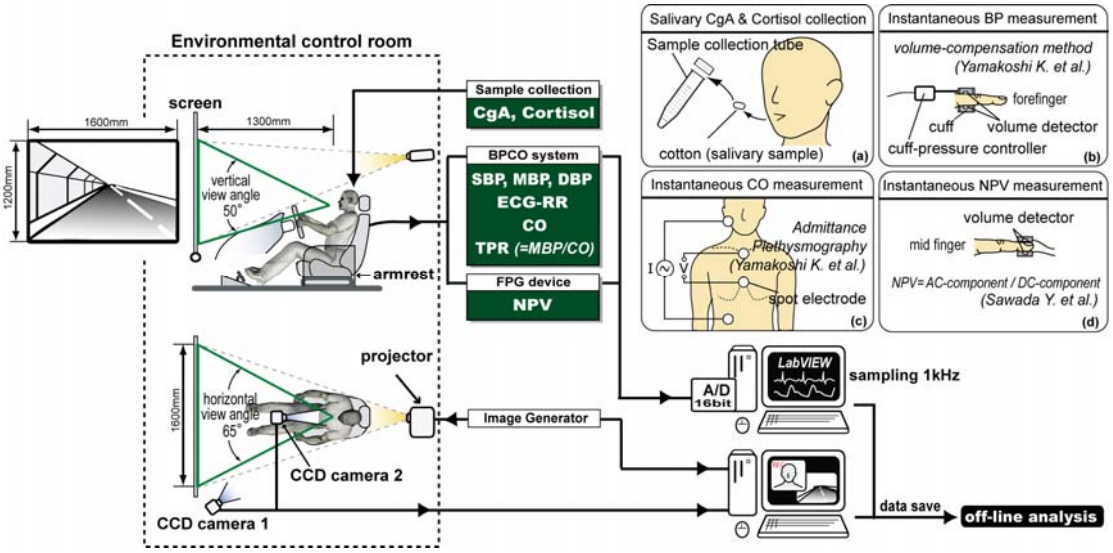
Fig. 2 Example of 135-min trend-charts of physiological variables (BP: blood pressure, CO: cardiac output, TPR: total peripheral resistance, NPV: normalized pulse volume) together with those of CgA and cortisol level, and reference of stress level (SRS: subjective rating of stress) obtained in one subject.

Fig. 3 Means±SDs of time course percent changes from baseline session during simulated

monotonous driving. The right-hand panel shows the means±SDs of the average values of the physiological measurements during the whole simulated driving session as a percentage change from the baseline obtained in all subjects. Asterisks indicate the statistical significance of the change according to the *Wilcoxon* test (*$p<0.05$, **$p<0.01$). See text for details.

Fig. 4 Model for the action of catestatin on CgA secretion from chromaffin cell. Upon stimulation by acetylcholine (Ach), nicotinic cholinergic receptor permitting influx of Na+, which depolarizes the cell membrane, permitting influx of Ca2+ through voltage gated calcium channels. CgA is an acidic protein co-released with catecholamines (CA) and catestatin during exocytosis from chromaffin cells. Consequently, secretion of CgA was blocked by the co-secreted catestatin from chromaffin granules.

Fig. 5 Schematic of circulation regulatory model and secretion regulatory mechanism of CgA. (a): Sympathetic activity and vasomotor constriction were accelerated by stimulated monotonous driving stress. Upon peripheral vessel constriction, an increase in TPR then increased BP. (b): Activation of catestatin was caused by the increase of BP. Catestatin inhibits CgA release from chromaffin cell by acting as non-competitive nicotinic cholinergic antagonist.



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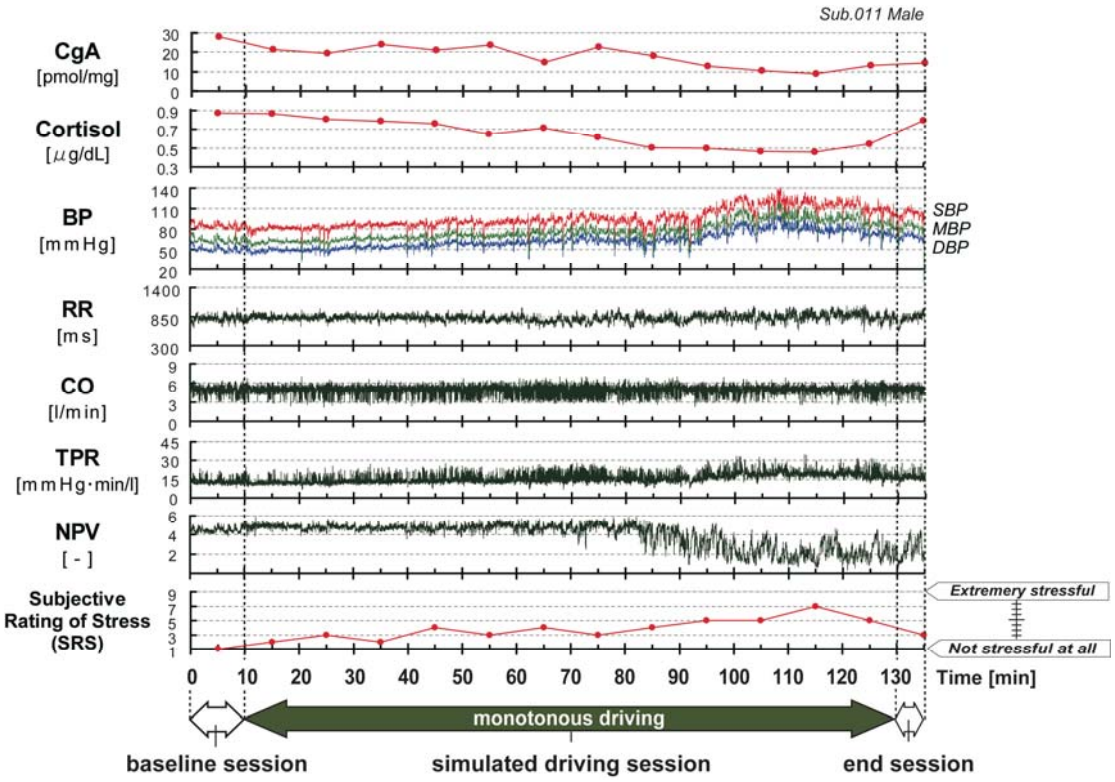

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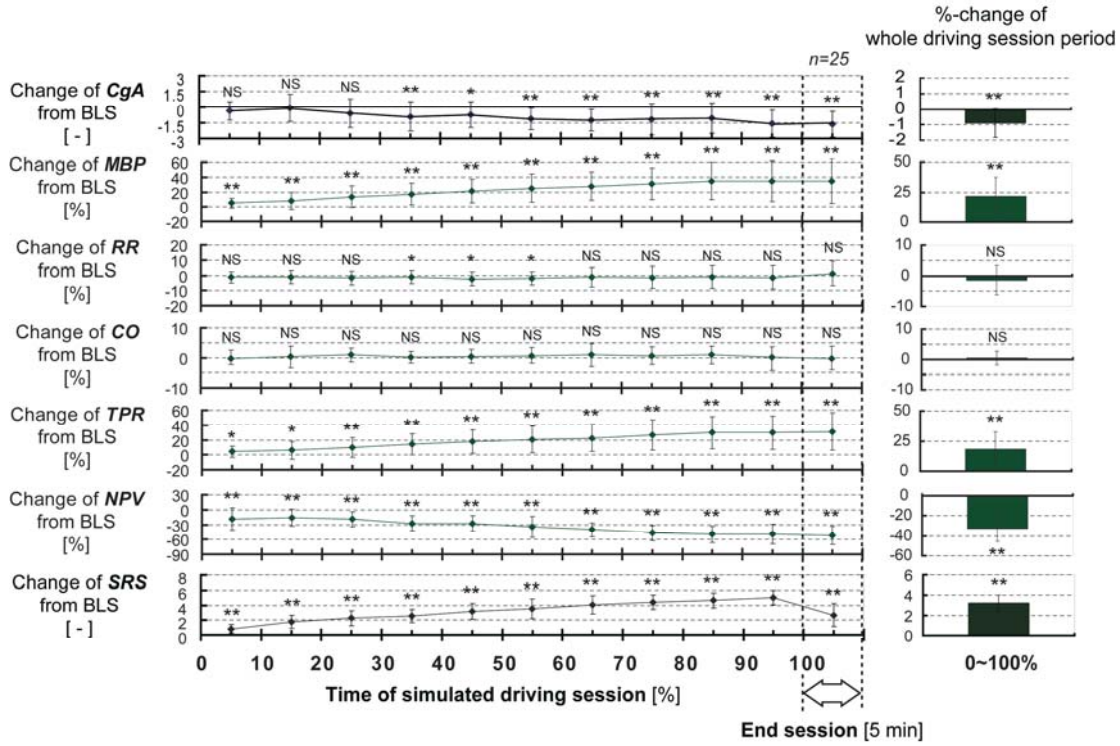

Fig. 3 Means±SDs of time course percent changes from baseline session during simulated monotonous driving. Right bar indicate means±SDs of average value of physiological measurement during whole simulated driving session from baseline obtained in all subjects. Asterisks indicate significant deviation according to the *Wilcoxon* test (*$p<0.05$, **$p<0.01$). See text for details.

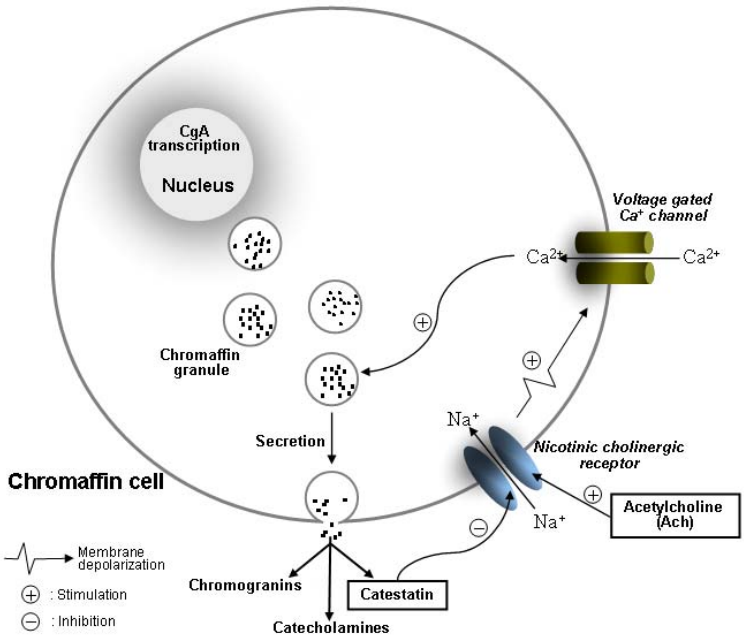

Fig. 4. Model for the action of catestatin on CgA secretion from chromaffin cell. Upon stimulation by acetylcholine (Ach), nicotinic cholinergic receptor permitting influx of Na+, which depolarizes the cell membrane, permitting influx of Ca2+ though voltage gated calcium channels. CgA is an acidic protein co-released with catecholamines (CA) and catestatin during exocytosis from chromaffin cells. Consequently, secretion of CgA was blocked by the co-secreted catestatin from chromaffin granules.

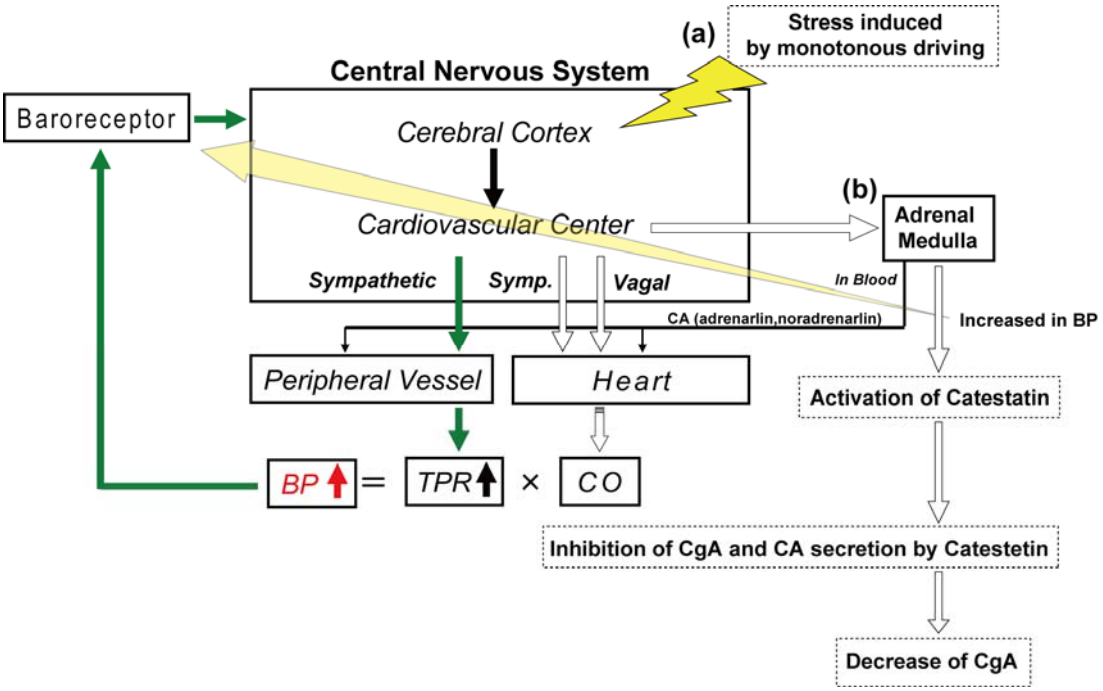

Fig. 5. Schematic of circulation regulatory model and secretion regulatory mechanism of CgA. (a): Sympathetic activity and vasomotor constriction were accelerated by stimulated monotonous driving stress. Upon peripheral vessel constriction, an increase in TPR then increased BP. (b): Activation of catestatin was caused by the increase of BP. Catestatin inhibits CgA release from chromaffin cell by acting as non-competitive nicotinic cholinergic antagonist.