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Contribution of central SGK-1 to the acute phase responses of mice to social isolation

Takao Kaji, Katsunori Nonogaki

Department of Lifestyle Medicine, Biomedical Engineering Center, Tohoku University and Metabolic Care Clinic, Tohoku University Hospital, 1-1 Seiryou-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

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1. ABSTRACT

Ghrelin is a hormone produced mainly by P/D1 cells which line the fundus of the stomach and epsilon cells of the pancreas that stimulate hunger. Ghrelin exists in an endocrinologicaly inactive (des-acyl ghrelin) and active (n-octanoyl-modified ghrelin) forms. The serum- and glucocorticoid-inducible kinase 1 (SGK-1) is a member of the AGC family of serine/threonine protein kinase. In this study, mice were isolated individually or in groups, and deprived from food supply for a period of 24-h. Despite decreases in plasma corticosterone levels and no changes in plasma des-acyl ghrelin, and the expression of hypothalamic neuropeptide Y and proopiomelanocortin, plasma active ghrelin levels and the expression of hypothalamic SGK-1 increased in the acute-isolated mice. Injection of SGK-1 small interfering RNA (siRNA) oligonucleotide into the third cerebral ventricle suppressed the acute social isolation-induced decreases in body weight and increases in plasma active ghrelin levels. Pretreatment with phentolamine (alpha-adrenergic receptor antagonist) but not alprenolol (beta-adrenergic receptor antagonist), partially but significantly suppressed the decreases in body weight induced by acute isolation stress. These finding suggest that isolation stress is a novel inducer of hypothalamic SGK-1 expression. SGK-1 might contribute to the isolation stress-induced body weight reductions and increases in plasma active ghrelin levels via, at least partly, altered central autonomic outflow in mice.

2. INTRODUCTION

The serum- and glucocorticoid-inducible kinase 1 (SGK-1), a member of the AGC family of serine/threonine protein kinases, was initially found as a gene transcriptionally induced by serum and glucocorticoids (1, 2). Its catalytic domain is homologous to the catalytic domains of other serine/threonine protein kinases, including Akt/protein kinase B, protein kinase A, and protein kinase C-zeta (1). SGK-1 is expressed in all tissues including the brain, pancreas, liver, heart, lung, muscle, intestine, and the ovary (3, 4). Various stimuli such as ischemia, restraint stress, water-immersion, and the elevated plus maze exposure increase SGK-1 gene expression in brain tissue (e.g. the hippocampus and cortex) (5-7).

We have previously reported that food deprivation for 24-h increases hypothalamic SGK-1 expression (8), and that social isolation affects the development of obesity due to the primary decreased energy expenditure (9) as well as the acute metabolic responses to fasting and re-feeding (10). However, the role of central SGK-1 in the regulation of energy balance and adaptive metabolic responses to isolation stress remains unknown.

We investigated the effects of acutely isolated individual- and group-housing on changes in body weight, epididymal white adipose tissue weight, plasma

corticosterone, active ghrelin and des-acyl ghrelin levels as well as the expression of other genes (hypothalamic SGK-1, neuropeptide Y (NPY), agouti-related protein (AGRP), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotrophin releasing hormone (CRH), melanin-concentrating hormone (MCH), and suppressor of cytokine signaling-3 (SOCS-3)) involved in the regulation of leptin signaling and energy balance (11) in mice deprived from food for 24-h. Also, we investigated the endogenous role of central SGK-1 in the regulation of acute isolation stress-induced energy expenditure food intake and energy balance. On the other hand, To determine the role of the sympathetic nervous system in acute isolation stress-regulated-body weight loss, we examined the effects of adrenergic receptor antagonists on acute isolation-induced body weight decrease in mice that were deprived from food for 24-h.

3. MATERIALS AND METHODS

3.1. Experimental procedures

4-week-old male C57BL/6J mice were purchased from Japan CLEA. Prior to experimentation, animals were housed in cages (21.5 X 32 X 14 cm) with free access to water and chow pellets in a light-(12 h on/12 h off; lights off at 2000 h) and temperature-(20-22°C) controlled environment. The experiment was performed between 1300 and 1500 h.

In the first experiment, all animals were housed in groups of 3 or 4 mice per cage. They were then deprived from food and housed in groups of 3to 4 per cage or individually for 24-h. At end of 24 h period, body and epididymal white adipose tissue weights were measured. The animals were sacrificed and the hypothalamus was removed for RNA extraction. Blood was collected for the measurement of corticosterone and ghrelin levels.

In the second experiment, all animals were housed in groups of 3 to 4 mice per cage. Specific siRNA oligonucleotides for SGK-1 or controls were injected into the third cerebral ventricle of the group-housed mice. Next day, animals were randomly transferred to individually-housed conditions. Animals were deprived from food and housed in groups of 3 to 4 per cage or individually after the icv injection. Twenty four hours later, body and epididymal white adipose tissue weight were measured. The animals were sacrificed and blood was collected for the measurement of ghrelin levels.

In the third experiment, all animals were individually-housed and were acclimatized to these conditions for 1 week prior to the experimentation. siRNA oligonucleotides Specific to SGK-1 or controls were injected into the third cerebral ventricle. Body and daily food intake were measured on the first and second days following the injection.

In the fourth experiment, all animals were housed in groups of 3 to 4 mice per cage. Animals were intra-peritoneally injected with phentolamine (5 mg/kg), alprenolol (6 mg/kg) or saline solution, and randomly transferred to individually-housed conditions. Animals were deprived from food and housed in groups of 3 to 4 per cage or individually for 24-h. Twenty four hours later, body weight was measured. Phentolamine was dissolved in 0.2 ml pyrogen-free water and alprenolol was dissolved in 0.2 ml 0.9 % saline solution. The doses of phentolamine and alprenolol eliminate adrenergic receptor function *in vivo* as described previously (12).

The animal studies were approved by the local university committee and carried out according to the institutional guidelines for animal experimentation at Tohoku University.

3.2. levels of ghrelin and cortisone in the blood

Plasma ghrelin levels were measured by ELISA kits for the active and des-acyl ghrelin (Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Corticosterone levels were measured using rat corticosterone radioimmunoassay kits (ICN Biomedicals, Costa Mesa, California). For the ELISA of active ghrelin, 1 N hydrogen chloride was added to the samples at a final concentration of 0.1 N, immediately after plasma separation.

3.3. Real-time quantitative RT-PCR

Total RNA was isolated from mouse hypothalamic tissue using the RNeasy Midi kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions as described previously (13,17,18). cDNA synthesis was performed using a Super Script III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Rockville, MD) using 1 μg total RNA. cDNA synthesized from total RNA was evaluated in a real-time PCR quantitative system (Light Cycler Quick System 350S; Roche Diagnostics, Mannheim, Germany). The primers used were as follows. For mouse POMC, sense, 5'-ATA GAT GTG TGG AGC TGG TG-3', antisense, 5'-GGC TGT TCA TCT CCG TTG-3'; for mouse CART, sense, 5'-CTG GAC ATC TAC TCT GCC GTG G-3', antisense, 5'-GTT CCT CGG GGA CAG TCA CAC AGC-3'; for mouse NPY, sense, 5'-GCT TGA AGA CCC TTC CAT TGG TG-3', antisense, 5'-GGC GGA GTC CAG CCT AGT GG-3'; for mouse AGRP, sense 5'-CAG ACC GAG CAG AAG AAG-3', antisense, 5'-GAC TCG TGC AGC CTT ACA-3'; for mouse CRH, sense, 5'-CCG GGC AGA GCA GTT AGC-3', antisense, 5'-CAA CAT TTC ATT TCC CGA TAA TCT C-3'; for mouse MCH, sense, 5'-TGA GTC TGG CTG TAA AAC CT-3', antisense, 5'-ACT CTT CCC AGC ATA CAC CT-3'; for mouse SOCS-3, sense 5'-GCG GGC ACC TTT CTT ATC C-3', antisense 5'-TCC CCG ACT GGG TCT TGA C-3'; for mouse SGK-1, sense, 5'-ACC CTT ACC TAC TCC AGA ATG-3', antisense, 5'-GCT GGC AAT CTT CTG AAT A-3'; and for mouse β-actin, sense, 5'-TTG TAA CCA ACT GGG ACG ATA TGG-3', antisense, 5'-GAT CTT GAT CTT CAT GGT GCT AGG-3'. The relative amount of mRNA was calculated using β-actin mRNA as the invariant control. The data are shown as the fold change of the mean value in the control group.

3.4. Small interfering RNA (siRNA)

The SGK-1 siRNA oligonucleotide was designed as follows: the specific SGK-1 siRNA oligonucleotide (targeting nucleotides 851-875) has the sequence of

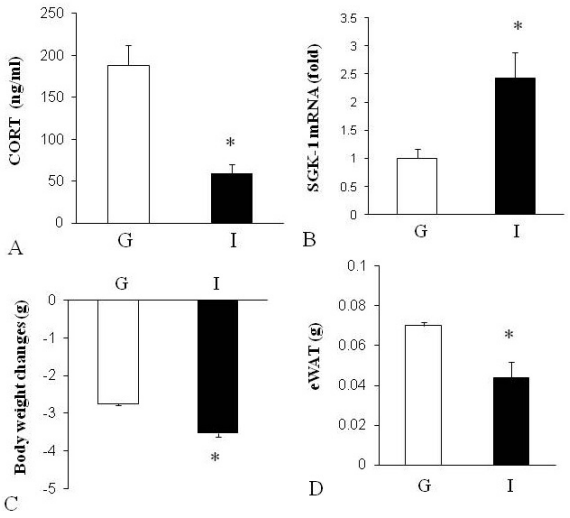

Figure 1. Plasma corticosterone levels (A), hypothalamic SGK-1 mRNA (B), body weight changes (C) epididymal white adipose tissue weight (D), plasma active ghrelin (E) and des-acyl ghrelin levels (F) in acute isolation individually (filled bars)- or group (open bars)-housed 5-week-old mice deprived a food supply for 24-h. Data are presented as the mean values ± SEM (n = 6-7). I; Individually-housed animals, G; Group-housed animals, CORT; corticosterone, eWAT; epididymal white adipose tissue. *P < 0.05

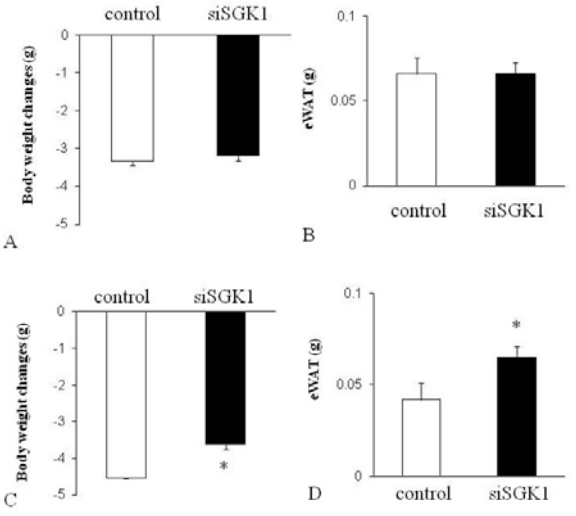

Figure 2. Body weight changes, epididymal white adipose tissue weight, and plasma active ghrelin after icv injection of SGK-1 siRNA oligonucleotide or control siRNA in group- and individually-housed 5-week-old mice deprived a food supply for 24-h (group-housed mice (A and B) and acute isolation individually-housed mice (C, D, E)). Data are presented as the mean values ± SEM (n = 12). C; icv injection of control siRNA, siSGK-1; icv injection of SGK-1 siRNA oligonucleotide, eWAT; epididymal white adipose tissue.*P < 0.05

antisense 5'-UAGAGCAUCUCAUACAGGACAGCCC-3' and sense 5'-GGGCUGUCCUGUAUGAGAUGCUCUA-3'. The stealth™ RNAi negative control Medium GC duplex (Invitrogen, Tokyo) was used as a control.. The siRNA particles were resuspended at 0.04 mM in 50 µl RNase-free saline water mixed with 1 µl lipofectamine (Invitrogen, Tokyo), and 10 µl resuspended SGK-1 siRNA was injected into the third cerebral ventricle of 5-week-old male C57BL6J mice, over 1 minute by stereotaxic surgery. Stereotaxic surgery was performed under anesthesia of the mouse using pentobarbital. For the intracerebroventricular (icv) injection, a microsyringe was placed on a stereotaxic frame into the following coordinates from the bregma: anteroposterior -0.5 mm; lateral 0 mm; vertical -2.5 mm, as described previously (13).

3.5. Data analysis

Data are presented as the mean values ± SEM (n=6-12). Comparisons between the two groups were performed using two-tailed unpaired Student's *t* test. Comparisons of than two groups were performed by analysis of variance using Bonferroni's test. The P value less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Effects of social isolation on plasma corticosterone and ghrelin levels, and the expression of hypothalamic genes

In comparison to group-housed mice, plasma corticosterone levels were significantly reduced in the acute individually-isolated mice compared to (Figure 1A). Hypothalamic SGK-1 expression was significantly increased in acutely isolated mice (2.4 fold; Figure 1B), whereas no significant differences in the expression of hypothalamic NPY, AGRP, POMC, CART, CRH, MCH, and SOCS-3 were observed (Table 1). The Body and epididymal white adipose tissue weights were significantly reduced in acutely isolated individually-housed mice after a 24-h food deprivation (Figure 1C and 1D) (basal body weight; group-housed mice 19.9±0.3 g and individually-housed mice; 19.1±0.4 g, no significant differences, n=6 for each group). Plasma active ghrelin levels were significantly increased in the acute individually-isolated mice (Figure 1E), whereas no differences in the plasma des-acyl ghrelin levels were observed (Figure 1F). Increases in hypothalamic SGK-1 expression and the acute phase responses to isolation stress under the 24-h fasting condition were not found in age-matched KK mice (data not shown).

4.2. Effects of SGK-1 siRNA oligonucleotide

Injection of SGK-1 siRNA oligonucleotide into the third cerebral ventricle had no significant effects on the weight decrease of the body and the epididymal white adipose tissue induced by 24-h fasting in the group-housed mice when compared to control siRNA group (Figure 2A and Figure 2B) (basal body weight; controls 19.6±0.1 g and mice treated with the icv injection of SGK-1 siRNA oligonucleotide; 19.6±0.3 g, no significant difference, n=12 for each group).

Table 1. Hypothalamic gene expression in individually- and group- housed 5-week-old mice

Gene	NPY	AGRP	POMC	CART	CRH	MCH	SOCS3
G	1±0.03	1±0.04	1±0.29	1±0.09	1±0.03	1±0.10	1±0.04
I	1±0.04	0.95±0.07	1.29±0.20	0.96±0.07	1.14±0.04	0.84±0.09	1±0.07

Hypothalamic NPY, AGRP, POMC, CART, CRH, MCH, and SOCS3 mRNA levels in individually- and group- housed 5-week-old C57BL6J mice deprived a food supply for 24-h. Data are presented as the mean values ± SEM (n = 6-7). I; Individually-housed animals, G; Group-housed

Animals

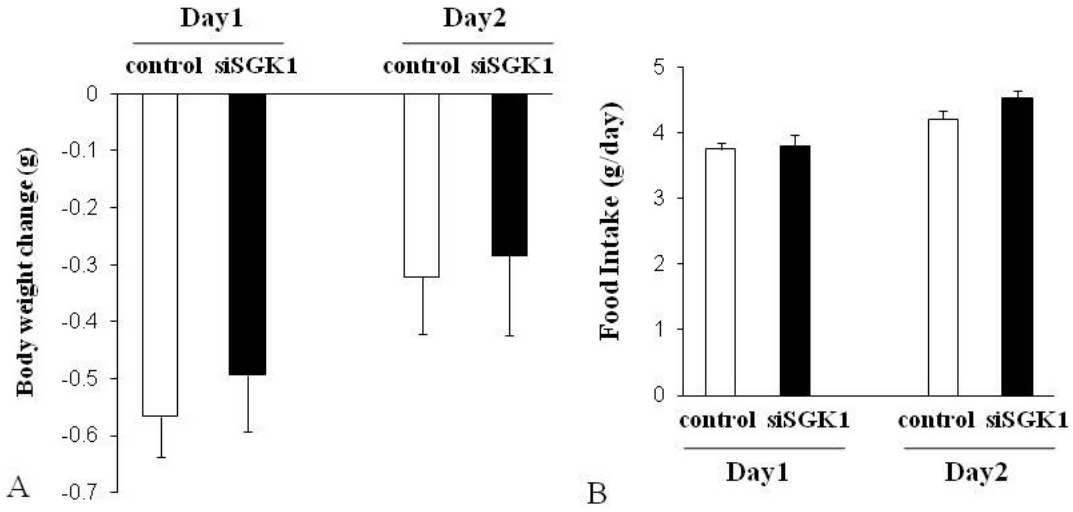

Figure 3. Body weight changes (A) and daily food consumption (B) after icv injection of SGK-1 siRNA oligonucleotide or control siRNA in 5-week-old mice. Data are presented as the mean values ± SEM (n = 6). C; icv injection of control siRNA, siSGK-1; icv injection of SGK-1 siRNA oligonucleotide.

On the other hand, SGK-1 siRNA oligonucleotide significantly suppressed the 24-h fasting-induced a decrease in body and epididymal white adipose weights in the acute individually-housed mice (Figure 2C and Figure 2D) (basal body weight; controls 19.3±0.3 g and mice treated with SGK-1 siRNA oligonucleotide; 18.9±0.3 g, no significant difference, n=12 for each group). Moreover, SGK-1 siRNA oligonucleotide significantly suppressed plasma active ghrelin levels in the acute individually-housed mice (Figure 2E).

Interestingly, SGK-1 siRNA treatment had no significant effects on body weight and daily food intake on the first or the second day post-treatment of5 wk-old C57BL6J mice (Figure 3A and Figure 3B) (basal body weight; controls 20.6±0.2 g and mice treated with SGK-1 siRNA oligonucleotide; 20.8±0.2 g, no significant difference, n=6 for each group).

4.3. Effects of adrenergic receptor antagonists on the acute isolation stress-reduced body weight

Pretreatment with phentolamine partially, but significantly suppressed acute isolation stress-reduced body weight, whereas pretreatment with alprenolol had no effect (Figure 4); These findings suggest that the sympathetic nervous system, contributes to the decrease in body weight induced by acute isolation stress *via* alpha-adrenergic receptors.

5. DISCUSSION

The present findings demonstrate that social isolation acutely increases the expression of hypothalamic SGK-1 and plasma active ghrelin levels under a 24-h fasting conditions. Interestingly, the isolation stress-induced increase in hypothalamic SGK-1 expression was independent of plasma corticosterone levels and the changes in the expression of hypothalamic neuropeptides, which are mainly involved in the leptin signaling required for energy balance. Social isolation is, therefore, a different inducer of hypothalamic SGK-1 expression than fasting and other forms of environmental stress.

CRH is found in the central amygdala and the hippocampus, which send heavy signal to the hypothalamus, particularly the paraventricular nucleus and the ventromedial hypothalamus (14, 15). In addition, CRH stimulates SGK-1 expression in cultured hippocampal neurons *via* CRH-R1 (16). Although the present findings show that isolation stress had no effects on hypothalamic CRH expression, CRH in the hippocampus and/or amygdala contributing to isolation stress-increased hypothalamic SGK-1 expression can not be ruled out.

Dysfunction of the ghrelin feedback system might be responsible for the pathophysiology of obesity and eating disorders (17). Fasting increases plasma ghrelin levels, while feeding reduces plasma ghrelin levels in normal animals and humans (17, 18). The efferent vagus

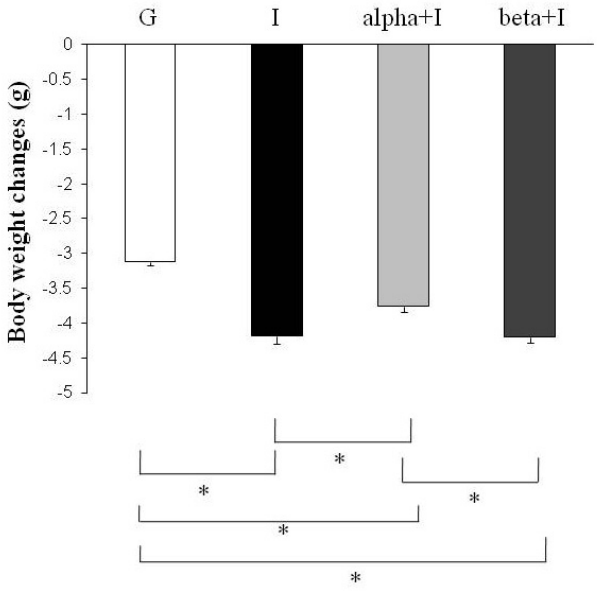

Figure 4. Effects of adrenoreceptor antagonists on body weight changes in acute isolation individually (filled bars)- or group (open bars)-housed 5-week-old mice deprived a food supply for 24-h. Data are presented as the mean values ± SEM (n = 12). I; Individually-housed animals, G; Group-housed animals, alpha; treatment with phentolamine, beta; treatment with alprenolol *P < 0.05

nerve contributes to the fasting-induced increase in ghrelin secretion (17, 18). A lean state and anorexia nervosa, plasma ghrelin levels are increased, whereas in obesity, plasma ghrelin levels are decreased, except in Prader-Willi syndrome (17, 18). However, ghrelin has two forms, active n-octanoyl-modified ghrelin and non-active des-acyl ghrelin (17, 18). The acute isolation stress-induced increases in plasma active-ghrelin levels and hypothalamic SGK-1 expression were not always accompanied by a decrease in body weight. In addition, the present findings indicate that the efferent vagus nerve, at least partly, contributes to the increase in plasma active ghrelin levels induced by acute isolation stress in fasting mice. We previously reported that plasma des-acyl ghrelin levels were inversely proportional to hyperphagia in KK and KKA^y mice (8, 19), whereas plasma active ghrelin levels were decreased in social isolation-induced obesity, due to the primary decreased energy expenditure (9). Thus, active ghrelin and des-acyl ghrelin display functionally different roles in the development of obesity *in vivo*. Plasma active ghrelin levels might be an indicator of energy expenditure, in addition to starvation.

The sympathetic nervous system stimulates lipolysis and adaptive thermogenesis, mainly *via* beta-adrenergic receptors (20). Interestingly, the present findings demonstrate that alpha-adrenergic receptors, but not beta-adrenergic receptors, might contribute to the adaptive metabolic responses to acute isolation stress. From these findings, factors other than the direct neural stimulation of adipose tissues might be involved in the mechanisms. The acute isolation stress decreased body weight in db/db mice with leptin receptor mutation (9), and had no effect on the expression of hypothalamic genes involved in the leptin signaling, which are required for energy balance. Taken together, central leptin-independent pathways might contribute to the acute isolation stress-induced energy expenditure *via* alpha-adrenergic receptors.

Moreover, the present findings show that the housing condition can alter the effects of SGK-1 siRNA treatment on the adaptive metabolic responses to fasting in mice. SGK-1 siRNA oligonucleotide suppressed the decrease in body weight and epididymal white adipose tissue weight induced by a 24-h fasting of acute individually-housed mice, although the same treatment had no effect on daily food intake and body weight of mice fed with standard diet. These results support the previous report that SGK-1-deficient mice display normal body weight (21, 22), suggesting that endogenous central SGK-1 has no direct effect on feeding behavior or energy balance. Central SGK-1 might, therefore, contribute to the acute isolation stress-induced energy expenditure, by stimulating central sympathetic outflow *via* alpha-adrenergic receptors.

In summary, the findings of the present study suggest that: 1) isolation stress is a novel inducer of hypothalamic SGK-1 expression; and 2) SGK-1 contributes to the acute social isolation-induced body weight decrease and plasma active ghrelin increase, *via,* at least partly, alpha-adrenergic receptors in mice.

6. ACKNOWLEDGMENTS

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Send correspondence to: Katsunori Nonogaki, Department of Lifestyle Medicine, Biomedical Engineering Center, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan, Tel: 81-22-717-7061, Fax: 81-22-717-7061, E-mail: knonogaki-tky@umin.ac.jp

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