



## PAPER

# Synergistic effect of polymorphisms of uncoupling protein 1 and $\beta$ 3-adrenergic receptor genes on autonomic nervous system activity

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**OBJECTIVE:** To investigate the association of the promoter region –3826 A to G polymorphism of the uncoupling protein 1 (UCP1) gene with autonomic nervous system (ANS) activity and the interaction of the polymorphism with the Trp64Arg polymorphism of the  $\beta$ 3 adrenergic receptor ( $\beta$ 3AR).

**SUBJECTS:** Three-hundred and forty-nine young (mean age  $20.4 \pm 2.1$  y old), healthy Japanese males.

**MEASUREMENTS:** DNA was extracted from whole blood and genotyped by polymerase chain reaction restriction fragment length polymorphism. Plasma glucose, plasma insulin and body mass index (BMI) were measured. Frequency of family history of diabetes or obesity was determined by interview. Subjects randomly chosen from each genotype were examined for ANS activity during supine rest and standing by electrocardiogram power spectral analysis of heart rate variability.

**RESULTS:** UCP1 or  $\beta$ 3AR polymorphism was not associated with BMI, plasma glucose, plasma insulin and frequency of family history of diabetes or obesity. The inhibitory effect of UCP1 polymorphism on ANS activity was observed only with occurrence of the variant of  $\beta$ 3AR. The very low frequency component associated with thermoregulation in the sympathetic nervous system of homozygotes of UCP1 (GG) at supine rest was significantly lower than normal (AA,  $203.2 \pm 50.3$  vs  $462.2 \pm 83.6$  ms<sup>2</sup>; mean  $\pm$  s.e.,  $P=0.021$ ). A higher response to postural change to standing was also observed in both sympathetic and parasympathetic nervous activities of AA than of GG.

**CONCLUSION:** While UCP1 polymorphism alone does not affect ANS activity, it has a synergistic effect with  $\beta$ 3AR polymorphism in decreasing sympathetic nervous system activity.

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**Keywords:**  $\beta$ 3-adrenergic receptor; uncoupling protein 1; polymorphism; autonomic nervous system activity; power spectral analysis

## Introduction

Obesity is known to be related to autonomic nervous system (ANS) activity. ANS activity is crucial in a wide range of physiological effects ranging from vascular tone and cardiac performance to metabolism and behavior.<sup>1</sup> In particular, the

sympathetic nervous system (SNS) regulates lipolysis and thermogenesis in brown adipose tissue.<sup>2,3</sup> Bray<sup>4</sup> has proposed that a relative or absolute suppression in the activity of the thermogenic component of the SNS is a primary factor of obesity.

Several polymorphisms of genes involved in thermogenic function through the SNS have been reported to be related to obesity. The Trp64Arg polymorphism of the  $\beta$ 3 adrenergic receptor ( $\beta$ 3AR) gene has been found to be associated with lower metabolic rate<sup>5</sup> or abdominal obesity.<sup>6</sup> The promoter region –3826 A to G polymorphism of the uncoupling

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protein 1 (UCP1) gene was identified and associated with body weight gain.<sup>7</sup> Furthermore, it has been reported that the UCP1 variant has an additive effect with the  $\beta$ 3AR variant on weight gain<sup>8</sup> or reduced basal metabolic rate.<sup>9</sup>  $\beta$ 3AR is a G protein receptor activated by catecholamines, and stimulates UCP1 through a cAMP metabolic pathway in brown adipose tissue.<sup>10,11</sup> UCP1 causes heat production through SNS modulation, which is a specific inner-mitochondrial component of brown adipose tissue that dissipates the electrochemical gradient generated in the electron transfer chain in the mitochondria, uncoupling the respiration as heat.<sup>11,12</sup>

We have already reported that a carrier of  $\beta$ 3AR variant had lower resting ANS activity than normal.<sup>13</sup> The previous data was derived by power spectral analysis of heart rate variability (HRV), a non-invasive and sensitive method to evaluate ANS activity.<sup>13-17</sup> In general, it has shown at least two distinct regions of periodicity in electrocardiogram (ECG) R-R intervals. The high frequencies (HI > 0.15 Hz) of HRV are associated solely with parasympathetic nervous system (PNS) activity and the low frequencies (LO < 0.15 Hz) of HRV may be associated with both PNS and SNS activities.

It has also been noted that the frequencies lower than 0.1 Hz may reflect an interaction of the renin-angiotensin system and thermoregulatory vasomotor control.<sup>14,18</sup> We have found that the very low frequencies (VLO) of HRV are associated especially with the thermoregulatory control in SNS activity.<sup>19</sup>

Accordingly, UCP1 variant may affect ANS activity, as does  $\beta$ 3AR variant. Moreover, because obesity is thought to depend on multiple genetic factors, co-occurrence of the two polymorphisms may have a co-operative effect on ANS activity. In this study, we have investigated the effect of UCP1 variant on ANS activity, especially the thermoregulatory function in SNS activity and the interaction of UCP1 variant with  $\beta$ 3AR variant, by ECG R-R interval power spectral analysis.

## Subjects and methods

### Subjects

Three-hundred and forty-nine male volunteers were studied after giving informed consent. All subjects were Japanese, and were determined by interview not to be taking any medications. The mean age of the subjects was  $20.4 \pm 2.1$  (s.d.) y. Blood sampling was carried out at 9–10 am after overnight fast. At the same time, height and body mass were measured, and family history was investigated by interview, including whether a subject had any relatives within the third degree who was diabetic or was significantly obese (body mass index, BMI > 30).

Fifty-three subjects screened for genotypes who approved our request to participate in additional experiment were examined for ANS activity by R-R interval power spectral analysis. The choice of subjects from each genotype group was completely random.

This study was approved by the ethics committees of Kyoto University School of Medicine.

### Measurement of plasma glucose and insulin

Plasma obtained by centrifugation was used for measurement of glucose and immunoreactive insulin. Plasma glucose was measured by the glucose-oxidase method.<sup>20</sup> Immunoreactive insulin was determined by radioimmunoassay using the polyethylene glycol method.<sup>21</sup>

### Determination of UCP1 and $\beta$ 3AR polymorphisms

Genomic DNA was extracted from peripheral blood cells using a DNA Extractor WB Kit (Wako, Japan). The UCP1 gene was amplified by polymerase chain reaction (PCR) with the forward primer 5'-CCAGTGGTGGCTAATGAGAGAA-3' and reverse primer GCACAAAGAAGAAGCAGAGAGG-3'. PCR amplification was conducted in a 20  $\mu$ l volume containing 50 ng genomic DNA, 5 pmol of each primer, 10 mmol/l Tris-HCl (pH 8.8), 50 mmol/l KCl, 1.5 mmol/l MgCl and Triton X-100, 0.25 units of DNA polymerase (Stratagene, USA) and 200  $\mu$ mol/l dNTP. The conditions of PCR were denaturation at 94°C for 3 min, followed 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, with final extension at 72°C for 4 min. The PCR products (279 bp) were digested with 60 units of *Bcl*I in a 100  $\mu$ l volume and separated on a 3% agarose gel. Digestion of the normal sequence (AA) yielded fragments of 157 and 122 bp in length, but the A to G variant eliminated the *Bcl*I site, yielding only a 279 bp product (GG). Samples from heterozygous (GA) subjects yielded the 279 bp as well as the 157 bp and 122 bp fragments.

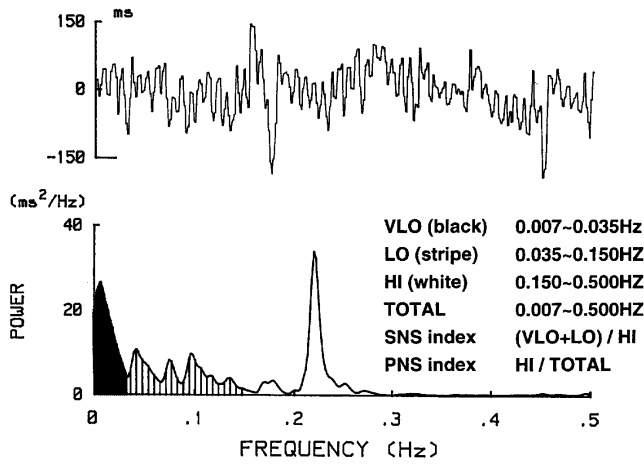
$\beta$ 3AR polymorphism was detected by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) assays as previously described.<sup>22</sup>

### R-R interval power spectral analysis

ANS activity was examined during supine rest and postural change to standing in the morning (9–11 am) after overnight fast. Subjects were at supine rest for 10 min, and then stood up by the bedside at standing rest for another 10 min at 25°C. The respiratory rate was controlled at 0.25 Hz by an electric metronome to avoid the parasympathetic component interfering with the low frequency component.

Our R-R interval power spectral analysis procedures have been fully described elsewhere.<sup>19,23-25</sup> Briefly, analog output of the ECG monitor (NEC Medical, Japan) was digitized via an analog-to-digital converter (Trans Era HTB 386, USA) at a sampling rate of 1000 Hz. The digitized ECG signal was differentiated, and the resultant QRS spikes and the intervals of the impulses (R-R intervals) were stored sequentially on a hard disk for later analysis.

Before R-R spectral analysis was performed, the stored R-R interval data were displayed and aligned sequentially to obtain equally spaced samples with an effective sam-



**Figure 1** A typical set of our computer-aided ECG R-R interval power spectral analysis results: raw R-R interval (top) and the corresponding spectrum (bottom) from which various ANS activity components are derived.

pling frequency of 2 Hz<sup>26</sup> and displayed on a computer screen for visual inspection. Then, the d.c. component and linear trend were completely eliminated by digital filtering for band-pass between 0.007 and 0.5 Hz. After passing through the Hamming-type data window, power spectral analysis by means of a fast Fourier transform was performed on the consecutive 480 s time series of R-R interval data obtained during the tests. We analyzed very low frequency (0.007–0.035 Hz, VLO), low frequency (0.035–0.15 Hz, LO), high frequency (0.15–0.5 Hz, HI), and total power (0.007–0.5 Hz, TOTAL) by integrating the spectrum for the respective band width. Figure 1 shows examples of raw R-R interval data (top) and the corresponding spectra (bottom). In this spectrum, the black area shows VLO, the striped area shows LO, and the white area shows HI. TOTAL reflects overall ANS activity. LO arises from com-

bined SNS and PNS function and HI largely from PNS activity. VLO has been shown to be closely associated with thermoregulatory control in SNS activity according to our recent study.<sup>19</sup> The ratio of (VLO + LO)/HI (SNS index) reflects SNS activity, and HI/TOTAL (PNS index) reflects PNS activity.<sup>27,28</sup>

**Statistical analysis**

Significant differences were evaluated by chi-square test, Student’s *t*-test or ANOVA, when appropriate.

**Results**

**Genotypes**

Seventy-one (20.4%) of the subjects were homozygous (GG) and 191 (54.7%) heterozygous (GA) for UCP1 variant, and 87 (24.9%) had the genotype AA. The frequency of the G allele was 0.48.

Three (0.8%) of the subjects were homozygous (Arg64Arg) for  $\beta$ 3AR variant and 84 (24.1%) were heterozygous (Arg64-Trp), and 262 (75.1%) were homozygous for the normal (Trp64Trp). The frequency of the Arg64 allele was 0.13.

These polymorphisms were in Hardy–Weinberg equilibrium.

**Clinical characteristics**

Table 1 shows the clinical characteristics of the subjects according to genotype. UCP1 polymorphism had no effect on any of the investigated characteristics ( $P > 0.05$ )—BMI ( $21.9 \pm 2.9$ ,  $21.5 \pm 2.8$  and  $21.3 \pm 2.6$  kg/m<sup>2</sup>; AA, GA and GG, respectively, means  $\pm$  s.d.), plasma glucose ( $5.3 \pm 0.3$ ,  $5.1 \pm 0.3$  and  $5.1 \pm 0.2$  mmol/l), insulin ( $47.3 \pm 7.6$ ,  $47.9 \pm 5.2$  and  $49.1 \pm 8.4$  pmol/l), or family history of diabetes or obesity (28.7, 24.6 and 21.1%). Similarly, subjects with Arg64Trp did not differ from subjects without the variant ( $P > 0.05$ ) in any investigated characteristic—BMI ( $21.8 \pm 3.4$  vs  $21.5 \pm 2.6$  kg/m<sup>2</sup>; Arg64Trp vs Trp64Trp, mean  $\pm$  s.d.), plasma glucose ( $5.2 \pm 0.3$  vs  $5.2 \pm 0.3$  mmol/l), insulin ( $47.1 \pm 6.5$  vs  $48.3 \pm 6.0$  pmol/l), or family history of diabetes

**Table 1** Clinical characteristics of subjects, according to genotype of the UCP1 and  $\beta$ 3AR

$\beta$ 3AR	Genotype						
	Trp64Trp			Arg64Trp + Arg64Arg			
	AA	GA	GG	AA	GA	GG	Total
UCP1							
<i>n</i>	59 (9)	145 (12)	58 (9)	25+3 (9)	46 (8)	13 (6)	349 (53)
BMI (kg/m <sup>2</sup> )	21.8 $\pm$ 2.4 (21.7 $\pm$ 2.0)	21.5 $\pm$ 2.6 (20.8 $\pm$ 2.0)	21.3 $\pm$ 2.7 (21.3 $\pm$ 1.9)	22.3 $\pm$ 3.9 (21.1 $\pm$ 2.6)	21.6 $\pm$ 3.7 (20.0 $\pm$ 2.3)	21.4 $\pm$ 2.6 (21.0 $\pm$ 2.3)	21.6 $\pm$ 2.8 (21.0 $\pm$ 2.0)
Plasma glucose (mmol/l)	5.3 $\pm$ 0.3 (5.2 $\pm$ 0.6)	5.1 $\pm$ 0.3 (5.4 $\pm$ 0.5)	5.1 $\pm$ 0.2 (5.1 $\pm$ 0.6)	5.3 $\pm$ 0.4 (5.2 $\pm$ 0.4)	5.2 $\pm$ 0.2 (5.3 $\pm$ 0.5)	5.1 $\pm$ 0.2 (5.2 $\pm$ 0.6)	5.1 $\pm$ 0.3 (5.3 $\pm$ 0.5)
Plasma insulin (pmol/l)	47.9 $\pm$ 6.1 (48.6 $\pm$ 6.5)	48.1 $\pm$ 4.8 (47.1 $\pm$ 6.3)	49.1 $\pm$ 8.7 (49.5 $\pm$ 8.9)	46.0 $\pm$ 7.2 (46.3 $\pm$ 6.4)	47.3 $\pm$ 5.9 (47.1 $\pm$ 5.9)	49.0 $\pm$ 7.2 (47.8 $\pm$ 7.4)	48.0 $\pm$ 6.1 (47.7 $\pm$ 7.6)
Family history of diabetes or obesity (%)	28.8	24.8	20.7	28.6	23.9	23.1	24.9

Values are mean  $\pm$  s.d. Values in parentheses show the data from R-R interval power spectral analysis subjects. The subjects were randomly chosen from those of each genotype group.

or obesity (24.8 vs 25.3%). In subjects of ECG R-R interval power spectral analysis, no significant difference in any of the clinical characteristics was observed between groups.

### Autonomic activities of subjects with and without the polymorphisms

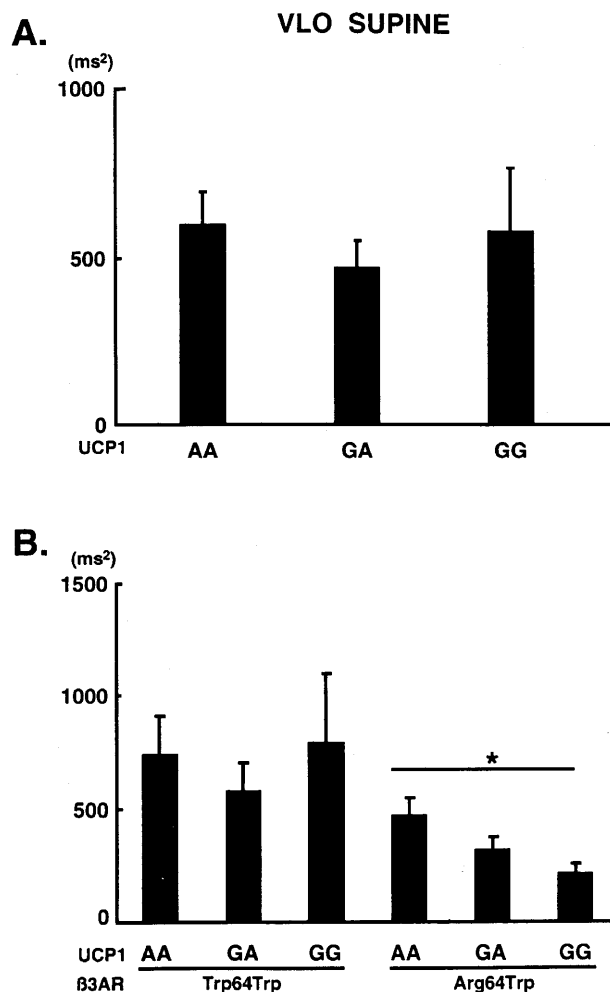
$\beta$ 3AR variant showed the effect on ANS activity. TOTAL of Arg64Trp at supine rest was significantly lower than that of Trp64Trp ( $1434.6 \pm 129.9$  vs  $2516.1 \pm 193.7$  ms<sup>2</sup> mean  $\pm$  s.e.,  $P=0.014$ ). With the postural change to standing, the SNS index and PNS index of Arg64Trp showed higher response than of Trp64Trp ( $16.6 \pm 2.0$  vs  $9.5 \pm 1.4$ ,  $P=0.004$ , SNS index;  $0.09 \pm 0.02$  vs  $0.14 \pm 0.02$ ,  $P=0.039$ , PNS index).

The effect of UCP1 variant on ANS activity was observed only in conjunction with  $\beta$ 3AR variant. In the carrier of Arg64Trp, VLO of GG at supine rest was significantly lower than that of AA ( $203.2 \pm 50.3$  vs  $462.2 \pm 83.6$  ms<sup>2</sup>; mean  $\pm$  s.e.,  $P=0.021$ ), while there were no significant differences in VLO at supine rest among the three groups separated only by UCP1 polymorphism (Figure 2). With the postural change to standing, the SNS index and PNS index of AA with Arg64Trp showed higher responses than those of GG with Arg64Trp ( $21.8 \pm 3.4$  vs  $9.5 \pm 2.8$ ,  $P=0.013$ , SNS index;  $0.06 \pm 0.01$  vs  $0.16 \pm 0.05$ ,  $P=0.025$ , PNS index; Figure 3). In subjects with Trp64Trp, no significant difference was found in the indices at standing among AA, GA and GG.

### Discussion

In this study we investigated the effect of UCP1 polymorphism on ANS activity by ECG R-R interval power spectral analysis. The frequency of G allele was 0.48, almost the same as the rate of previous reports in Japan,<sup>29,30</sup> but higher than in other studies in Canada,<sup>7</sup> France,<sup>8</sup> Finland,<sup>9,31,32</sup> and Denmark.<sup>33</sup> This polymorphism is not associated with the clinical features of abnormality. In ANS activity, Arg64Trp had lower TOTAL at supine rest and higher response in both PNS and SNS activities with the postural change to standing. These results accorded with our previous study.<sup>13</sup> Only together with the occurrence of the  $\beta$ 3AR variant was VLO of GG at supine rest significantly lower than that of AA, and a higher response to the postural change to standing was observed in both the PNS and SNS activities of AA when compared to those of GG.

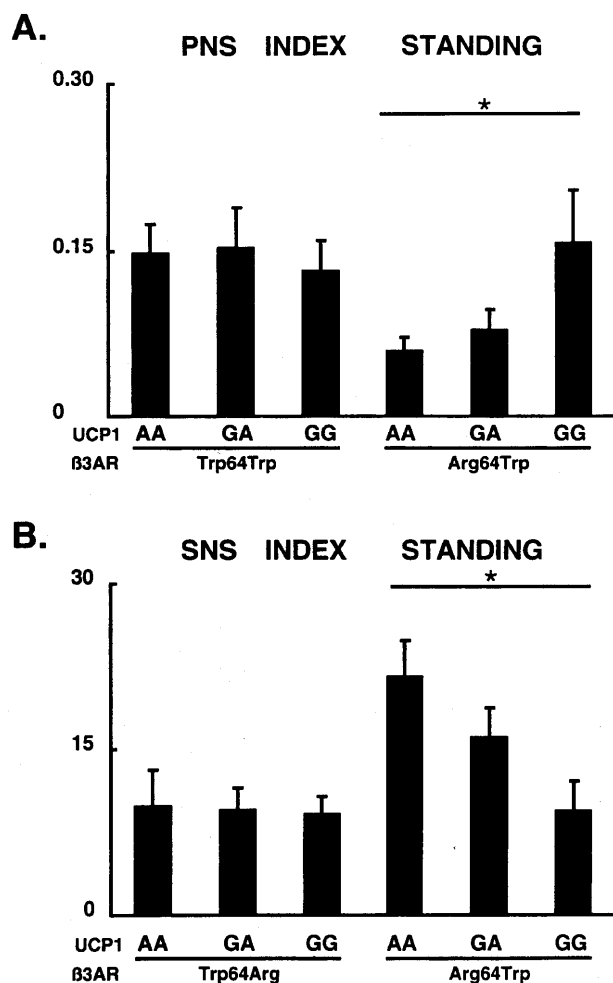
An important role of UCP1 is thermogenesis in brown adipose tissue. The significant decrease observed in GG was in VLO, the spectral region especially associated with thermoregulatory control. The defect of UCPI polymorphism was found to be in the main function of UCP1 through analysis of the ANS activity. We have reported that the VLO is lower in obese women upon acute cold exposure.<sup>19</sup> Valve *et al*<sup>9</sup> reported that UCP1 polymorphism did not have an independent effect on basal metabolic rate (BMR), but that its simultaneous occurrence with  $\beta$ 3AR variant resulted in



**Figure 2** Autonomic nervous system activity by R-R interval power spectral analysis. R-R interval power spectra were integrated for three band widths: very low frequency (0.007–0.035 Hz, VLO); low frequency (0.035–0.15 Hz, LO); and high frequency (0.15–0.5 Hz, HI). VLO is associated with thermoregulatory control in SNS activity. (A) shows VLO at supine rest separated by only UCP1 genotype, and (B) separated by both UCP1 and  $\beta$ 3AR genotypes. Values are mean  $\pm$  s.e.

much lower BMR than did  $\beta$ 3AR variant alone. This finding partly concurs with their report. Dysfunction of the ANS, especially the sympathetic responsiveness associated with thermoregulatory function, may affect BMR, and low BMR is one of the risk factors for weight gain.<sup>34</sup>

We have previously described the higher response to postural change to standing in both PNS and SNS activities in subjects with Arg64Trp<sup>13</sup> and got the same results in this study. This response was considered a compensatory action for low resting ANS activity. In the present study, a higher response was observed in both PNS and SNS activities of AA than of GG only with the occurrence of the  $\beta$ 3AR variant. The A to G allele may reduce up-regulation of the SNS only in a state of SNS activation, such as, for example, during exercise.<sup>17</sup> Therefore, the compensation for the effect of the



**Figure 3** Standing (A) PNS index, the ratio of HI/TOTAL which reflects parasympathetic nervous system activity. (B) SNS index, the ratio of (VLO + LO)/HI which reflects sympathetic nervous system activity. Values are mean  $\pm$  s.e.

$\beta$ 3AR variant may be abolished by the reduced increase in SNS activity induced by UCP1 polymorphism. Previous investigation<sup>29</sup> found that, during a combined low calorie diet and exercise regimen, obese subjects with both GG and Arg64Trp lost less weight than those with either GG of UCP1 polymorphism alone or Arg64Trp alone. In such a case, the effect of exercise on activation of SNS activity might be reduced, resulting in a lower expenditure of fat.

Our results show that UCP1 variant had an influence on the ANS activity only in the presence of  $\beta$ 3AR variant. Esterbauer *et al*<sup>35</sup> reported that the UCP1 variant caused a lower UCP1 mRNA level than normal in human intraperitoneal fat. In rodents, UCP gene expression is regulated via adrenergic mechanisms including the  $\beta$ 3AR,<sup>36</sup> so the  $\beta$ 3AR variant might well induce low expression of UCP1. When the effect of  $\beta$ 3AR variant is added to that of UCP1 variant, the UCP1 expression level could be so low that the defect of

low expression of UCP1 only then becomes detectable in ANS activity or BMR. Without  $\beta$ 3AR variant or another complementary factor, UCP1 polymorphism alone may have so little effect that another pathway could mask it. Although UCP1 polymorphism may have less importance in metabolic function than  $\beta$ 3AR polymorphism, the occurrence of UCP1 variant with  $\beta$ 3AR variant has more influence on ANS activity than does  $\beta$ 3AR variant alone. Because obesity is derived from multiple genetic factors, both UCP1 and  $\beta$ 3AR polymorphisms should be examined when genotypes are tested for risk factors of metabolic disorder.

In this study, there were no significant differences in the clinical features in any of the groups. However, the subjects were young and in good health at the time of the experiments, and their mean BMI was almost ideal. It has been reported that ANS activity declines with age.<sup>37</sup> The defect in ANS activity may only then become obvious in clinical features when the influence of aging is added to the effect of the polymorphisms.

In conclusion, UCP1 variant has a synergistic effect with  $\beta$ 3AR variant in decreasing SNS activity. It is likely that certain effects of the low SNS activity caused by UCP1 variant in conjunction with  $\beta$ 3AR variant become more obvious with aging or stressful alteration of environmental factors. A longitudinal study of the same subjects will be conducted to ascertain the increasing influence of UCP1 and  $\beta$ 3AR polymorphism with age.

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