Table 1. Basal characteristics of subjects.

Age	62.0±0.9 (178)
Male %	47% (83)
BMI (kg/m²)	24.5±0.3 (174)
LDL-C (mg/dL)	153.4±3.3 (174)
HDL-C (mg/dL)	57.8±1.4 (134)
TG (mg/dL)	154.7±5.5 (175)
TC (mg/dL)	242.2±3.6 (174)
RLP-C (mg/dL)	7.9±0.5 (63)
A1C (%)	7.2±0.2 (139)
FPG (mg/dL)	126.0±3.9 (116)
hs-CRP (mg/L)	0.69 (0.33-1.36) (31)
Diabetes mellitus %	58% (103)
Hypertension %	35% (62)
Peripheral arterial disease %	4% (7)
Cerebral infarction %	7% (12)
Coronary heart disease %	18% (32)

Data are presented as the mean±S.E.M. Numbers of subjects are shown in parentheses. Abbreviations: TC: total cholesterol, FPG: fasting plasma glucose.

and 12 months, respectively (**Fig. 1C**). Furthermore, serum RLP-C levels were significantly decreased by 14.0%, 20.2% and 22.8% after 3, 6 and 12 months, respectively (**Fig. 1D**).

Serum hs-CRP levels were significantly decreased in 31 subjects after 12 months (median: 0.69 mg/L to 0.45 mg/L, −34.8%, $p<0.01$, **Fig. 2A**). Pitavastatin similarly decreased hs-CRP levels in subjects with diabetes mellitus (median: 0.59 mg/L to 0.36 mg/L, −39.0%, $p<0.05$, **Fig. 2B**).

There was no significant correlation between changes in LDL-C and hs-CRP levels even after transformation of hs-CRP values into a logarithm after 12 months ($r=0.124$, $p=0.39$). We also found no significant correlation between changes in hs-CRP and changes in serum TG or serum RLP-C levels during the study (data not shown) and there were no serious adverse events. In a subgroup of diabetic patients, there was a slight and statistically insignificant decrease in A1C after pitavastatin treatment for 12 months (7.0% to 6.9%, $n=80$, $p=0.07$).

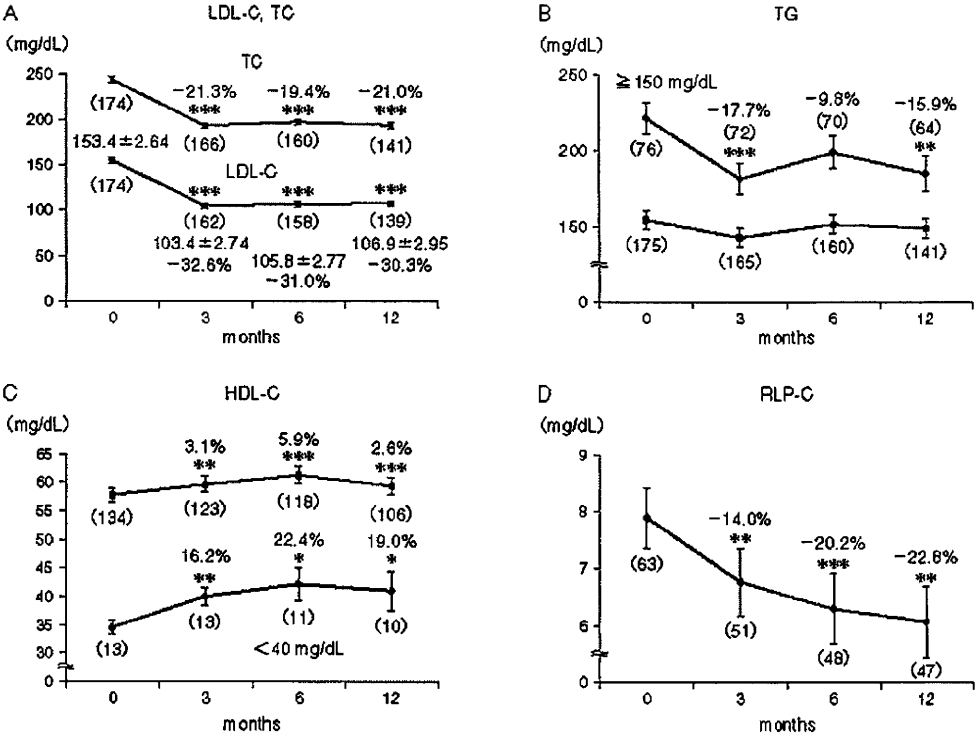

Fig. 1. Effect of pitavastatin on lipid levels in all subjects.

A: Effect of pitavastatin on total cholesterol (TC) and LDL cholesterol (LDL-C) levels. B: Effect of pitavastatin on triglyceride (TG) levels in all subjects and in subjects with basal TG levels of more than 150 mg/dL. C: Effect of pitavastatin on HDL cholesterol (HDL-C) levels. Data in subjects with basal HDL-C levels less than 40 mg/dL are indicated separately. D: Effect of pitavastatin on remnant-like particle cholesterol (RLP-C) levels.

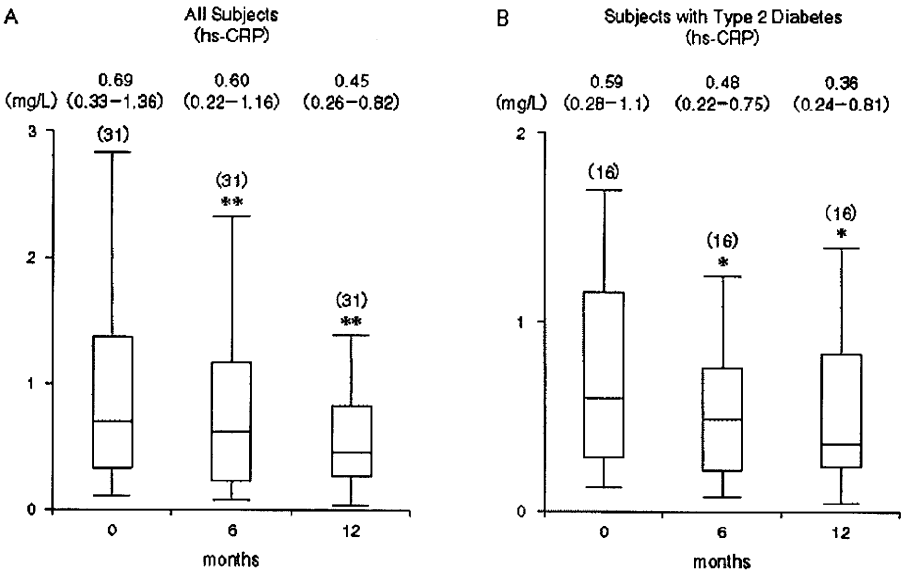

Fig. 2. Effect of pitavastatin on hs-CRP levels in all patients (panel A) and patients with type 2 diabetes (panel B) after 12 months.

Data are presented as the mean ± S.E.M. Numbers of subjects are shown in parentheses. $^{*}p<0.05$ vs. basal values, $^{**}p<0.01$ vs. basal values.

Discussion

The present study demonstrated that pitavastatin improved serum lipid profiles in Japanese subjects with hypercholesterolemia: a decrease in LDL-C, an increase in HDL-C, a decrease in RLP-C, and a decrease in TG among those with higher basal TG levels. The improvement in lipid profiles was compatible with a recent report on pitavastatin in Japanese subjects[16], although, in the present study, LDL-C levels before the administration of pitavastatin were lower (153 ± 3 mg/dL) than in the previous report (183 ± 21 mg/dL)[16].

Like other statins, pitavastatin has been indicated to have pleiotropic effects on vascular cells *in vitro*. In cell culture experiments, for example, pitavastatin is reported to reduce osteopontin expression in rat vascular smooth muscle cells[17], suppress interleukin-8 (IL-8), monocyte chemoattractant protein-1 and endothelin-1 expression, enhance endothelial nitric oxide synthase expression[7], and inhibit CRP-induced IL-8 production[8], thus showing its direct ant-inflammatory and anti-atherogenic effect. However, there have been no reports about anti-inflammatory effects of pitavastatin *in vivo* in humans.

The present study showed that pitavastatin lowered hs-CRP in patients with hypercholesterolemia. A recent study in Korea has shown that pitavastatin reduced mean hs-CRP levels from 24.6 to 16.5 mg/L by 8-week treatment[18]. In the present study, pitavastatin, at similar doses, significantly reduced hs-CRP levels in hypercholesterolemic subjects, including type 2 diabetes, with lower basal hs-CRP levels. These findings have been supported by previous reports which indicated anti-inflammatory effects of pitavastatin *in vitro*[6, 8]. There have been conflicting reports about the effects of statins on hs-CRP *in vivo* in diabetic subjects; one report did not show a significantly decrease in hs-CRP after atorvastatin[14]. Therefore, it is possible that diabetic patients are resistant to the inhibitory effects of statins on hs-CRP, although diabetic patients showed a significant decrease in hs-CRP after atorvastatin in a recent paper[19]. The present as well as the previous study[18], also demonstrated that pitavastatin, a new synthetic lipophilic strong statin, lowered hs-CRP levels in hypercholesterolemic subjects, including type 2 diabetes. There was no significant correlation between pitavastatin-induced decreases in hs-CRP and LDL-C, in the present study with pitavastatin, as previously reported with other statins[9, 10, 12, 19]. We also found no significant correlation between pitavastatin-induced decrement in hs-CRP and those in RLP-C in this study cohort. Thus, it is suggested that pitavastatin may have direct anti-inflammatory effects which are independent of improved lipid profiles. A previous study showed thst non-responders,

whose LDL-C levels were above 100 mg/dL after atorvastatin treatment, tender to be resistant to hs-CRP reduction by statin treatment[19]; however, the present study did not show such a tendency but hs-CRP reduction to be independent of LDL-C levels after pitavastatin treatment (data not shown).

There were no serious adverse events in the present study, including A1C changes in subjects with diabetes mellitus. In contrast, atorvastatin has been reported to result in a slight but significant increase of A1C in Caucasian diabetic subjects, as shown by the Collaborative Atorvastatin Diabetes Study (CARDS)[20]. It has been shown that Japanese subjects with type 2 diabetes are mainly insulin-deficient rather than insulin-resistant[21]. In addition, Asians have higher plasma levels of statins than Caucasians[22]. Therefore, it is possible to speculate that Japanese subjects with type 2 diabetes may be more prone to worsening glycemic control due to, if any, adverse effects of statins on insulin secretion; however, the persent study indicated that pitavastatin did not significantly worsen glycemic control in Japanese diabetic subjects.

This study is limited by the fact that it was an uncontrolled study with a moderate number of subjects, especially a relatively small number with hs-CRP measurement. It is to be noted, however, that the subjects showed lower basal hs-CRP levels before pitavastatin treatment (median: 0.69 mg/L) than those in previous studies of Caucasians (median: about 3 mg/L)[5, 9]. In the Pravastatin Inflammation/CRP Evaluation (PRINCE) study, baseline hs-CRP levels were the major determinants of the change in hs-CRP levels after pravastatin[12], indicating that anti-inflammatory effects of statins would be more pronounced in subjects with higher hs-CRP levels. It is intriguing that pitvastatin was able to reduce hs-CRP even in subjects with lower basal hs-CRP levels in the present study. Previous large scale cardiovascular event outcome studies with other statins have been conducted in Caucasians with high basal hs-CRP levels; therefore, it is of clinical importance to investigate whether pitavastatin can similarly reduce cardiovascular events among Japanese subjects with lower basal hs-CRP values than Caucasians[10, 23, 24].

Conclusions

It is concluded that pitavastatin improves lipid profiles, including the reduction of RLP-C levels, and that it decreases hs-CRP levels independently of improved lipid profiles, without any adverse effects, in Japanese subjects with hypercholesterolemia, including type 2 diabetes.

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Association of estrogen receptor-α gene polymorphisms with cardiac autonomic nervous activity in healthy young Japanese males

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ABSTRACT

Background: Estrogens exert beneficial effects on the cardiovascular system that are mediated by estrogen receptors. We examined the association between the estrogen receptor α gene (*ESR1*) *Pvu*II and *Xba*I polymorphisms and cardiac autonomic nervous function in Japanese males.
Methods: We examined 252 young healthy males for association of *ESR1 Pvu*II and *Xba*I polymorphisms and short-term heart rate variability (HRV) during supine rest and in a standing position. The very low frequency (VLF), low frequency (LF), and high frequency (HF) components of HRV were quantified by frequency domain analysis.
Results: Carriers of the *ESR1 Pvu*II C allele had higher mean blood pressure (BP), while the *Xba*I GG genotype was significantly associated with higher diastolic and mean BP, but lower HR. In the haplotype analysis, carriers of the *ESR1* haplotype 2 (*Pvu*II C and *Xba*I A) allele had a higher systolic and mean BP, and lower HRV spectral powers (total power, VLF, LF, and HF components) in a supine rest compared with those of non-carriers.
Conclusions: The *ESR1 Pvu*II and *Xba*I haplotype is associated with BP variation and the reduction in cardiac autonomic nervous activity in young Japanese males, which may be precursors of future pathological episodes of cardiovascular diseases.

1. Introduction

The risk of cardiovascular diseases (CVD) is greater in males than in premenopausal women, which can be attributed, in part, to the sex difference in estrogen concentrations [1]. Circulating estrogen levels are quite low in males, however, males generate estrogen by direct synthesis from testosterone in target tissues, and estrogens also have protective effects against the development of CVD in males [2,3]. These beneficial effects occur mainly through activation of the estrogen receptor α (ERα), directly and indirectly resulting in numerous physiological processes, including vasodilation, protection against vascular injury, and favorable influence to the lipidemic profile [2,4]. The ERα acts as an estrogen-activated transcription factor, and is expressed in various tissues including endothelial, smooth muscle, and myocardial cells in the cardiovascular system [1,4].

The human ERα gene (*ESR1*) is located on chromosome 6 (6q25.1) and consists of approximately 140 k base pairs (bp) including 8 exons separated by 7 intronic regions. Numerous polymorphisms have been identified in *ESR1* [5], and the associations between these polymorphisms and CVD were investigated in several populations [6,7]. Of the common polymorphisms of *ESR1*, *Pvu*II (c.454–397T/C: rs2234693) and *Xba*I (c.454–351A/G: rs9340799), which are located in the first intron and separated by only 46 bp, have been the most extensively examined. A number of studies have shown that these two polymorphisms are associated with CVD or related physiological conditions, including myocardial infarction [8–10], coronary artery disease [11,12], increased blood pressure [13,14], arterial stiffness [15], and stroke [16,17]. However, the physiological mechanism for those associations remains unclear.

Previous studies have suggested that some of the protective cardiovascular effects of estrogen may relate to beneficial effects on cardiac

Abbreviations: *ESR1*, estrogen receptor-α gene; HRV, heart rate variability; VLF, very low frequency; LF, low frequency; HF, high frequency; BP, blood pressure; CVD, cardiovascular diseases; ER, estrogen receptor; bp, base pair; CNS, central nervous system; SBP, systolic blood pressure; DBP, diastolic blood pressure; ECG, electrocardiogram; MBP, mean blood pressure.

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autonomic nervous function. As well as its peripheral cardiovascular expression pattern, ERα is found in autonomic centers of the brain stem involved in cardiovascular regulation, and estrogen can access to autonomic preganglionic cells in the central nervous system (CNS) [18]. Treatment with estrogen has also been shown to improve cardiac autonomic control and reduce sympathetic drives [19–22]. Heart rate variability (HRV) provides a noninvasive quantitative phenotypic marker of cardiac autonomic nervous activity, and has been used as an indicator of cardiovascular health [23,24]. Abnormalities of cardiac autonomic control reflected by a reduced HRV were showed to be a powerful predictor of cardiac events [25,26], sudden cardiac death [27], pathogenesis of hypertension [28], and overall mortality [29,30]. Previous studies have shown that chronic or short-term estrogen treatment increases HRV and decreases HRV sympathetic indices [19–22], in addition to reducing blood pressure (BP) [21,31]. Our main aim in the present study was to determine the association of *ESR1 PvuII* and *XbaI* polymorphisms with cardiac autonomic nervous function by HRV analysis in young healthy Japanese males.

2. Materials and methods

2.1. Subjects

Two hundred and fifty-two young healthy Japanese males, recruited at random from Kyoto University, participated in each examination after written informed consent was obtained. The subjects ranged in age from 18 to 28 years (mean and SEM., 21.7 ± 0.1 years). All subjects were normotensive (causal supine BP<140/90 mm Hg) and non-obese (body mass index [BMI]<30 kg/m^2). It was determined by interview that the subjects were not taking any medication, and had no history of organic diseases such as CVD, metabolic disorder, renal disease, or neuropathy. BMI, BP (systolic [SBP] and diastolic [DBP]), and HR (at supine rest and standing) were measured as baseline characteristics, and third-degree family history of hypertension, diabetes, or obesity was investigated by interviews. All subjects underwent electrocardiogram (ECG) recording and power spectral analysis of HRV. However, HRV could not be determined for three subjects in the supine position, ten subjects in the standing position, and for one subject in both postures. The study protocol was reviewed by the appropriate institutional review committee of Kyoto University Graduate School of Human and Environmental Studies, and the guidelines of the Declaration of Helsinki (2000) of the World Medical Association were followed.

2.2. Genotyping

Genomic DNA was extracted from whole blood (DNA Extractor WB Kit; Wako, Osaka, Japan) or buccal cells (BuccalAmp™ DNA Extraction Kit; EPICENTRE Biotechnologies, Madison, WI). The *ESR1 PvuII* (c.454–397T/C: rs2234693) and *XbaI* (c.454–351A/G: rs9340799) polymorphisms were genotyped as previously described [32].

2.3. Electrocardiogram R–R interval power spectral analysis

Details of the HRV analysis methodology used have been well reviewed [23,24]. Each subject was studied in a quiet room at an ambient temperature of 25 °C. Subjects rested in a supine position for at least 20 min before ECG recording. The CM5 lead ECG was continuously recorded during supine rest and postural change to a standing position. After 10 min of supine rest, the subjects stood up by the bedside and remained at standing rest for another 10 min. During the test, the respiratory rate was controlled at 0.25 Hz (15 breaths/min) by means of an electric metronome to reduce significant variations in HRV spectral powers resulting from individual variations in breathing frequency and to avoid interference with the low frequency component by the parasympathetic component [33]. The R–R interval power spectral analysis procedures have been described previously [34]. Briefly, the ECG R–R interval data obtained from the CM5 lead was digitized at 1000 Hz, and the derived R–R interval time series were then aligned in a 2 Hz sequence for power spectral analysis. The DC component and linear trend were completely eliminated by digital filtering for a band-pass between 0.007 and 0.5 Hz. After passing through the Hamming-type data window, power spectral analysis was performed using a fast Fourier transform on the consecutive 480 s time series of R–R interval data obtained during the tests. The very low frequency (0.007–0.035 Hz, VLF), low frequency (0.035–0.15 Hz, LF), high frequency (0.15–0.5 Hz, HF), and total power (0.007–0.5 Hz) were evaluated by integrating the spectrum for the respective band width. The ratio of LF to HF (LF/HF) was also calculated as an index of sympathetic modulation [23,24]. The average HR in beats per minute in each position (supine rest and standing) was derived from the R waves of the ECG.

2.4. Statistical analysis

Hardy–Weinberg equilibrium was verified by comparison of the observed and expected genotype frequency using the χ^2 test. Linkage disequilibrium analysis was performed with the Haploview program [35]. In line with previous studies [23], a natural logarithmic transformation was used to normalize the distribution of HRV power spectral indices, as these data showed a distribution skewed to the right. After testing the homoscedasticity with the Levene's test, differences among the genotypes in clinical characteristics and log-transformed values (ln) of HRV indices were evaluated by Student's *t*-test, one-way ANOVA, or one-way ANCOVA, with Bonferroni's method for *post-hoc* multiple comparisons, where appropriate. For comparison of ln HRV indices, *P* values were provided after adjustment of age, BMI, and family history of hypertension, diabetes, or obesity. Data were expressed as mean ± SEM. The χ^2 test was performed for analysis of the relationship of genotype distributions to family history of hypertension, diabetes, or obesity. Statistical analyses were performed using the SPSS 11.0 software (SPSS, Chicago IL). Significant differences were considered to be present at $P<0.05$.

3. Results

3.1. Characteristics of the study subjects

The frequencies of the *ESR1 PvuII* C allele and the *XbaI* G allele were 36.5% and 17.3%, respectively, similar to those reported in the Japanese population [36]. There was no detectable deviation from the Hardy–Weinberg equilibrium for the *PvuII* T/C polymorphism ($\chi^2=0.15$, $P=0.701$), but there was a slight deviation for the *XbaI* A/G polymorphism ($\chi^2=3.92$, $P=0.048$). The clinical characteristics of the subjects were compared with reference to the *ESR1 PvuII* and *XbaI* genotype groups, as shown in Table 1. The *ESR1 PvuII* C allele carriers had a significantly higher mean BP (MBP) and a tendency for association with higher SBP and DBP than carriers of the TT genotype in the C allele dominant model (SBP $P=0.054$, DBP $P=0.057$). On the other hand, *XbaI* AA genotype carriers had a higher body weight compared with G allele carriers in the G allele dominant model. Additionally, homozygotes for the *XbaI* G allele had significantly higher DBP and MBP, but lower HR (supine rest and standing), compared with those of AA or AG genotype carriers.

3.2. ESR1 polymorphisms and HRV indices

The power spectral parameters of HRV with respect to the *ESR1 PvuII* or *XbaI* polymorphisms are shown in Table 2. As described [23,24], power spectral analysis of HRV generally shows two major distinct regions of periodicity in ECG R–R intervals: a HF component (>0.15 Hz) and a LF component (<0.15 Hz). Previous studies demonstrated that the HF component is mediated solely by parasympathetic nervous system activity, while the LF component arises from both sympathetic and parasympathetic nervous activities [23,24]. In addition, we evaluated a VLF component (<0.035 Hz) which is a powerful independent predictor

Table 1
Clinical characteristics of study subjects according to the genotypes of *ESR1 Pvu*II and *Xba*I polymorphisms.

Characteristics	*ESR1 Pvu*II T/C genotype			*P* value			*ESR1 Xba*I A/G genotype			*P* value		
	T/T	T/C	C/C	Codominant	Dominant	Recessive	A/A	A/G	G/G	Codominant	Dominant	Recessive
Number	103	114	35				177	63	12			
Age (year)	21.7±0.2	21.7±0.2	21.9±0.4	NS	NS	NS	21.8±0.2	21.1±0.2	22.8±1.0	**0.026**	NS	NS
Height (cm)	172.8±0.6	172.5±0.5	171.7±1.0	NS	NS	NS	172.8±0.4	172.1±0.7	170.9±1.8	NS	NS	NS
Body weight (kg)	62.7±0.9	63.6±0.8	61.1±1.1	NS	NS	NS	63.6±0.7	61.1±0.9	61.1±2.0	NS	**0.033**	NS
BMI (kg/m^2)	21.0±0.3	21.3±0.2	20.7±0.3	NS	NS	NS	21.3±0.2	20.6±0.3	20.9±0.6	NS	NS	NS
SBP (mm Hg)	109.8±0.9	111.9±0.8	112.8±1.7	NS	NS	NS	111.4±0.7	109.4±1.3	114.8±2.1	NS	NS	NS
DBP (mm Hg)	61.6±0.9	63.5±0.7	64.0±1.5	NS	NS	NS	62.9±0.7	61.3±0.9	67.9±2.1	**0.030**	NS	**0.020**
MBP (mm Hg)	77.7±0.8	79.7±0.7	80.3±1.4	NS	**0.028**	NS	79.1±0.6	77.3±0.8	83.6±1.8	**0.021**	NS	**0.020**
HR (supine rest, bpm)	61.2±0.8 (n=101)	61.2±0.8 (n=113)	60.2±1.7 (n=34)	NS	NS	NS	61.7±0.7 (n=175)	60.4±1.0 (n=62)	55.1±3.2 (n=11)	**0.039**	NS	**0.020**
HR (standing, bpm)	81.5±1.1 (n=99)	82.7±1.1 (n=108)	80.8±2.0 (n=35)	NS	NS	NS	82.2±0.8 (n=169)	82.6±1.4 (n=61)	74.3±3.9 (n=12)	**0.050**	NS	**0.015**
Family history of HT, DM, or obesity (%)	30.1	25.4	31.4	NS	NS	NS	27.7	28.6	33.3	NS	NS	NS

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; HT, hypertension; DM, diabetes mellitus.
Values are mean±SEM and were compared by one-way ANOVA or Student's *t*-test.
Chi-square test was performed for analysis of genotype distributions as to family history.
P values<0.05 are shown in boldface type.

of cardiac events in patients with congestive heart failure [25] and all-cause mortality in patients with myocardial infarction [29]. The ratio of the LF to HF component is considered as an index of sympatho-vagal balance or sympathetic activity [23,24].

There was no statistically significant association between the *ESR1 Pvu*II genotypes and HRV indices. On the other hand, in a supine rest position, VLF power was marginally lower in *Xba*I AA genotype carriers compared with G allele carriers in the dominant model, although statistical significance was not obtained ($P = 0.052$). In a standing position, there were no significant differences in HRV indices for both *ESR1* polymorphisms (data not shown).

3.3. *ESR1 PvuII–XbaI haplotype analysis*

A high degree of linkage disequilibrium was observed between the *ESR1 Pvu*II T/C and *Xba*I A/G polymorphisms ($|D'| = 0.978$), resulting in 3 common and 1 rare haplotypes: haplotype 1 (T–A) 63.3%, haplotype 2 (C–A) 19.5%, haplotype 3 (C–G) 17.0%, and one subject with haplotype 4 (T–G, 0.2%). We evaluated the association of *ESR1 Pvu*II–*Xba*I haplotype with clinical characteristics and HRV parameters. The prevalence of none, 1, or 2 copies of haplotype 2 (C–A) were 64.7%, 31.7%, and 3.6%, respectively. Clinical characteristics and HRV parameters of the subjects according to the *ESR1* haplotype 2 are presented in Table 3. As the number of subjects with the two copies of haplotype 2 was limited, statistical comparison was conducted according to the presence or absence of the copy of haplotype 2. Interestingly, the *ESR1* haplotype 2 was significantly associated with both clinical and HRV parameters. Carriers of 1 or 2 copies of haplotype 2 had higher SBP and MBP values compared with those with non-carriers.

HRV indices were compared with reference to the *ESR1* haplotype 2, and all of the HRV spectral powers at supine rest, i.e., total power, VLF, LF, and HF, were significantly lower in haplotype 2 carriers than in non-carriers, suggesting that simultaneous presence of the *ESR1 Pvu*II–*Xba*I haplotype 2 may be associated with reduced overall cardiac autonomic nervous system activity. Association with *ESR1* haplotype 1 and haplotype 3 was similar to those observed in the association with *ESR1 Pvu*II and *Xba*I polymorphisms, respectively.

4. Discussion

We demonstrated that *ESR1 Pvu*II and *Xba*I polymorphisms were associated with variation of BP and HRV in young healthy Japanese males. The *Pvu*II C allele and homozygote of the *Xba*I G allele were associated with relatively higher BP, while *Xba*I G allele carriers had significantly lower HR than non-carriers. Subjects with the *Xba*I AA genotype had marginally lower VLF power of HRV than G allele carries. Haplotype analysis revealed that carriers of the haplotype 2 (*Pvu*II C and *Xba*I A) allele had the lower HRV indices (total power, VLF, LF, and HF) with the higher BP (SBP and MBP) compared with those of non-carriers. These data suggest that the *ESR1 Pvu*II and *Xba*I polymorphisms or haplotype may play a role in BP variation, as well as the attenuation of cardiac autonomic nervous activity.

ERα has been identified in the brain stem centers involved in autonomic cardiovascular regulation [18]. Administered estrogen

Table 2
Power spectral parameters of HRV at supine rest according to the genotypes of *ESR1 Pvu*II and *Xba*I polymorphisms.

Parameters	*ESR1 Pvu*II T/C genotype			*P* value			*ESR1 Xba*I A/G genotype			*P* value		
	T/T	T/C	C/C	Codominant	Dominant	Recessive	A/A	A/G	G/G	Codominant	Dominant	Recessive
Number	101	113	34				175	62	11			
Total power, ln ms^2 (geometric mean)	7.56±0.09 (1916.4)	7.46±0.09 (1736.3)	7.45±0.12 (1715.8)	NS	NS	NS	7.44±0.07 (1707.3)	7.64±0.09 (2088.6)	7.56±0.17 (1911.3)	NS	NS	NS
VLF, ln ms^2 (geometric mean)	6.36±0.10 (578.1)	6.35±0.09 (570.9)	6.20±0.13 (494.8)	NS	NS	NS	6.26±0.08 (522.8)	6.49±0.10 (659.1)	6.61±0.19 (743.7)	NS	NS	NS
LF, ln ms^2 (geometric mean)	6.51±0.10 (673.0)	6.38±0.09 (589.4)	6.38±0.14 (591.3)	NS	NS	NS	6.39±0.08 (593.5)	6.57±0.11 (710.4)	6.44±0.22 (628.3)	NS	NS	NS
HF, ln ms^2 (geometric mean)	6.21±0.09 (499.6)	6.05±0.10 (426.0)	6.17±0.15 (480.5)	NS	NS	NS	6.08±0.07 (437.7)	6.31±0.11 (549.1)	6.03±0.23 (415.7)	NS	NS	NS
LF/HF, ln (geometric mean)	0.30±0.07 (1.35)	0.32±0.07 (1.38)	0.21±0.14 (1.23)	NS	NS	NS	0.30±0.06 (1.36)	0.26±0.10 (1.29)	0.41±0.24 (1.51)	NS	NS	NS

VLF, very low frequency, LF, low frequency; HF, high frequency; LF/HF indicates ratio of LF to HF power.
Values are mean±SEM; geometric means are given between parentheses.
P values were obtained by ANCOVA adjusted for age, BMI, and family history of hypertension, diabetes, or obesity.

Table 3
Association of *ESR1 Pvu*II–*Xba*I C–A haplotype with clinical characteristics and HRV parameters at supine rest.

	Copies of *ESR1* haplotype 2 (C–A)		
	No copies	1 or 2 copies	*P* value
Clinical characteristics			
Number	163	89	
Age (year)	21.6 ± 0.2	21.9 ± 0.3	0.442
BMI (kg/m^2)	20.9 ± 0.2	21.5 ± 0.3	0.060
SBP (mm Hg)	110.0 ± 0.7	113.2 ± 1.0	**0.009**
DBP (mm Hg)	62.2 ± 0.7	63.9 ± 0.9	0.128
MBP (mm Hg)	78.1 ± 0.6	80.3 ± 0.8	**0.028**
HR (supine rest, bpm)	60.5 ± 0.7 (n = 159)	62.1 ± 1.0 (n = 89)	0.154
HR (standing, bpm)	81.4 ± 0.9 (n = 157)	82.9 ± 1.2 (n = 85)	0.310
Family history of HT, DM, or obesity (%)	30.1	24.7	0.368
HRV parameters			
Number	159	89	
Total power, ln ms^2 (geometric mean)	7.60 ± 0.06 (1989.9)	7.32 ± 0.10 (1515.4)	**0.017**
VLF, ln ms^2 (geometric mean)	6.44 ± 0.07 (626.5)	6.14 ± 0.11 (464.4)	**0.020**
LF, ln ms^2 (geometric mean)	6.54 ± 0.08 (693.3)	6.24 ± 0.11 (513.3)	**0.024**
HF, ln ms^2 (geometric mean)	6.23 ± 0.07 (506.2)	5.97 ± 0.12 (392.7)	**0.042**
LF/HF, ln (geometric mean)	0.31 ± 0.06 (1.37)	0.27 ± 0.08 (1.31)	0.717

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; HT, hypertension; DM, diabetes mellitus.
VLF, very low frequency, LF, low frequency; HF, high frequency; LF/HF indicates ratio of LF to HF power.
Values are mean ± SEM; geometric means are given between parentheses in HRV parameters.
Differences in clinical characteristics were compared by Student's *t*-test.
Chi-square test was performed for analysis of genotype distributions as to family history.
P values in HRV parameters were obtained by ANCOVA adjusted for age, BMI, and family history of HT, DM, or obesity.
P values < 0.05 are shown in boldface type.

can directly affect the autonomic preganglionic cells in the CNS, and estrogen modulates autonomic nervous activity via the CNS [18]. Previous studies suggest that the cardiovascular protective effects of the ERα may be explained, at least in part, by improvement in cardiac autonomic functions [19–22]. Estrogen supplementation can increase vagal activity reflected in increased HRV spectral power, and administration of 17-β-estradiol has been shown to increase HRV in postmenopausal women [19–22]. In the present study, the *ESR1 Xba*I AA genotype and *ESR1* haplotype 2 (C–A) were significantly associated with lower VLF in HRV and overall HRV indices, respectively, indicating that the *ESR1* polymorphisms may contribute to reduced cardiac autonomic control. Thus, the contribution of the *ESR1* polymorphisms to increased risk of CVD may be partly explained by its association with impairment of the cardiac autonomic nervous system. Impaired autonomic function is closely associated with the pathophysiology of hypertension and CVD [37,38]. Reduced HRV or HRV spectral indices (VLF, LF, and HF) are associated with an increased risk of cardiac or overall mortality [25,27,29,30], and predict risk of the future onset of CVD [30]. Among normotensive healthy subjects, lower HRV was also associated with a greater risk of developing CVD or hypertension [28,30]. Thus, in the present study the subjects with cardiac autonomic dysfunction characterized by low HRV may be already at risk for pathogenesis of CVD. It should be considered, however, lifestyle factors such as alcohol intake and smoking habit can modify cardiac autonomic nervous activity. Indeed, previous studies have indicated the relation of drinking or smoking to HRV modulation [39,40]. Thus, these environmental factors might modify the association between the *ESR1* polymorphisms and cardiac autonomic nervous activity.

ERα are expressed in cardiomyocytes and have a cardioprotective function through direct action on cardiovascular tissues and also by systemic effects [1,4], these data are partly supported by the case of 31 year-old man with a disruptive mutation of *ESR1* who exhibits endothelial dysfunction and coronary artery disease [41,42]. Genetic variations in *ESR1*, especially the *Pvu*II (c.454–397T/C: rs2234693) and *Xba*I (c.454–351A/G: rs9340799) polymorphisms, are linked to the severity and risk of cardiovascular diseases such as myocardial infarction, heart failure, and ischemic heart diseases in prospective, cross-sectional, or retrospective studies [6,7]. In the Framingham Heart Study, of 1739 unrelated males and females, there was an association between *Pvu*II CC genotypes and an increase of atherothrombotic cardiovascular disease in males [10]. In addition, the *ESR1 Pvu*II T>C variation was reported to be associated with an increased risk of myocardial infarction [9], coronary artery disease [12], and stroke [17] in males. The *ESR1 Xba*I polymorphism was also reported to be associated with cardiovascular diseases and related pathological phenotypes such as an increased risk of myocardial infarction and ischemic heart disease in postmenopausal females [8] and onset of stroke in males [16]. A more recent study by Lazaros et al. showed that the *Xba*I AA genotype was associated with the onset of stroke at a younger age in male patients with metabolic syndrome [16]. Thus, our data provide indirect support for the concept that *ESR1 Pvu*II and *Xba*I polymorphisms may modify the cardiovascular health, resulting in various pathological phenotypes including attenuation of cardiac autonomic nervous activity.

The present study showed a significant association between *ESR1* polymorphisms and BP variation in young healthy males. The *ESR1 Pvu*II C allele was associated with higher SBP, DBP, and MBP than for TT carriers, whereas homozygous for the *Xba*I G allele was associated with higher DBP and MBP. Furthermore, haplotype analysis demonstrated that haplotype 2 (C–A) carriers had a higher SBP and MBP than non-carriers. Several reports suggest an important role for estrogen in the regulation of vasodilation and BP [21,31,43]. Administration of estrogen can improve vasodilation in postmenopausal females [43], while aromatase inhibition that lowers estrogen levels induces a substantial reduction in flow-mediated vasodilation in young healthy men [44], suggesting a role of estrogen in vasodilation in both sexes. In the present study, the peripheral vasodilator effects of estrogen through the ERα may have contributed to the observed decreased BP, and indeed, estrogen has been previously shown to reduce SBP and DBP in males [31]. An association between *ESR1* polymorphisms and blood pressure has also been previously demonstrated, where *Pvu*II CC carriers had higher SBP and pulse pressure than non-carriers in males [14], while there was an association of the *Pvu*II T allele with higher SBP in healthy males, but not in females [13]. Thus, even though these data require further confirmation in large-scale study, our results provide suggestive evidence for a role of *ESR1 Pvu*II and *Xba*I polymorphisms in BP variation.

There are some limitations in the present study. First, our study subjects were composed of only young Japanese males, and therefore, our conclusions cannot be applied to females and other racial/ethnic group. Previous studies have shown that normal ERα function is required for normal cardiovascular development in males [2,3,41,42]. Thus, a modest effect of *ESR1* polymorphisms on ERα function may have larger clinical importance under conditions of very low levels of circulating estrogens, such as in males. Second, although the *Xba*I GG genotype was significantly associated with higher BP but lower HR, there was a slight deviation from the Hardy–Weinberg equilibrium for *Xba*I polymorphism, with only 12 carriers of the *Xba*I GG genotype due to the low abundance of the *Xba*I G allele. Further large-scale studies are required to replicate our preliminary observations in other populations. Third, exact functional mechanism of the *ESR1 Pvu*II and *Xba*I haplotypes in relation to related pathological phenotypes is uncertain, as both polymorphisms are present in the intronic region. As such, we cannot exclude the possibility that both *Pvu*II and *Xba*I polymorphisms may be in linkage disequilibrium with other functional

genetic variants affecting the expression or functional properties of ERα. Finally, we do not completely exclude the possibility that the reported findings may be false-positive because of the multiple testing (2 genetic markers, 5HRV indices, and 2 postural positions). However, the *ESR1 Pvu*II and *Xba*I polymorphisms are in strong linkage disequilibrium, and HRV measures are correlated. Therefore, in this case, each new test would not have provided a completely independent opportunity for a type I error, and the Bonferroni correction for multiple testing would have been too conservative [45]. In the present study, the simple original statistical results are adopted with the aim of reporting some potentially important associations that are likely to be worthwhile pursuing further.

In conclusion, this study demonstrated an association of the *ESR1 Pvu*II and *Xba*I genotype or haplotype with HRV in young healthy males, suggesting an important role of *ESR1* genetic variation in cardiac autonomic nervous activity. These observations support the hypothesis that the *ESR1 Pvu*II and *Xba*I polymorphisms are potentially clinically important for risk of CVD in males.

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Association of *UCP2* and *UCP3* polymorphisms with heart rate variability in Japanese men

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Objectives **The mitochondrial uncoupling proteins UCP2 and UCP3 are implicated in energy metabolism and regulation of reactive oxygen species, which are closely involved in autonomic nervous system function. Heart rate variability (HRV) reflects cardiac autonomic regulation and has been used to evaluate dysfunction of the autonomic nervous system in hypertension and cardiovascular diseases. We examined the association between polymorphisms in the *UCP2* and *UCP3* genes and HRV in healthy young Japanese men.**

Methods **The 45 bp insertion/deletion polymorphism in exon8 of *UCP2* and the −55C/T polymorphism in the *UCP3* promoter region were genotyped ($n = 255$). Cardiac autonomic function was evaluated by power spectral analysis of HRV during supine rest and in a standing position. Low-frequency (<0.15 Hz) and high-frequency (>0.15 Hz) components of HRV were quantified by frequency domain analysis.**

Results **The I/I genotype of the *UCP2* 45 bp insertion/deletion polymorphism was associated with relatively higher blood pressure and HRV sympathetic indices (low frequency percentage and low frequency:high frequency ratio) at supine rest. For the −55C/T polymorphism of *UCP3*, individuals carrying the −55T allele had significantly lower HRV sympathetic indices, but higher HRV parasympathetic indices (high frequency and high frequency percentage), than carriers of the C/C genotype at standing. Both *UCP2* and *UCP3* polymorphisms were significantly associated with a third-degree family history of hypertension, diabetes, and obesity. Additionally, carriers of the *UCP2* 45 bp I allele −*UCP3* −55C/C combined genotype had the lowest HRV sympathetic and the highest HRV parasympathetic indices at standing among the combined genotypes.**

Conclusion ***UCP2*** **and *UCP3* polymorphisms were associated with HRV in young and healthy states, suggesting a significant relationship between autonomic cardiovascular regulation and UCP2/UCP3 polymorphisms.** *J Hypertens* **27:305–313 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: autonomic nervous system, heart rate variability, polymorphism, UCP2, UCP3

Abbreviations: ACR, urinary albumin-to-creatinine ratio; AI, augmentation index; AIc, aortic (central arterial) augmentation index; AIp, peripheral augmentation index; APOGH, African Project on Genes on Hypertension; BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; DM, diabetes mellitus; E/A, early-to-late transmitral velocity; HbA_{1c}, glycated haemoglobin; LV, left ventricular; LVM, left ventricular mass; LVMI, left ventricular mass indexed to height$^{2.7}$; MWT, mean wall thickness; PWV, carotid-femoral pulse wave velocity; SBP, systolic blood pressure; TOD, target organ changes

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Introduction

Uncoupling proteins (UCPs) are a family of mitochondrial transporters that can uncouple oxidative phosphorylation through a dissipation of the proton gradient across the inner mitochondrial membrane. Apart from the roles of both UCPs in energy and fatty acid metabolism [1,2], UCP2 and UCP3 are also involved in preventing the formation of reactive oxygen species (ROS) [3,4]. ROS are produced by the mitochondria during mitochondrial respiration, and lowering of the proton gradient across the inner mitochondrial membrane by UCPs results in a reduction in ROS generation [3,4]. Indeed, superoxide or ROS production was increased in macrophages, pancreatic islets, or skeletal muscle mitochondria from UCP2-deficient or UCP3-deficient mice, respectively [5–7].

UCP2 is widely distributed in human tissues (e.g. adipose tissue, skeletal muscle, heart, placenta, liver, kidney, pancreas, and brain), and UCP3 is predominantly expressed in the skeletal muscle and the heart, whereas both UCPs are also expressed in the central nervous system (CNS) or dorsal root ganglion [8,9]. It has been shown that UCP2 plays neuromodulatory/neuroprotective roles in the CNS, and UCP3 protects neurons from glucose-induced degeneration by preventing ROS

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formation [8,9]. Recent studies have demonstrated that ROS have a sympathoexcitatory effect at the central, cardiac, and peripheral tissues [10,11]. These data suggest a possible association between UCPs and autonomic nervous system (ANS) activity through ROS regulation.

Many genetic polymorphisms have been identified in the *UCP2* and *UCP3* genes (http://www.ncbi.nlm.nih.gov/projects/SNP/). Of these, a 45 bp insertion/deletion polymorphism (rs1800795) in the 3′ untranslated region (UTR) of exon 8 in *UCP2* and a −55C/T polymorphism (rs1800849) in the *UCP3* promoter region have been intensively studied in association with various pathological phenotypes [12,13]. The biological or functional relevance of both polymorphisms of *UCPs* have been previously shown. The *UCP2* 45 bp insertion/deletion polymorphism was reported to be related to metabolic rate [14] and increased BMI [15–17], whereas the *UCP3* −55T allele was associated with increased expression of *UCP3* mRNA in the skeletal muscle [18]. However, the (patho)physiological impact of both polymorphisms remains unknown.

Heart rate variability (HRV) analysis provides useful indirect markers of cardiac autonomic modulation and is an early and sensitive indicator of the pathological states of hypertension or cardiovascular disease (CVD) [19–22]. Impairment of autonomic balance is closely involved in the pathophysiology of hypertension and CVD [23,24]. Using HRV analysis, sympathovagal imbalance, especially sympathetic predominance or decreased vagal activity, was often observed in patients with hypertension or CVD [25–28], whereas some HRV indices could predict the future onset of these diseases [20,21] and cardiac or all-cause mortality after myocardial infarction [29–32]. Recent reports suggest that heritable variations may play critical roles in ANS function as reflected in HRV [33–35]. Although UCP2 and UCP3 are involved in the ANS either directly by effects on autonomic pathway or indirectly through ROS regulation, the contribution of genetic polymorphisms of *UCPs* to ANS function remains unclear. Thus, in the present study, we assessed the association of *UCP2* 45 bp insertion/deletion and *UCP3* −55C/T polymorphisms with cardiac ANS by HRV analysis in young Japanese men.

Methods

Participants

Two hundred and fifty-five young, healthy Japanese men, recruited at random from Kyoto University, participated in each examination after written informed consent was obtained. The ages of participants ranged from 18 to 28 years (21.5 ± 0.1 years). All participants were normotensive [causal supine blood pressure (BP) <140/90 mmHg] and nonobese (BMI <30 kg/m^2). It was determined by interview that participants were not taking any medication and had no history of organic diseases such as CVD, metabolic disorder, renal disease, or neuropathy. BMI, BP [systolic BP (SBP) and diastolic BP (DBP)], and heart rate (HR) (at supine rest and standing) were measured as baseline characteristics, and family history (including whether participants had relatives within the third degree who had hypertension, diabetes, or obesity) was investigated by interviews. All participants underwent ECG recording and power spectral analysis of HRV. However, HRV could not be determined for three participants in the supine position, 10 participants in the standing position, and for one participant in both postures. The study protocol was reviewed by the appropriate institutional review committee of Kyoto University Graduate School of Human and Environmental Studies, and the guidelines of the Declaration of Helsinki were followed.

Genotyping

Genomic DNA was extracted from whole blood (DNA Extractor WB Kit; Wako, Osaka, Japan) or buccal cells (BuccalAmpTM DNA Extraction Kit; EPICENTRE Biotechnologies, Madison, Wisconsin, USA). *UCP2* 45 bp insertion/deletion and *UCP3* −55C/T polymorphisms were genotyped as previously described [14,36]. The genotype of the *UCP2* 45 bp insertion/deletion polymorphism of one participant could not be determined because of an inadequate sample of buccal cells.

ECG R–R interval power spectral analysis

Details of the HRV analysis methodology used have been well reviewed [37,38]. Each participant was studied in a quiet room at an ambient temperature of 25°C. Participants rested in a supine position for at least 20 min before ECG recording. CM5 lead ECG was continuously recorded during supine rest and postural change to a standing position. After 10 min of supine rest, participants stood up by the bedside and remained at standing rest for another 10 min. During the test, the respiratory rate was controlled at 0.25 Hz (15 breaths/min) by means of an electric metronome to reduce significant variations in HRV spectral powers resulting from individual variations in breathing frequency and to avoid interference with the low-frequency component by the parasympathetic component [39]. The R–R interval power spectral analysis procedures have been described previously [34,35]. Briefly, ECG R–R interval data obtained from the CM5 lead were digitized at 1000 Hz, and the derived R–R interval time series were then aligned in a 2 Hz sequence for power spectral analysis. Direct current component and linear trend were completely eliminated by digital filtering for a band-pass between 0.007 and 0.5 Hz. After passing through the Hamming-type data window, power spectral analysis was performed using a fast Fourier transform on the consecutive 480-s time series of R–R interval data obtained during the tests. Very low frequency (VLF, 0.007–0.035 Hz), low frequency (0.035–0.15 Hz), high frequency (0.15–0.5 Hz),

and total power (0.007–0.5 Hz) were evaluated by integrating the spectrum for the respective bandwidth. Low-frequency and high-frequency powers were expressed in both absolute units (ms^2) and in normalized units (%). The normalized low-frequency or high-frequency powers were calculated as follows: low-frequency power (%) = (low-frequency/total power − VLF) × 100 and high-frequency power (%) = (high-frequency/total power − VLF) × 100 [25]. The ratio of low-frequency to high-frequency powers (low-frequency power/high-frequency power) was also calculated as an index of sympathetic modulation [37,38]. The average HR in beats per minute in each position (supine rest/standing) was derived from the R waves of the ECG.

Statistical analysis

Hardy–Weinberg equilibrium was verified by comparison of the observed and expected genotype frequency using the χ^2 test. Linkage disequilibrium analysis was performed with the Haploview program [40]. In line with previous studies [25,37], a natural logarithmic transformation was used to normalize the distribution of HRV power spectral indices, as these data showed a distribution skewed to the right. Differences in clinical characteristics and log-transformed values (ln) of HRV indices were evaluated by Student's *t*-test or one-way analysis of variance with Bonferroni's method for post-hoc multiple comparisons, whenever appropriate. For comparison of HRV indices, adjusted *P* values were also provided after adjustment of potential confounding factors (age, BMI, and family history of hypertension, diabetes, or obesity). Data were expressed as mean ± standard error of mean. The χ^2 test was performed for analysis of the relationship of genotype distributions with family history of hypertension, diabetes, or obesity. Statistical analysis was performed using the Statview Statistical Package (SAS Institute Inc.; Cary, North Carolina, USA). Significant differences were considered to be present at a *P* value of less than 0.05.

Results

Characteristics of study participants

The general clinical characteristics of study participants are shown in Table 1. All participants were in good health with almost ideal BMI and BP. Frequencies of the *UCP2* 45 bp I allele and the *UCP3* −55T allele were 20.7 and 31.0%, respectively, similar to that reported previously in the Japanese population [41,42]. There were no significant deviations from the Hardy–Weinberg equilibrium for *UCP2* 45 bp insertion/deletion ($\chi^2 = 1.45$, $P = 0.23$) or *UCP3* −55C/T ($\chi^2 = 1.04$, $P = 0.31$) polymorphisms.

The clinical characteristics of study participants classified by the *UCP2* 45 bp insertion/deletion or the *UCP3* −55C/T polymorphisms are shown in Table 2. Carriers of the *UCP2* 45 bp I/I genotype had significantly higher SBP and DBP than the 45 bp D/D or insertion/deletion carriers. A significantly higher third-degree family history of hypertension, diabetes, and obesity were also observed for the 45 bp I allele carriers of *UCP2*. On the contrary, the *UCP3* −55T allele carriers had significantly lower BMI than carriers of the C/C genotype, and body weight also tended to be relatively lower in the −55T allele carriers. Although not statistically significant, the −55T allele carriers tended to have lower mean BP than the C/C carriers. Additionally, a significantly less common family history of hypertension, diabetes, or obesity was seen for carriers of the −55T allele.

Table 1 Clinical characteristics of study participants ($n = 255$)

Characteristics	Mean ± SEM
Age (years)	21.5 ± 0.1
Height (cm)	172.4 ± 0.3
Body weight (kg)	62.5 ± 0.5
BMI (kg/m^2)	21.0 ± 0.2
SBP (mmHg)	111.2 ± 0.6
DBP (mmHg)	63.0 ± 0.6
MBP (mmHg)	79.0 ± 0.5
HR (supine rest, bpm; $n = 251$)	61.0 ± 0.5
HR (standing, bpm; $n = 244$)	81.4 ± 0.7
Third-degree family history of HT, DM, or obesity (%)	27.1

bpm, beats per minute; DBP, diastolic blood pressure; DM, diabetes mellitus; HR, heart rate; HT, hypertension; MBP, mean blood pressure; SBP, systolic blood pressure; SEM, standard error of mean.

Association of UCP2 and UCP3 polymorphisms with heart rate variability indices

Power spectral parameters of HRV according to the *UCP2* 45 bp insertion/deletion or the *UCP3* −55C/T polymorphisms are shown in Tables 3 and 4. As described [37,38], power spectral analysis of HRV has generally shown two major distinct regions of periodicity in ECG R–R intervals: a high-frequency component (>0.15 Hz) and a low-frequency component (<0.15 Hz). Previous studies have shown that the high-frequency component is mediated solely by parasympathetic nervous system activity, and that the low-frequency component arises from both sympathetic and parasympathetic nervous activities [37,38]. In addition, the ratio of the low-frequency to the high-frequency component has been considered as an index of sympathovagal balance or sympathetic activity [37,38]. Low-frequency and high-frequency components of HRV were quantified by frequency domain analysis and expressed in absolute and normalized units.

At supine rest, carriers of the *UCP2* 45 bp I/I genotype had significantly higher HRV sympathetic indices (low frequency percentage and low frequency/high frequency) and a lower parasympathetic index (high frequency percentage) than 45 bp insertion/deletion carriers, although there was no significant difference when compared with the 45 bp D/D genotype. For the −55C/T polymorphism of *UCP3*, in a standing position, the *UCP3* −55T allele carriers had a significantly higher high frequency and high frequency percentage than the C/C carriers. On the

Table 2 Clinical characteristics of study participants according to the genotypes of *UCP2* or *UCP3* polymorphisms

	UCP2 45 bp I/D genotype			*P*			*UCP3* −55 C/T genotype			*P*		
Characteristics	D/D	I/D	I/I	Codominant	Dominant	Recessive	C/C	C/T	T/T	Codominant	Dominant	Recessive
Number	163	77	14				118	116	21			
Age (years)	21.4 ± 0.2	21.7 ± 0.3	21.9 ± 0.7	0.506	0.259	0.514	21.7 ± 0.2	21.4 ± 0.2	21.5 ± 0.5	0.703	0.405	0.904
Height (cm)	172.0 ± 0.4	173.2 ± 0.6	172.3 ± 1.2	0.317	0.159	0.938	172.5 ± 0.5	172.0 ± 0.5	173.7 ± 1.3	0.390	0.740	0.235
Body weight (kg)	62.3 ± 0.6	63.2 ± 1.0	62.0 ± 2.2	0.725	0.522	0.802	63.6 ± 0.8	61.4 ± 0.7	62.5 ± 1.6	0.142	0.056	0.969
BMI (kg/m^2)	21.0 ± 0.2	21.0 ± 0.3	20.9 ± 0.8	0.990	0.955	0.884	21.4 ± 0.2	20.7 ± 0.2	20.7 ± 0.4	0.116	**0.038**	0.482
SBP (mmHg)	110.3 ± 0.7	111.7 ± 1.1	117.3 ± 2.9	**0.014**	0.051	**0.007**	112.3 ± 1.0	110.1 ± 0.8	110.7 ± 2.2	0.201	0.079	0.829
DBP (mmHg)	62.4 ± 0.7	63.3 ± 1.2	66.6 ± 1.3	0.166	0.193	0.080	64.0 ± 0.8	62.4 ± 0.8	61.2 ± 1.6	0.238	0.110	0.317
MBP (mmHg)	78.4 ± 0.6	79.5 ± 1.1	83.5 ± 1.6	**0.038**	0.082	**0.017**	80.1 ± 0.8	78.3 ± 0.7	77.7 ± 1.6	0.164	0.060	0.401
HR (supine rest, bpm)	61.3 ± 0.7 (n = 160)	61.1 ± 0.9 (n = 76)	57.8 ± 2.4 (n = 14)	0.363	0.536	0.157	61.3 ± 0.8 (n = 115)	60.7 ± 0.9 (n = 115)	60.8 ± 1.9 (n = 21)	0.876	0.608	0.923
HR (standing, bpm)	82.2 ± 0.9 (n = 156)	80.4 ± 1.1 (n = 73)	78.1 ± 2.7 (n = 14)	0.231	0.125	0.219	81.5 ± 0.9 (n = 115)	80.8 ± 1.1 (n = 111)	84.2 ± 1.6 (n = 18)	0.442	0.871	0.238
Family history of HT, DM, or obesity (%)	22.7	33.8	42.9	0.079	**0.032**	0.175	33.1	20.7	28.6	0.103	**0.046**	0.871

Values are mean ± SEM and were compared by one-way ANOVA or Student's *t*-test. Chi-square test was performed for analysis of genotype distributions as to family history. *P* values of less than 0.05 are shown in boldface type. ANOVA, analysis of variance; bpm, beats per minute; DBP, diastolic blood pressure; DM, diabetes mellitus; HR, heart rate; HT, hypertension; MBP, mean blood pressure; SBP, systolic blood pressure; SEM, standard error of mean.

Table 3 Power spectral parameters of heart rate variability according to the genotypes of *UCP2* 45 bp insertion/deletion polymorphism

	UCP2 45 bp I/D genotype			*P*			Adjusted *P*		
Parameters	D/D	I/D	I/I	Codominant	Dominant	Recessive	Codominant	Dominant	Recessive
Supine rest									
Number	160	76	14						
LF, ln ms^2 (geometric mean)	6.43 ± 0.07 (618.7)	6.25 ± 0.12 (517.6)	6.72 ± 0.30 (828.7)	0.179	0.412	0.191	0.172	0.325	0.230
LF (%)	56.6 ± 1.3	52.6 ± 2.0	63.6 ± 3.7	**0.041**	0.295	0.067	**0.038**	0.207	0.089
HF, ln ms^2 (geometric mean)	6.14 ± 0.08 (462.0)	6.13 ± 0.11 (459.5)	6.10 ± 0.29 (447.0)	0.992	0.936	0.905	0.992	0.931	0.907
HF (%)	43.4 ± 1.3	47.4 ± 2.0	36.4 ± 3.7	**0.041**	0.295	0.067	**0.038**	0.207	0.089
LF/HF, ln (geometric mean)	0.29 ± 0.06 (1.34)	0.12 ± 0.09 (1.13)	0.62 ± 0.17 (1.85)	**0.039**	0.323	0.059	**0.037**	0.228	0.078
Standing									
Number	156	73	14						
LF, ln ms^2 (geometric mean)	6.26 ± 0.06 (523.2)	6.18 ± 0.08 (484.6)	6.49 ± 0.15 (659.3)	0.332	0.779	0.199	0.333	0.624	0.243
LF (%)	83.9 ± 1.0	85.6 ± 1.0	90.0 ± 1.0	0.090	0.089	0.062	0.131	0.121	0.085
HF, ln ms^2 (geometric mean)	4.38 ± 0.08 (79.8)	4.25 ± 0.09 (70.3)	4.23 ± 0.18 (68.6)	0.565	0.286	0.660	0.553	0.262	0.673
HF (%)	16.1 ± 1.0	14.4 ± 1.0	10.0 ± 1.0	0.090	0.089	0.062	0.131	0.121	0.085
LF/HF, ln (geometric mean)	1.88 ± 0.07 (6.59)	1.93 ± 0.08 (6.89)	2.26 ± 0.11 (9.61)	0.218	0.330	0.091	0.278	0.401	0.119

LF (%) and HF (%) indicate percentage of LF or HF power in the range between 0.035 and 0.05 Hz (see methods for details). LF/HF indicates ratio of LF to HF power. Values are mean ± SEM; geometric means are given between parentheses. *P* values were obtained by one-way ANOVA or Student's *t*-test. Adjusted *P* values were calculated after adjusting for age, BMI, and family history of hypertension, diabetes, or obesity. *P* values <0.05 are shown in boldface type. ANOVA, analysis of variance; HF, high frequency; I/D, insertion/deletion; LF, low frequency; SEM, standard error of mean.

Table 4 Power spectral parameters of heart rate variability according to the genotypes of *UCP3* −55C/T polymorphism

	UCP3 −55C/T genotype			*P*			Adjusted *P*		
Parameters	C/C	C/T	T/T	Codominant	Dominant	Recessive	Codominant	Dominant	Recessive
Supine rest									
Number	115	115	21						
LF, ln ms^2 (geometric mean)	6.45 ± 0.10 (635.9)	6.31 ± 0.09 (551.3)	6.50 ± 0.21 (663.4)	0.473	0.354	0.608	0.530	0.398	0.629
LF (%)	57.3 ± 1.5	54.5 ± 1.6	55.1 ± 3.3	0.419	0.191	0.839	0.408	0.182	0.792
HF, ln ms^2 (geometric mean)	6.13 ± 0.09 (459.3)	6.11 ± 0.08 (451.9)	6.27 ± 0.20 (530.3)	0.777	0.945	0.484	0.775	0.870	0.475
HF (%)	42.7 ± 1.5	45.5 ± 1.6	44.9 ± 3.3	0.419	0.191	0.839	0.408	0.182	0.792
LF/HF, ln (geometric mean)	0.33 ± 0.07 (1.38)	0.20 ± 0.07 (1.22)	0.22 ± 0.15 (1.25)	0.415	0.187	0.820	0.402	0.178	0.773
Standing									
Number	115	111	18						
LF, ln ms^2 (geometric mean)	6.26 ± 0.07	6.22 ± 0.07 (502.2)	6.40 ± 0.16 (604.7)	0.592	0.872	0.351	0.659	0.935	0.369
LF (%)	86.9 ± 0.8	82.5 ± 1.2	85.1 ± 1.7	**0.009**	**0.003**	0.916	**0.007**	**0.002**	0.974
HF, ln ms^2 (geometric mean)	4.19 ± 0.08 (66.1)	4.44 ± 0.09 (84.5)	4.56 ± 0.15 (95.3)	0.069	**0.024**	0.271	**0.034**	**0.011**	0.236
HF (%)	13.1 ± 0.8	17.5 ± 1.2	14.9 ± 1.7	**0.009**	**0.003**	0.916	**0.007**	**0.002**	0.974
LF/HF, ln (geometric mean)	2.07 ± 0.07 (7.91)	1.78 ± 0.08 (5.94)	1.85 ± 0.13 (6.35)	**0.022**	**0.006**	0.679	**0.016**	**0.004**	0.590

LF (%) and HF (%) indicate percentage of LF or HF power in the range between 0.035 and 0.05 Hz (see methods for details). LF/HF indicates ratio of LF to HF power. Values are mean ± SEM; geometric means are given between parentheses. *P* values were obtained by one-way ANOVA or Student's *t*-test. Adjusted *P* values were calculated after adjusting for age, BMI, and family history of hypertension, diabetes, or obesity. *P* values of less than 0.05 are shown in boldface type. ANOVA, analysis of variance; HF, high frequency; LF, low frequency; SEM, standard error of mean.

contrary, both low frequency percentage and low frequency/high frequency of the −55T allele carriers were significantly lower than those of the C/C carriers. These results suggested that the −55T allele was associated with a relatively lower sympathetic and a higher parasympathetic activity upon postural change to standing. These differences remained significant after adjustment for age, BMI, and family history of hypertension, diabetes, or obesity.

Combined effects of UCP2 and UCP3 polymorphisms

Modest linkage disequilibrium was seen between the *UCP2* 45 bp insertion/deletion and the *UCP3* −55C/T polymorphisms ($|D'| = 0.74$). Table 5 shows the combined effects of both polymorphisms on clinical characteristics. The comparison was conducted among four combined *UCP2–UCP3* genotype groups according to participants being with or without the *UCP2* 45 bp I allele or the *UCP3* −55 T allele (I allele + C/C, I allele + T allele, D/D + C/C, D/D + T allele). No significant interaction of *UCP2* and *UCP3* polymorphisms with BMI and BP was found. However, carriers of the *UCP2* 45 bp I allele–*UCP3* −55C/C combined genotype had the highest family history of hypertension, diabetes, and obesity among the combined genotype groups. On the other hand, some HRV parameters were significantly different between the groups (Table 6). In a standing position, carriers of the *UCP2* 45 bp D/D–*UCP3* −55T allele had the lowest sympathetic indices (low frequency percentage and low frequency/high frequency), with an accompanying increased parasympathetic index (high frequency percentage), suggesting that *UCP2* and *UCP3* polymorphisms have an additive effect on HRV.

Discussion

In the present study, in young Japanese men, we found a significant association of the *UCP2* 45 bp insertion/deletion or the *UCP3* −55C/T polymorphisms with HRV, indicating the contribution of both *UCP* polymorphisms to cardiac autonomic regulation. Autonomic imbalances are significantly involved in the pathophysiology of metabolic syndrome, CVD, and hypertension [43,44]. Previous studies have shown a sympathetic predominance reflected in increased HRV sympathetic indices (low frequency percentage or low frequency/high frequency) or reduced HRV parasympathetic indices (high frequency, or high frequency percentage) in patients with hypertension or CVD [25–28]. In this context, pharmacological interventions (beta-blocker treatment) in patients with cardiac diseases have been shown to improve cardiac autonomic balance in which HRV sympathetic indices were reduced [45,46]. In the present study, participants carrying the *UCP3* −55T allele showed lower HRV sympathetic indices and higher HRV parasympathetic indices than carriers of the C/C genotype in a standing position, which was independent of age, BMI, and the family history of hypertension,

Table 5 Association of combined *UCP2–UCP3* genotypes with clinical characteristics

Characteristics	*UCP2–UCP3* combined genotypes				*P*
	D/D–T allele	D/D–C/C	I allele–T allele	I allele–C/C	
Number	98	65	39	52	
Age (years)	21.4 ± 0.2	21.5 ± 0.3	21.6 ± 0.4	21.9 ± 0.3	0.604
BMI (kg/m^2)	20.9 ± 0.2	21.3 ± 0.3	20.4 ± 0.3	21.5 ± 0.4	0.118
SBP (mmHg)	109.7 ± 0.9	111.1 ± 1.4	111.3 ± 1.6	113.9 ± 1.4	0.103
DBP (mmHg)	61.6 ± 0.7	63.7 ± 1.2	63.6 ± 1.8	64.3 ± 1.2	0.277
MBP (mmHg)	77.6 ± 0.7	79.5 ± 1.1	79.5 ± 1.6	80.8 ± 1.2	0.132
Family history of HT, DM, or obesity (%)	20.4	26.2	25.6	42.3	**0.039**

Values are mean ± SEM and were compared by one-way ANOVA. Chi-square test was performed for analysis of genotype distributions as to family history. *P* values of less than 0.05 are shown in boldface type. ANOVA, analysis of variance; DBP, diastolic blood pressure; DM, diabetes mellitus; HT, hypertension; MBP, mean blood pressure; SBP, systolic blood pressure; SEM, standard error of mean.

diabetes, and obesity. For the 45 bp insertion/deletion polymorphism of *UCP2*, the 45 bp I/I genotype was associated with relatively higher BP and HRV sympathetic indices at supine rest. These findings suggest that the studied *UCP* polymorphisms are associated with sympathovagal balance in resting or sympathetic activated (standing) conditions.

ROS-induced autonomic dysregulation can be related to hypertension, cardiac diseases, and neuropathy [10,11, 47–49]. Apart from roles in energy metabolism [1,2], UCP2 and UCP3 play an important role in the prevention of mitochondrial ROS formation [3,4] and have been implicated in the pathophysiology of hypertension and CVD [50–53]. Recent studies also suggest that ROS is closely related to ANS activity, with, for instance, a sympathoexcitation effect of ROS at the central, cardiac, and peripheral levels [10,11]. UCP families are expressed in various human tissues, including the CNS and dorsal root ganglion [8,9]. UCP3 has been shown to protect neurons from glucose-induced neuronal oxidative stress [9], whereas UCP2 exhibits both neuromodulatory and neuroprotective roles in the CNS [8,54].

Previous reports have shown increased UCP3 mRNA levels in the skeletal muscle in *UCP3* −55T allele carriers [18]; speculatively, the higher expression of *UCP3* in the −55T allele carriers may prevent or reduce oxidative stress in the ANS, which may protect from excessive cardiac sympathetic activation. On the other hand, UCP2 also plays a crucial role in decreasing ROS formation, which can affect BP or cardiovascular regulation [50–53]. Thus, the *UCP2* polymorphism may affect BP regulation through ROS or autonomic nervous modulation. In this context, significant associations between the *UCP2* −866G/A polymorphism, which was in linkage disequilibrium with the 45 bp insertion/deletion polymorphism [55], and hypertension [56], coronary artery disease [57], and oxidative stress [58] have been reported. However, in the present study, carriers of the 45 bp I/I genotype were limited because of the low abundance of the 45 bp I allele. Further large-scale studies are required to detect the precise impact of this polymorphism on BP variation.

Recent data suggest that UCP2 and UCP3 might be associated with obesity or obesity-related phenotypes. In the present study, carriers of the *UCP3* −55T allele

Table 6 Association of combined *UCP2–UCP3* genotypes with power spectral parameters of heart rate variability

Parameters	*UCP2–UCP3* combined genotypes				*P*	Adjusted *P*
	D/D–T allele	D/D–C/C	I allele–T allele	I allele–C/C		
Supine rest						
Number	98	62	38	52		
LF, ln ms^2 (geometric mean)	6.35 ± 0.09 (574.2)	6.55 ± 0.11 (696.2)	6.31 ± 0.15 (549.6)	6.33 ± 0.16 (562.3)	0.539	0.510
LF (%)	55.2 ± 1.6	58.8 ± 2.0	52.9 ± 3.0	55.4 ± 2.3	0.335	0.265
HF, ln ms^2 (geometric mean)	6.12 ± 0.10 (455.7)	6.16 ± 0.12 (472.0)	6.18 ± 0.12 (483.0)	6.09 ± 0.15 (439.8)	0.996	0.948
HF (%)	44.8 ± 1.6	41.2 ± 2.0	47.1 ± 3.0	44.6 ± 2.3	0.335	0.265
LF/HF, ln (geometric mean)	0.23 ± 0.07 (1.26)	0.39 ± 0.09 (1.47)	0.13 ± 0.13 (1.14)	0.25 ± 0.10 (1.28)	0.351	0.279
Standing						
Number	92	64	37	50		
LF, ln ms^2 (geometric mean)	6.23 ± 0.08 (508.7)	6.30 ± 0.09 (544.7)	6.28 ± 0.11 (532.1)	6.20 ± 0.10 (492.8)	0.885	0.769
LF (%)	82.0 ± 1.3*	86.5 ± 1.3	85.0 ± 1.7	87.3 ± 0.9	**0.016**	**0.014**
HF, ln ms^2 (geometric mean)	4.48 ± 0.10 (88.5)	4.23 ± 0.12 (68.7)	4.38 ± 0.11 (79.8)	4.15 ± 0.12 (63.6)	0.145	0.077
HF (%)	18.0 ± 1.3*	13.5 ± 1.3	15.0 ± 1.7	12.7 ± 0.9	**0.016**	**0.014**
LF/HF, ln (geometric mean)	1.75 ± 0.09† (5.75)	2.07 ± 0.10 (7.93)	1.90 ± 0.11 (6.67)	2.05 ± 0.08 (7.75)	**0.044**	**0.038**

LF (%) and HF (%) indicate percentage of LF or HF power in the range between 0.035 and 0.05 Hz (see methods for details). LF/HF indicates ratio of LF to HF power. Values are mean ± SEM; geometric means are given between parentheses. *P* values were obtained by one-way ANOVA. Adjusted *P* values were calculated after adjusting for age, BMI, and family history of hypertension, diabetes, or obesity. *P* values of less than 0.05 are shown in boldface type. ANOVA, analysis of variance; HF, high frequency; LF, low frequencyz; SEM, standard error of mean. *$P < 0.05$, compared with D/D–C/C and I allele–C/C (Bonferroni post-hoc test in adjusted model). †$P = 0.054$, compared with D/D–C/C (Bonferroni post-hoc test in adjusted model).

had a lower BMI than the C/C carriers, consistent with previous reports in Japanese and Caucasian individuals [41,59,60]. UCP3 plays a role in uncoupling oxidative phosphorylation in the skeletal muscle, heart, and adipose tissues and is thought to mediate the increase in energy expenditure [1,2]. Mice overexpressing UCP3 were shown to be hyperphagic but lean and resistant to diet-induced obesity [61]. Furthermore, UCP3 mRNA expression was positively correlated with sleeping metabolic rate [62]. Thus, with respect to these data, the increased UCP3 expression in the −55T allele carriers could be associated with increased energy expenditure, causing a body weight reduction. However, other studies found no association or opposite results in different populations [36,63]. Further studies are needed to confirm the direct effect of this polymorphism on body weight regulation.

The *UCP2* gene is adjacent to the *UCP3* gene. In the present study, modest linkage disequilibrium was found between the *UCP2* 45 bp insertion/deletion and the *UCP3* −55C/T polymorphisms. Although synergistic interaction effects between the studied polymorphisms were not observed in our populations, an additive effect was found in some HRV indices. In a standing position, participants with the D/D−T allele combined genotype had the lowest HRV sympathetic (low frequency percentage and low frequency/high frequency) and the highest HRV parasympathetic (high frequency percentage) indices among the combined genotypes, suggesting that the *UCP2* 45 bp insertion/deletion and the *UCP3* −55C/T combined genotypes could have additive effects on autonomic cardiovascular regulation. In addition, a significant association was found between the D/D−T allele genotype and a family history of hypertension, diabetes, and obesity. Follow-up studies are required to evaluate the future prevalence of these diseases in the carriers of each genotype. Recently, some studies indicated an association of variation of the *UCP2–UCP3* gene cluster with very low calorie diet-induced body fat reduction [55] and type 2 diabetes [64]. Thus, we cannot exclude the possibility that the *UCP2* 45 bp insertion/deletion or *UCP3* −55C/T polymorphisms are in linkage disequilibrium with other responsible functional polymorphisms. Therefore, haplotype or diplotype analyses for the *UCP2–UCP3* gene cluster may provide a more precise understanding of the contribution of these polymorphisms to the related (patho)physiological phenotypes.

To our knowledge, this is the first study to examine the association of the *UCP2* 45 bp insertion/deletion or *UCP3* −55C/T polymorphisms with HRV. However, there are some limitations. First, although HRV is of clinical importance in overall stability of cardiac ANS, we did not investigate the direct physiological effect of the studied *UCP* polymorphisms, such as plasma catecholamine levels. Thus, further studies combining biochemical testing with HRV analysis will help in understanding the association between *UCP2* and *UCP3* polymorphisms and ANS activity. Secondly, an interesting association was found between the *UCP2* and *UCP3* polymorphisms and a family history of hypertension, diabetes, and obesity. It is possible that these polymorphisms of *UCPs* may account for a part of the causes of these diseases. However, the present study did not use a familial-based approach, and the findings were an indirect association, which did not provide direct evidence for a heritable role of the studied *UCP* polymorphisms in the prevalence of these diseases. A further familial-based study to specifically confirm the inheritance of the *UCP2* and *UCP3* polymorphisms for the related diseases would be beneficial.

In conclusion, the present study found that the *UCP2* 45 bp insertion/deletion and the *UCP3* −55C/T polymorphisms were significantly associated with HRV as well as BMI and BP in young Japanese men. These phenotypes, according to *UCP2* and *UCP3* polymorphisms, may be potential risk factors for future pathological episodes of UCP-related diseases such as hypertension and CVD. As *UCP2* and *UCP3* expression is altered with aging [65], prospective cohort studies are necessary to investigate the long-term contributions of *UCP2* and *UCP3* polymorphisms to related (patho)physiological phenotypes.

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GLUCOSE REGULATION OF DIPEPTIDYL PEPTIDASE IV GENE EXPRESSION IS MEDIATED BY HEPATOCYTE NUCLEAR FACTOR-1α IN EPITHELIAL INTESTINAL CELLS

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SUMMARY

1. Dipeptidyl peptidase IV (DPP-IV) is a new drug target in the treatment of Type 2 diabetes. Dipeptidyl peptidase IV enzyme activity is significantly altered in Type 2 diabetic patients with hyperglycaemia, but the underlying molecular mechanisms remain unclear.

2. The first aim of the present study was to clarify whether glucose regulates DPP-IV enzyme activity. To address this, DPP-IV gene expression and enzyme activity were measured in Caco2 cells cultured in the presence of low (2.5 mmol/L) or high (16.7 mmol/L) concentrations of glucose. We observed that high glucose inhibited DPP-IV gene expression and enzyme activity.

3. The second aim of the present study was to investigate whether hepatocyte nuclear factor (HNF)-1α contributes to glucose regulation of DPP-IV gene expression. To explore this question, associations between the gene expression of DPP-IV and HNF-1α were examined in Caco-2 cells cultured in the presence of low (2.5 mmol/L) or high (16.7 mmol/L) glucose. We found that the pattern of glucose-regulated DPP-IV gene expression is similar to that of HNF-1α. Moreover, to elucidate whether glucose regulation of DPP-IV gene expression is affected when HNF-1α is inhibited, we produced two stable cell lines in which a dominant-negative mutant HNF-1αR271G or basic vectors were stably expressed. We found that glucose regulation of DPP-IV gene expression was compromised in HNF-1αR271G cells, but was well maintained in basic vector cells.

4. These results suggest that glucose regulation of DPP-IV gene expression is mediated by HNF-1α.

Key words: Caco-2, dipeptidyl peptidase IV, glucose regulation, hepatocyte nuclear factor-1α.

INTRODUCTION

Dipeptidyl peptidase IV (DPP-IV/CD26) is a multifunctional glycoprotein expressed both in soluble form and on the cell surface of various tissues, including the liver, small intestine, kidney and pancreas.[1] Dipeptidyl peptidase IV enzyme activity depends on a serine protease, which acts to remove N-terminal proline and alanine dipeptides from many biologically active polypeptides, cytokines and chemokines, such as glucagon-like peptide (GLP-1).[2] Glucagon-like peptide-1 is mainly secreted by the small intestine after ingestion of a meal, enhancing glucose-induced insulin secretion[3] and inducing satiety.[4] The action of GLP-1 is a key to normal post-prandial glucose homeostasis and constitutes the functional activity of the enteroinsular axis.[5] In Type 2 diabetic patients, DPP-IV degrades GLP-1 in response to meal ingestion,[6] resulting in a reduction of early post-prandial insulin secretion.[7] Prevention of inactivation of GLP-1 by direct inhibition of DPP-IV enzyme activity has emerged recently as a new treatment for Type 2 diabetes.[8]

Recent clinical studies have reported that DPP-IV enzyme activity is significantly altered in Type 2 diabetic patients with hyperglycaemia.[6,9] Enhanced DPP-IV enzyme activity may cause a deficiency of early post-prandial insulin secretion and aggravate the diabetic condition. However, until now, it was not known whether glucose regulates DPP-IV enzyme activity. Furthermore, it is unclear that DPP-IV enzyme activity is altered in Type 2 diabetic patients, because the molecular mechanism underlying DPP-IV gene expression has not been elucidated completely. A previous study found that hepatocyte nuclear factor (HNF)-1α contributes to the regulation of DPP-IV gene expression.[10] Hepatocyte nuclear factor-1α, the causal gene of maturity onset diabetes of the young (MODY) 3,[11] is expressed in the small intestine, pancreas, liver and kidneys.[12] Hepatocyte nuclear factor-1α is composed of three functional domains: the dimerization domain, the DNA-binding domain and the transcriptional domain.[13] Mutant HNF-1α results in synthesis of truncated proteins with simple loss of function or with dominant-negative effects.[14]

In the present study, we investigated whether glucose regulates DPP-IV gene expression and enzyme activity in Caco2 cells, which belong to an epithelial intestinal cell line[15] In addition, we investigated whether HNF-1α contributes to glucose regulation of DPP-IV gene expression in Caco-2 cells, in which a dominant-negative mutant HNF-1αR271G and basic vector (as a control) were stably expressed.

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We measured DPP-IV gene expression and enzyme activity in Caco2 cells cultured in the presence of low or high concentrations of glucose.

METHODS

Cell culture

Caco-2 (clone TC7) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 16.7 mmol/L glucose (Sigma, St Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin and 100 mg/L streptomycin (Sigma) and maintained in 5% CO_2 at 37°C. For growth under low glucose conditions, DMEM devoid of glucose was used and was supplemented with 20% heat-inactivated FBS. Because of the serum, the glucose concentration in this solution was 2.5 mmol/L. All cells were cultured in medium containing 16.7 mmol/L glucose until the 1st day of confluence; thereafter, cells were cultured in medium containing 2.5 glucose or 16.7 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol (as an osmolality control for 16.7 mmol/L glucose) until the Day 14 of confluence.

Plasmid constructs

For the luciferase assay, wild-type HNF-1α and mutant HNF-1αR271G cDNA, provided by Dr Jun Takeda (Gifu University, Gifu, Japan), were subcloned into pCMV6b vector. The promoter region (nucleotides (nt) –473 to +2 bp relative to the cap site) of the DPP-IV gene, including the HNF-1α-binding sites (nt –147 to –135), was subcloned into pGL3 basic reporter vector (Promega, Madison, WI, USA). For the establishment of a stable cell line, a part of the HNF-1α promoter site (nt –781 to +6 bp) was combined with mutant HNF-1αR271G cDNA and subcloned into pIRESneo2 vectors (Clontech, Mountain View, CA, USA).

Transfection analysis

Caco-2 cells were plated 24 h before transfection and grown to 70% confluence. Transfection was performed with Lipofect AMINE Reagent (Invitrogen) according to the manufacturer's instructions. For transient transfection, cells were harvested 24 h after the start of transfection and cell extracts were prepared for luciferase and β-galactosidase assays. Luciferase activity was normalized against β-galactosidase activity. For the transfection of a stable line, all cells were harvested and subcultured into fresh complete medium 24 h after the start of transfection and 1.0 mg/mL G418 (Invitrogen) was added after 48 h to select for the expression of resistance gene. Individual clones appeared after approximately 2 weeks and selected clones were maintained in 0.5 mg/mL G418.

RNA isolation and reverse transcription–polymerase chain reaction

Total RNA was extracted using Trizol reagents (Invitrogen). First-strand cDNA was synthesised from total RNA using Superscript-II enzyme (Invitrogen). Real-time polymerase chain reaction (PCR) was performed on the ABI7900 using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the protocol provided by the manufacturer. Respective primer sets (forward and reverse) were as follows: DPP-IV, 5′-CCTTCTACTCTGATGAGTCACTGC-3′ and 5′-GTGCCACTAAGCTCCATCTTC-3′; HNF-1α, 5′-TACACCTGGTACGTCCGCAA-3′ and 5′-CACTTGAAACGGTTCCTCCG-3′; sucrase-isomaltase (SI), 5′-CATCCTACCATGTCAAGAGCCA-3′ and 5′-GCTTGTTAAGGTGGTCTGGTTT-3′; β-actin, 5′-AGTACTCCGTGTGGATCGGC-3′ and 5′-GCTGATCCACATCTGCTGGA-3′. Levels of HNF-1α, DPP-IV and SI mRNA were normalized against the amount of β-actin mRNA.

Preparation of protein and immunoblots

Protein was prepared as described by Oliver *et al.*[10] Protein samples (50 μg) were electrophoresed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) by electroblotting. For immunological blots, membranes were incubated with anti-HNF-1α antibody (N-19; Santa Cruz, Santa Cruz, CA, USA) and anti-actin (Sigma). Bands were visualized with ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA).

Determination of DPP-IV and SI activity

All cells were washed twice with phosphate-buffered saline (PBS) and then lysed in M-Per Mammalian Protein Extraction Regent (Pierce, Rockford, IL, USA). In the present study, DPP-IV activity was assayed using the DPPIV-Glo™ Protease Assay Kit (Promega), according to the instructions provided by the manufacturer. Sucrase-isomaltase activity was assayed as described previously.[16] Protein contents were determined using the BCA protein assay kit (Pierce).

Statistical analysis

Statistical analyses were performed by Student's *t*-test using the Statview Statistical Package (SAS Institute, Cary, NC, USA). All data are shown as the mean±SEM of six independent experiments and statistical significance was set at $P < 0.05$.

RESULTS

Effects of glucose concentration on DPP-IV and HNF-1α gene expression

We observed that DPP-IV gene expression and enzyme activity were lower on Day 1 of confluence in Caco-2 cells (Fig. 1). With differentiation of Caco-2 cells, DPP-IV gene expression and enzyme activity were significantly increased in the presence of both concentrations of glucose (Fig. 1). However, DPP-IV gene expression was markedly increased in Caco-2 cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol (as an osmolality control for 16.7 mmol/L glucose) and twofold higher than that in cells cultured in the presence of 16.7 mmol/L glucose on Day 14 of confluence (Fig. 1a). Moreover, this increased DPP-IV gene expression in Caco-2 cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol resulted in a threefold increase in DPP-IV activity (Fig. 1b). This indicates that high concentrations of glucose suppress DPP-IV gene expression, resulting in decreased DPP-IV activity in Caco-2 cells.

In addition, HNF-1α gene expression was increased with the differentiation of Caco-2 cells in the presence of both low and high concentrations of glucose (Fig. 2). On Day 14 of confluence, HNF-1α gene expression was higher in cells cultured in the presence of 2.5 mmol/L glucose or 2.5 mmol/L glucose plus 14.2 mmol/L mannitol than in cells cultured in the presence of 16.7 mmol/L glucose (Fig. 2). The pattern of gene expression of DPP-IV and its regulation by glucose is similar to that for HNF-1α, suggesting that HNF-1α may be involved in glucose regulation of DPP-IV gene expression.

Effects of mutant HNF-1αR271G on wild-type HNF-1α

Mutant HNF-1αR271G has been identified as a causal mutation for MODY3 subjects.[11] In the present study, we tested whether