we identified SNPs on the human SHIP2 gene promoter and 5′-untranslated region (5′-UTR) and investigated their relationship with impaired fasting glycemia (IFG) in a Japanese cohort. Furthermore, the effect of the SNPs on promoter activity was examined in HeLa and HL60 cells.

MATERIALS AND METHODS

Subjects of a Cohort Study

The subjects (2178 individuals) consisted of 1096 males and 1082 females between 40 and 79 years of age. This cohort study was approved by the National Institute for Longevity Sciences–Longitudinal Study of Aging. All participants were independent residents in Ohu and Hiragashima in the Aichi prefecture in central Japan and were randomly selected from the resident register in cooperation with the local government. Written informed consent was obtained from each participant. Venous blood was collected early in the morning after at least a 12-hour fast for the measurement of serum lipids and plasma glucose. The medical history and prescribed drugs were examined in an interview by medical doctors. IFG was defined as HbA1c $\geq$ 5.8 or a fasted blood glucose $\geq$ 110 mg/dL, but not the use of insulin and/or hypoglycemic drugs. Subjects with treatment (treated) were defined by their use of insulin and/or hypoglycemic drugs.

Cell Culture

The human acute myelocytic leukemia cell line HL60 and the human cervix adenocarcinoma cell line HeLa were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO_2 at 37°C.

Identification of SNPs in the Human SHIP2 Gene Promoter and 5′-UTR

Blood samples were obtained from all subjects, and genomic DNA was prepared from peripheral leukocytes according to established procedures.[19] The polymerase chain reaction (PCR) and direct sequencing were performed to identify SNPs in the SHIP2 gene promoter and 5′-UTR. As a primary study, we examined genomic DNAs from 68 Japanese patients with diabetes, 25 Japanese patients with pancreatic adenocarcinoma and/or cholecystolithiasis, and 33 healthy Japanese volunteers. Primer pairs used for amplification of SHIP2 gene promoter and 5′-UTR are as follows: −571, 5′-TTGCACACTCCTACTACAACTGTCC-3′ and −44, 5′-GCGTCTCCGGGGAGTCGTCTTCCTG-3′; −92, 5′-CGGCTGGAGGCCTGCGCCTTTAAGG-3′ and +228, 5′-ACGAAGGTGACAGCTGTGTAAACTG-3′; +199, 5′-GTGTCAATAATACCCGTTCGTTCCG-3′ and +379, 5′-CGTCGGCTTCCCGCTCTGGAAAATG-3′. Primer sequences are based on the human chromosome 11 sequence found in GenBank/EMBL/DDBJ Data Bank (accession no. AP000593), and these primers amplify nucleotides from −571 to +379 (numbered from the most upstream TSS[18]). The PCR reaction was carried out in a 50-μL mixture containing 20 ng of genomic DNA, 400 nmol/L of each primer, 200 nmol/L of each dNTP, and 1.25 U of Pyrobest DNA polymerase with 1× PCR buffer supplied by the manufacturer (Takara, Tokyo, Japan). PCR conditions were 35 cycles of 98°C for 5 seconds, 65°C for 30 seconds, and 72°C for 35 seconds. The PCR products were purified and SNPs were identified by direct sequencing with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Genotyping of SNPs in the Human SHIP2 Gene Promoter

For the detection of SNP (+334 C/T) of SHIP2, dynamic allele-specific hybridization was performed as previously described[20,21] at Toyobo Gene Analysis (Tsuruga, Japan). Two PCR primers (5′-GCCGAGCGCGGTACGAG-3′ and 5′-CCGCTCTGGAAAATGGGGAT-3′) and one dynamic allele-specific hybridization probe (5′-TGTGGCGAGGGC CGGCTCTG-3′) labeled at the 3′ end with Texas Red were designed. Double-strand DNA-specific intercalating dye (SYBR Green I) was used as the fluorescence resonance energy transfer donor. PCR amplifications were performed under the conditions recommended by the enzyme supplier. In brief, a 25-μL aliquot containing 20 ng of genomic DNA, the enzyme supplier reaction buffer, 3.5 mmol/L of $MgCl_2$, 0.2 mmol/L of dNTP, and 1.25 U of rTaq containing Taqplus High (Toyobo, Osaka, Japan). Cycling parameters were as follows: denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 67.5°C for 30 seconds, primer extension at 72°C for 30 seconds for 45 cycles, and postextension at 72°C for 2 minutes in a thermal cycler PE9700 (PerkinElmer Life and Analytical Sciences, Inc, Boston, MA). Five microliters of reaction mixture containing 40 mmol/L of ethylenediaminetetraacetic acid, 10 pmol of probe, and SYBR Green I (final concentration × 25,000) was added to the PCR products. The mixture was put into the ABI PRIZM 7700 (Applied Biosystems), and melting temperature was measured. The program for analytical melting was 95°C for 30 seconds, 40°C for 1 minutes, increasing to 80°C by 10 minutes. The fluorescence signal was detected at excitation and emission wavelengths of 485 and 612 nm.

Statistical Analyses

Statistical differences between the groups were assessed using Fisher exact test. An odds ratio (OR) with 95% confidence intervals (CI) was calculated to evaluate the difference in genotype frequency between the groups. Probability differences of $P < 0.05$ were considered as statistically significant.

Northern Blotting

Northern blotting was performed as described.[19] Briefly, total RNAs (HeLa; 20 μg, HL60; 4 μg) isolated from cultured cells by acid phenol-guanidinium thiocyanate-chloroform extraction method were subjected to an RNA blot analysis. The probes were human SHIP2 cDNA fragment and human β-actin cDNA fragment.

Luciferase Activity Assay

The reporter gene used in these studies was firefly luciferase in a pGL3-Basic vector (Promega, Madison, WI). A

TABLE 1. SNPs Identified in the Promoter and 5′-UTR (exon1) of the Human SHIP2 Gene

This Work				Kaisaki et al[16]			
SNP*	Position[18]	Variants	Allele Frequency†	SNP	Position[16]	Variants	Allele Frequency‡
snpA	−405	C/A	0.024 A; 0.976 C	snp1	3021	C/A	0.035A; 0.965 A
snpB	−227	G/T	0.008 T; 0.992 G	—	3199	—	—
snpC	−208	C/A	0.004 A; 0.992 C	—	3218	—	—
—	−43	—	—	snp2	3383	C/T	rare
snpD	1	C/T	0.004 T; 0.992 C	—	3426	—	—
snpE	57	G/A	0.024 A; 0.976 G	snp3	3482	G/A	intermediate
—	308	—	—	rs4329713	3733	C/T	frequent
snpF	334	C/T	0.024 T; 0.976 C	snp4	3759	C/T	rare

*Three SNPs (snpA, snpE, snpF) formed the major haplotype (−405 C, +57 G, +334 C) and the minor haplotype (−405 A, +57 A, +334 T).
†Allele frequencies were calculated from panels of 126 individuals.
‡Based on Kaisaki et al[16]: rare ≤ 1–3%, intermediate ≥ 3–6%, frequent ≥ 6%.

genomic DNA fragment including part of the first exon and the 5′-flanking sequence (from −111 to +380) was amplified by PCR with the upstream primers (5′-CGACGCGTGGAGG CCTGCGCCTTTAA-3′) tagged with an *Mlu*I site and the downstream primer (5′-CGAGATCTGCGTCGGCTTCCCG CTCTG-3′) tagged with a *Bgl*II site (restriction enzyme sequences are underlined). The amplified fragments were digested with *Mlu*I and *Bgl*II and ligated into the *Mlu*I/*Bgl*II sites of the pGL3-Basic vector. Two kinds of polymorphic plasmids, pGL3-GC (+57G, +334C) and pGL3-AT (+57A, +334T), were obtained using polymorphic individual DNA as a template. HL60 cells were transfected with 0.5 μg of pRL-TK plasmid (Promega) as an internal standard along with 10 μg of pGL3-basic, pGL3-GC, or pGL-AT plasmid by electroporation at 250 V, 960 μF. HeLa cells were transfected using cationic liposome Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. At 24 hours after transfection, luciferase reporter assays were performed using the luciferase assay kit (Toyo Ink, Tokyo, Japan). Transfections were performed in triplicate and repeated 3 times to ensure reproducibility.

TABLE 2. Genotype Frequency of the +334C to T Polymorphism Among the IFG, Treated, and Normal Groups

		Genotype (+334)		
Group		CC	CT and TT	OR (95% CI)*
Normal	N (%)	1580 (76.4%)	76 (3.7%)	—
IFG†	N (%)	373 (18.0%)	40 (1.9%)	2.23 (1.50–3.32)‡
Normal + IFG	N (%)	1953 (89.7%)	116 (5.3%)	—
Treated§	N (%)	102 (4.7%)	7 (0.3%)	1.16 (0.53–2.54)‖
Normal	N (%)	1580 (72.5%)	76 (3.5%)	—
IFG + Treated	N (%)	475 (21.8%)	47 (2.2%)	2.06 (1.41–3.00)¶

*Ratio of odds (+334 CT, TT vs. +334 CC) and 95% confidence interval.
†Impaired fasting glycemia: FBG(fasted blood glucose) ≥ 110 mg/dL and/or HbA1c ≥ 5.8.
‡$P < 0.0001$ (+334 CT, TT vs. +334 CC) when analyzed by Fisher's exact test.
§Treated with insulin and/or hypoglycemic drugs.
‖$P = 0.72$ (+334 CT, TT vs. +334 CC) when analyzed by Fisher's exact test.
¶$P = 0.0001$ (+334 CT, TT vs. +334 CC) when analyzed by Fisher's exact test.

RESULTS

Identification of SNPs in the Human SHIP2 Gene Promoter and 5′-UTR

The 6 SNPs in the human SHIP2 gene promoter and the 5′-UTR (exon 1) were detected in a panel of 126 Japanese, consisting of 68 patients with diabetes, 25 patients with pancreatic adenocarcinoma and/or cholecystolithiasis, and 33 healthy volunteers (Table 1). These SNPs did not fall into the TSSs and the functional Sp1 binding sequence (Sp1a; −85–68) except snpD (+1 C/T), which hit the most upstream TSS.[18] As the most downstream TSS locates at position +356,[18] the functional class of 3 SNPs (snpD, snpE, snpF) may be a promoter and a 5′-UTR. The 3 SNPs [snpA (−405 C/A), snpE (+57 G/A) and snpF (+334 C/T)] were found in relatively high frequency in Japanese population and formed haplotypes CGC and AAT, as determined by the PCR-cloning method using the sense primer (−571) and antisense primer (+379) and DNA sequencing.

Association of Human SHIP2 Gene Polymorphisms and IFG

A cohort study in a Japanese population was employed. The subjects consist of 1102 males and 1087 females between 40 and 79 years of age. The snpF (+334 C/T) has chosen to investigate, as its allele frequency is relatively high in Japanese (Table 1) and it locates in the core promoter sequence.[18] The frequency of genotypes (+334 CT and TT, compared with CC) were significantly higher in the IFG group than the normal group ($P < 0.0001$, OR = 2.23, 95% CI = 1.50–3.32) (Table 2, upper row). This association was not affected by age and gender. When we compared between the normal and IFG subjects and treated subjects, no significance in genotype distribution was observed (Table 2, middle row). We were unable to understand the reason, but the number of treated subjects may be too low to compare with the other subjects. When we compared between the IFG and treated groups and the normal group, the frequency of genotypes (+334 CT and TT, compared with CC) were significantly higher in the IFG and treated groups than in the normal

TABLE 3. Characteristics Related to Obesity and Insulin Resistance According to +334C/T Polymorphism

	Genotype (+334)		
	CC	CT and TT	P*
Age, y	58.8 ± 0.2	60.0 ± 1.0	NS
Weight, kg	57.3 ± 0.2	56.5 ± 0.9	NS
BMI, kg/m^2	22.9 ± 0.1	22.7 ± 0.3	NS
Percent body fat by DXA, %	26.4 ± 0.2	26.1 ± 0.6	NS
Leptin, ng/mL†	5.7 ± 0.1	5.6 ± 0.4	NS
FBG, mg/dL†	100.9 ± 0.4	103.8 ± 1.5	0.07
Insulin, μU/mL†	8.2 ± 0.1	8.7 ± 0.5	NS
HbA1c, %†	5.19 ± 0.01	5.31 ± 0.05	0.02
HOMA-R†	2.12 ± 0.04	2.29 ± 0.18	NS

Data are expressed as means ± SE.
*P values are by Fisher exact test.
†Values are calculated from the groups excluded the individuals with treatment.
BMI indicates body mass index; FBG, fasted blood glucose; NS, not significant.

group (P = 0.0001, OR = 2.06, 95% CI = 1.41–3.00) (Table 2, lower row). As expected, the genotype distribution of snpE was the same as that of snpF in this cohort also, indicating complete linkage (data not shown). However, there was no significant association in the indexes related to obesity, such as body mass indexes, serum leptin level, and dual energy x-ray absorptiometry (DXA) fat, and the indexes related to insulin secretion and insulin resistance accessed by homeostasis model assessment (HOMA) classified age and gender (Table 3).

Effect of SNPs on Human SHIP2 Promoter Activity

To examine the contribution of the SNPs to the expression of human SHIP2, we constructed luciferase reporter plasmids containing a part of the promoter and 5′-UTR (−111 to +380). We chose the region because it exhibited nearly the maximum promoter activity[18] and contained 2 major SNPs (snpE and snpF) found as the haplotypes GC and AT. Then, we checked SHIP2 mRNA in the human cervix adenocarcinoma cell line, HeLa, and human acute myelocytic leukemia cell line, HL60, as they were reported to express significant levels of SHIP2 mRNA (Fig. 1A).[6] Two luciferase reporter plasmids (pGL3-GC, pGL3-AT), which were the same except the snpE (+57 G/A) and the snpF (+334 C/T), were transfected into HeLa and HL60 cells. The HeLa cells transfected with the pGL3-AT construct showed significantly higher promoter activity than the cells transfected with pGL3-GC (Fig. 1B). Also, the HL60 cells transfected with the pGL3-AT construct showed relatively higher promoter activity than the cells transfected with pGL3-GC, but the difference was not significant (Fig.1C).

DISCUSSION

We hypothesized that IFG may be partially explained by the expression level of SHIP2 gene, as the enhanced expression of SHIP2 was observed in the skeletal muscle and fat tissue of diabetic db/db mice[9] and the mice lacking the SHIP2 gene revealed an increased sensitivity to insulin.[10] Therefore, we focused on the regulatory mechanism of the human SHIP2 gene in the previous study.[18] The 6 SNPs in the human SHIP2 gene promoter and the 5′-UTR (exon 1) were detected in a panel of 126 Japanese. Three of the previously described SNPs in this region[16] were the same as the SNPs (snpA, snpE, snpF) detected in the present work. The frequency of the SNPs (snpA, snpE, snpF) is suggested to be relatively high in the British and French, as well as Japanese (Table 1).

To access the relation between human SHIP2 gene polymorphisms in the promoter and the 5′-UTR (exon 1) and physiological abnormality of the metabolic syndrome, a cohort study in a Japanese population was employed. As a result, a

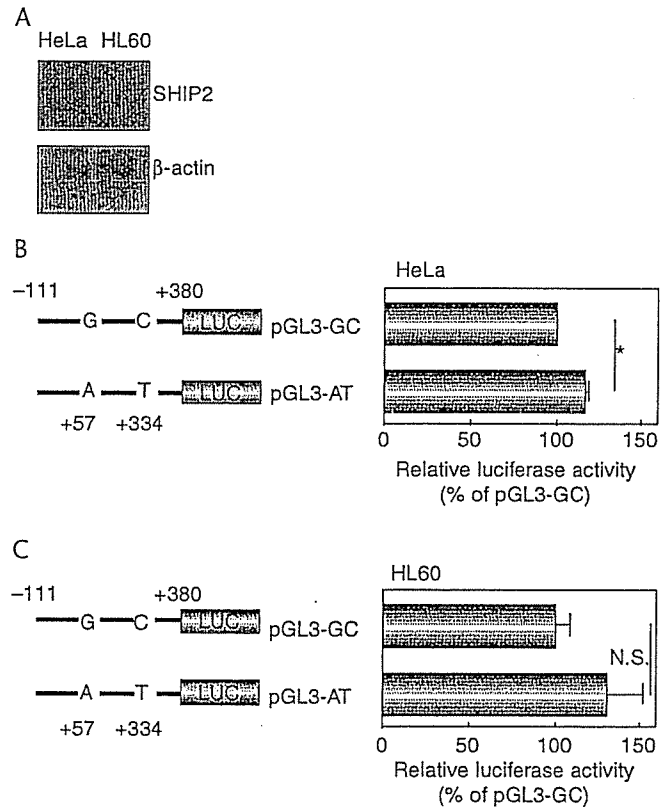

FIGURE 1. Effect of SNPs on human SHIP2 gene promoter activity. A, SHIP2 mRNA expression in HeLa cells and HL60 cells. Northern blotting was performed as described.[19] Total RNA (HeLa; 20 μg, HL60; 4 μg) was hybridized with a SHIP2 cDNA probe. A single 4.7-kb signal was observed in both cell lines. Hybridization to the human β-actin cDNA probe is shown as the control. Each SHIP2 fragment-luciferase plasmid was transiently transfected into HeLa cells (B) and HL60 cells (C). The diagram on the left shows the structure of each human SHIP2 fragment-luciferase construct. Promoter activities are shown as relative luciferase activities on the right. (pGL3-GC was regarded as 100%.) Values are expressed as means and standard errors of 3 independent experiments. * $P < 0.05$ compared to pGL3-GC (by Student t test). N.S., not significant.

significant association between the frequency of a SNP and IFG was observed. Furthermore, the effect of the SNP on promoter activity was examined in human cells, and the direction of the change of promoter activity met the results of the cohort study. However, there was no significant association in the indexes related to obesity, such as body mass index, serum leptin level, and DXA fat (Table 3). We are unable to understand the reason. Considering the SHIP2 knockout mice phenotypes, the increase in SHIP2 expression may result in hyperglycemia and/or obesity.[10,11] Probably, this is due to the difference of species and/or the slightness of the change of promoter activity. Also, there was no significant association in the indexes related to insulin secretion and insulin resistance assessed by HOMA classified by age and gender (Table 3). Although the SNPs (snpE, snpF) in the present work affected the promoter activity of SHIP2, other SNPs and mutations were reported to affect the SHIP2 activity in the different manners. An SNP adjacent to the NPXY motif decreased tyrosine phosphorylation of SHIP2 and subsequent association with Shc.[17] A deletion in the 3′-UTR of SHIP2 gene resulted in reporter messenger RNA and protein overexpression in cell culture.[15]

In conclusion, the SNPs in the SHIP2 gene promoter and the 5′-UTR (exon 1) may account partly for the IFG and may be a marker for the risk of diabetes, at least in the Japanese.

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International Journal of Obesity (2006) 30, 1609–1614
 0307-0565/06 $30.00
www.nature.com/ijo

ORIGINAL ARTICLE

Preproghrelin Leu72Met variant contributes to overweight in middle-aged men of a Japanese large cohort

M Kuzuya[1], F Ando[2], A Iguchi[1] and H Shimokata[2]

[1]*Department of Geriatrics, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Japan and* [2]*Department of Epidemiology, National Institute for Longevity Sciences, Obu, Aichi, Japan*

Objective: To investigate whether Leu72Met polymorphism of the preproghrelin gene is associated with overweight/obesity in middle-aged and older Japanese.
Design: Cross-sectional analysis.
Subjects: A total of 2238 community-dwelling middle-aged and older Japanese people (age: 40–79 years) who participated in the first wave of examinations in the National Institute for Longevity Sciences – Longitudinal Study of Aging from April 1998 to March 2000.
Measurements: The Leu72Met polymorphism of prepoghrelin gene, anthropometric variables including body weight, body mass index (BMI), waist circumference, waist-to-hip ratio and whole-fat mass and biochemical variables including serum lipid levels, fasting plasma glucose, insulin and homeostasis model assessment for insulin resistance.
Results: The frequencies of the Leu72Leu, Leu72Met and Met72Met alleles were 63.4, 32.7 and 4.0%, respectively. No differences in the genotype distributions of the Leu72Met polymorphism were found between genders or age groups, and no significant associations were observed between polymorphism and anthropometric variables in women and older men. However, middle-aged men who were 72Met allele carriers showed a higher body weight change from body weight at 18 years of age, as well as a higher waist circumference and a tendency to a higher waist-hip-ratio than noncarriers. Although there were no significant differences in the genotype distribution according to BMI in women and older men, a significantly higher frequency of the 72Met allele was found in the higher BMI group (BMI $\geqslant$ 25 kg/m^2) of middle-aged men than in the normal-weight group. No significant associations were observed between polymorphism and serum lipid, glucose or insulin levels.
Conclusions: These results suggest that the 72Met allele of the preproghrelin gene is a contributing factor for midlife weight change in men.
International Journal of Obesity (2006) **30**, 1609–1614. doi:10.1038/sj.ijo.0803296; published online 25 July 2006

Keywords: ghrelin; polymorphism; preproghrelin; lipid metabolism; glucose metabolism

Introduction

Ghrelin has been shown to be the natural ligand of the previously identified 'orphan' growth hormone secretagogue receptor.[1] Although widely expressed in many tissues, ghrelin is most abundantly produced by the stomach.[1] Ghrelin is much more than a mere natural growth hormone secretagogue, however: it has been found to have profound growth hormone-independent weight- and appetite-increasing effects.[2] Ghrelin stimulates food intake in both rodents and humans,[2,3] and is strongly involved in the regulation of energy homeostasis.[4] This suggests that derangement in the ghrelin system could play a role in obesity. In addition, ghrelin may affect carbohydrate and lipid metabolisms.[5,6]

Recently, three major polymorphisms in the human ghrelin gene were described.[7] One of these nucleotide changes, a single-base substitution C214A with Met replacing Leu at codon 72 in the preproghrelin amino-acid sequence, seems to be associated with an earlier onset of obesity,[7–9] but it has also been proposed that 72Met could provide protection against the accumulation of fat.[10] Thus, previous studies on preproghrelin genetic variants have arrived at contradictory findings as to their role in

Correspondence: Dr M Kuzuya, Department of Geriatrics, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan.
E-mail: kuzuya@med.nagoya-u.ac.jp
Received 13 April 2005; revised 24 January 2006; accepted 4 February 2006; published online 25 July 2006

obesity. Additionally, most studies have had only child and adolescent subjects, whereas few studies have targeted the middle aged or elderly, or randomly sampled community-dwelling individuals.

The aim of the present study was to test whether genetic variants in the preproghrelin gene (Leu72Met) could play a role in predisposing carriers to overweight/obesity or be associated with anthropometric data, serum lipid levels and values related with glucose metabolisms in a middle-aged to elderly Japanese population.

Materials and methods

Subjects

The present study consisted of a cross-sectional analysis of 1110 women and 1128 men who participated in the first wave of examinations in the National Institute for Longevity Sciences – Longitudinal Study of Aging (NILS-LSA) from April 1998 to March 2000. The subjects of the NILS-LSA were male and female residents 40–79 years old. The population of Obu city and Higashiura town in the Aichi prefecture in central Japan was stratified by both age and gender, and randomly selected from resident registrations in cooperation with the local governments. The number of male and female participants was to be the same to test gender difference. Age at the base line is to be 40–79 years and the number of participants in each decade (1940s, 1950s, 1960s, 1970s) is to be the same. The examinations include various areas of gerontology and geriatrics such as medical examinations, anthropometry, body composition, physical functions, physical activities, psychological assessments, nutritional analysis and molecular epidemiology. The subjects will be followed up every 2 years. The details of the NILS-LSA have been described elsewhere.[11] Randomly selected men and women were invited by mail to attend an explanatory meeting. At that meeting, the procedures for each examination and the follow-up schedule were fully explained. Written, informed consent to the entire procedure was obtained from each participant. The study was approved by the Ethics Committee of the National Institute for Longevity Sciences.

Anthropometric variables

Body weight was measured to the nearest 0.01 kg using a digital scale, height was measured to the nearest 0.1 cm using a wall-mounted stadiometer and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference and waist-to-hip ratio (WHR) were used as the indices for body fat distribution in this study. Waist-to-hip ratio was calculated as the ratio of waist circumference measured at the mid-point between the anterior superior iliac crest and the lowest rib-to-hip circumference. Whole-body fat mass, assessed by dual-energy X-ray absorptiometry (QDR-4500; Hologic, Madison, OH, USA), was used as an index for determining body composition. The subjects' weight at 18 years of age was obtained by questionnaire. Weight change was defined as the current weight minus the weight at 18 years of age.

Biochemical assays of blood

An antecubital blood sample was drawn from each subject after an overnight fast. Serum total cholesterol, triglycerides and low-density lipoprotein cholesterol were determined enzymatically, serum high-density lipoprotein cholesterol was measured by the heparin–manganese precipitation method and fasting plasma glucose was assayed by the glucose oxidase method. Lipoprotein (a) was measured in plasma using a commercially available ELISA. Plasma insulin was measured by radioimmunoassay. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5.[12]

Determination of preproghrelin genotypes

Genotypes were determined using a fluorescence-based allele-specific DNA primer assay system (Toyobo Tsuruga Gene Analysis, Tsuruga, Japan). The polymorphic regions (Leu72Met (C214A)) of preproghrelin were amplified by polymerase chain reaction with allele-specific sense primers labeled at the 5′-end with either fluorescein isothiocyanate (5′-CCG ACC CGG ACT TCC XTT-3′) or Texas red (5′-GTA CCG ACC CGG ACT TCC XG-3′) and with an antisense primer labeled at the 5′-end with biotin (5′-GGC TCC GCC CGG AAG ATG-3′). The reaction mixtures (25 μl) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/l of each deoxynucleoside triphosphate, 2.5 mmol/l $MgCl_2$ and 1 U of rTaq DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) in polymerase buffer. The amplification protocol consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 30 s; a final extension was conducted at 68°C for 2 min. Further details are provided elsewhere.[13]

Data analysis

Quantitative data were compared among three groups by one-way analysis of variance and the Tukey–Kramer *post hoc* test, and between two groups by the unpaired Student's *t*-test. Allele frequencies were estimated by the gene-counting method, and the χ^2 test was used to identify any significant departure from Hardy–Weinberg equilibrium. In the analyses to examine the association between genotypes and lipid or glucose metabolisms, participants who were being treated with lipid-lowering medications or oral hypoglycemic agents or insulin were excluded. Unless indicated otherwise, a *P*-value of <0.05 was considered to be statistically significant. The data were analyzed with the Statistical Analysis System (SAS), release 8.2.

Results

Genotype frequencies for the preproghrelin Leu72Met polymorphism were CC (Leu72Leu) 0.634, CA (Leu72Met) 0.327 and AA (Met72Met) 0.04. These frequencies are consistent with those expected under Hardy–Weinberg equilibrium. There were no significant differences in the genotype distributions of preproghrelin Leu72Met polymorphism between men and women, or among the different age groups (Table 1).

As shown in Table 2, although there were no differences in current body weight and body weight at 18 years of age between genotypes, middle-aged men who were 72Met allele carriers showed both a higher body weight change from body weight at 18 years of age ($P=0.013$, CC vs CA/AA) and higher waist circumference ($P=0.038$, CC vs CA/AA) than noncarriers. Among the middle-aged men in the present study, the Leu72Leu genotype was associated with the lowest BMI (trend, $P=0.049$), and the 72Met allele carriers tended to have a higher WHR ($P=0.062$, CC vs CA/AA) than subjects with the Leu72Leu genotype. However, no differences in anthropometric measurements among Leu72Met genotypes were observed in older men, or in female cohorts (Table 3).

In order to assess the association of the Leu72Met polymorphism with overweight or obesity, genotype and allele frequencies were compared among normal-weight ($BMI<25\,kg/m^2$) and overweight/obese ($BMI\geqslant25\,kg/m^2$) groups (Table 4). Although there were no significant differences in the genotype distribution according to BMI in women and older men, a significantly higher frequency of CA, AA or CA/AA was found in the higher BMI group than in the normal-weight group among middle-aged men.

No significant association was observed between the three genotypes and serum lipid, fasting glucose, insulin, HbA1c or HOMA-IR levels in men and women (Table 5). The preproghrelin Leu72Met genotypes showed similar allele frequencies in diabetic individuals and in non-diabetic controls (data not shown).

Discussion

We observed that the frequency of the 72Met allele of the present cohort was 36.6%. It has been demonstrated that

Table 1 Distribution of Leu72Met genotype of preproghrelin gene of the subjects

	n	CC		CA		AA		CA/AA	
		n	%	n	%	n	%	n	%
Total	2228	1412	63.4	728	32.7	88	4.0	816	36.6
Men*†	1121	709	63.3	371	33.1	41	3.7	412	36.8
Women	1107	703	63.5	357	32.3	47	4.3	404	36.5
Age (years)‡§									
40–49	562	364	64.8	177	31.5	21	3.7	198	35.2
50–59	556	357	64.2	177	31.8	22	4.0	199	35.8
60–69	560	359	64.1	180	32.1	21	3.8	201	35.9
70–79	550	332	60.4	194	35.3	24	4.4	218	39.6

*CC, CA, AA, men vs women, $\chi^2=0.6159$, $P=0.7350$; †CC, CA/AA, men vs women, $\chi^2=0.0160$, $P=0.8995$; ‡CC, CA, AA, age groups, $\chi^2=2.9716$, $P=0.8124$; §CC, CA/AA, age groups, $\chi^2=2.9149$, $P=0.4049$.

Table 2 Anthropometric variable of men according to age group and Leu72Met polymorphism of preproghrelin gene

	Middle aged (n = 563)								*Older* (n = 556)							
	CC		CA		AA		CA/AA		CC		CA		AA		CA/AA	
	Mean	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Weight (kg)	64.6	0.5	65.6	0.7	67.5	2.0	65.8	0.6	59.7	0.5	59.1	0.6	57.2	1.9	58.9	0.6
Weight at 18 years (kg)	56.9	0.4	56.4	0.5	57.2	1.5	56.5	0.5	55.3	0.4	54.5	0.5	55.4	1.5	54.6	0.5
Weight change from 18 years (kg)	7.7	0.4	9.2	0.6	10.3	1.7	9.3	0.5*	4.6	0.5	4.9	0.7	1.8	2.1	4.6	0.7
Height (cm)	167.0	0.3	167.2	0.4	166.3	1.4	167.2	0.4	162.0	0.3	161.9	0.4	161.7	1.2	161.9	0.4
BMI (kg/m^2)	23.1	0.1	23.4	0.2	24.4	0.6	23.5	0.2†	22.7	0.2	22.5	0.2	21.9	0.6	22.4	0.2
Waist circumference (cm)	82.2	0.4	83.4	0.6	84.9	1.8	83.6	0.6‡	82.4	0.5	82.1	0.6	80.5	1.9	81.9	0.6
Hip circumference (cm)	92.2	0.3	92.7	0.4	93.4	1.1	92.8	0.3	90.0	0.3	89.6	0.4	88.7	1.1	89.5	0.3
Waist-hip-ratio	0.891	0.003	0.899	0.004	0.907	0.012	0.899	0.004§	0.913	0.003	0.914	0.005	0.904	0.014	0.913	0.004
Fat mass (kg)	20.6	0.2	21.2	0.3	20.8	1.0	21.2	0.3	21.9	0.2	22.0	0.3	22.1	0.9	22.0	0.3

Except for *, †, ‡ and §, no significant trends and differences were detected among three groups (CC, CA and AA) and between two groups (CC and CA/AA). *$P=0.013$ (CC vs CA/AA); †$P=0.049$ (trend); ‡$P=0.038$ (CC vs CA/AA); §$P=0.062$ (CC vs CA/AA). Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. BMI = Body mass index.

Table 3 Anthropometric variable of women according to age group and Leu72Met polymorphism of preproghrelin gene

	Middle aged (n = 553)								*Older (n = 552)*							
	CC		CA		AA		CA/AA		CC		CA		AA		CA/AA	
	Mean	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Weight (kg)	53.9	0.4	53.9	0.6	54.4	1.6	54.0	0.6	50.8	0.4	50.9	0.6	52.4	1.7	51.1	0.6
Weight at 18 years (kg)	48.8	0.3	49.1	0.4	49.2	1.2	49.1	0.4	47.9	0.4	47.7	0.5	49.2	1.4	47.8	0.5
Weight change from 18 years (kg)	5.2	0.4	4.8	0.6	5.2	1.6	4.9	0.5	3.0	0.5	3.2	0.7	3.1	1.9	3.2	0.7
Height (cm)	154.0	0.3	154.1	0.4	154.4	1.0	154.1	0.3	148.5	0.3	148.6	0.4	147.9	1.2	148.5	0.4
BMI (kg/m^2)	22.7	0.2	22.7	0.2	22.8	0.7	22.7	0.2	23.0	0.2	23.0	0.2	24.1	0.7	23.2	0.2
Waist circumference (cm)	73.5	0.5	73.4	0.6	73.4	1.7	73.4	0.6	76.4	0.5	77.5	0.7	77.8	2.0	77.5	0.7
Hip circumference (cm)	91.5	0.3	91.5	0.4	90.9	1.1	91.4	0.4	89.8	0.3	89.9	0.4	90.8	1.2	90.0	0.4
Waist-hip-ratio	0.802	0.003	0.801	0.005	0.806	0.012	0.802	0.004	0.849	0.004	0.860	0.005	0.855	0.014	0.860	0.005
Fat mass (kg)	30.7	0.3	30.3	0.4	30.5	1.0	30.3	0.3	32.3	0.3	32.7	0.4	33.3	1.1	32.7	0.4

No significant trends and differences were detected among three groups (CC, CA and AA) and between two groups (CC and CA/AA). Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. BMI = Body mass index.

Table 4 Distribution of Let72Met genotype of preproghrelin gene

	n	CC		CA		AA		CA/AA		P	P*
		n	%	n	%	n	%	n	%		
All age groups											
Men											
BMI < 25 kg/m^2	854	546	63.9	280	32.8	28	3.3	308	36.1	0.411	0.393
BMI ⩾ 25 kg/m^2	267	163	61.1	91	34.1	13	4.9	104	39.0		
Women											
BMI < 25 kg/m^2	866	558	64.4	273	31.5	35	4.0	308	35.6	0.454	0.224
BMI ⩾ 25 kg/m^2	241	145	60.2	84	34.9	12	5.0	96	39.8		
Middle ages (younger than 60 years)											
Men											
BMI < 25 kg/m^2	413	280	67.8	123	29.8	10	2.4	133	32.2	0.032	0.036
BMI ⩾ 25 kg/m^2	151	88	58.3	54	35.8	9	6.0	63	41.7		
Women											
BMI < 25 kg/m^2	446	288	64.6	139	31.2	19	4.3	158	35.4	0.694	0.395
BMI ⩾ 25 kg/m^2	108	65	60.2	38	35.2	5	4.6	43	39.8		
Older (60 years or older)											
Men											
BMI < 25 kg/m^2	441	266	60.3	157	35.6	18	4.1	175	39.7	0.692	0.394
BMI ⩾ 25 kg/m^2	116	75	64.7	37	31.9	4	3.5	41	35.3		
Women											
BMI < 25 kg/m^2	420	270	64.3	134	31.9	16	3.8	150	35.7	0.604	0.389
BMI ⩾ 25 kg/m^2	133	80	60.2	46	34.6	7	5.3	53	39.9		

P-value by the χ^2 analysis among groups CC, CA and AA. *P**-value by the χ^2 analysis between groups CC and CA/AA. BMI = Body mass index.

the frequency of the 72Met allele of the preproghrelin gene is approximately 8% in the Caucasian population and approximately 2% in the black population in three different cohorts.[10] Compared with these previous studies, the frequency of the 72Met allele in our Japanese cohort was much higher than that observed in Caucasian or African populations, probably reflecting genetic/ethnic heterogeneity.

The Leu72Met polymorphism of preproghrelin was previously found in a group of obese French children and adolescents.[9] In this case, a significant association was observed between the 72Met allele and earlier age of onset of obesity. Additionally, obese Italian children and adolescents with the preproghrelin 72Met allele have also been reported to become obese earlier than homozygous patients for the wild Leu72 allele, even though 72Met allelic frequency was similar between obese and control groups.[8] These findings were not confirmed, however, in a group of extremely obese German children.[14] In addition, one report

Table 5 Metabolic variables and Leu72Met polymorphism of preproghrelin gene

	Men									Women								
	n	CC		CA		AA		CA/AA		n	CC		CA		AA		CA/AA	
		Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.		Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.
Total cholesterol (mM)[a]	1044	5.48	0.03	5.49	0.05	5.42	0.14	5.48	0.04	996	5.83	0.03	5.92	0.05	5.83	0.14	5.91	0.05
Triglyceride (mM)[a]	1027	1.48	0.04	1.53	0.06	1.32	0.16	1.51	0.10	977	1.20	0.03	1.23	0.04	1.21	0.10	1.23	0.04
HDL-C (mM)[a]	1044	1.49	0.01	1.48	0.02	1.49	0.06	1.48	0.02	996	1.71	0.02	1.71	0.02	1.71	0.06	1.71	0.02
LDL-C (mM)[a]	1035	3.40	0.03	3.42	0.05	3.36	0.13	3.42	0.04	980	3.57	0.03	3.63	0.05	3.62	0.14	3.63	0.05
Lipoprotein (a) (mM)[a]	1034	0.39	0.02	0.37	0.03	0.35	0.07	0.37	0.02	980	0.40	0.02	0.46	0.03	0.33	0.07	0.44	0.02
Glucose (mM)[b]	1049	5.71	0.04	5.74	0.05	5.91	0.15	5.75	0.05	1051	5.51	0.03	5.52	0.05	5.20	0.13	5.49	0.04
Insulin (μU/ml)[b]	1048	8.28	0.22	8.21	0.31	7.63	0.91	8.15	0.29	1050	8.23	0.19	8.57	0.27	8.02	0.74	8.51	0.25
HbA1c (%)[b]	1064	5.21	0.02	5.26	0.03	5.41	0.10	5.28	0.03	1071	5.16	0.02	5.15	0.03	5.06	0.07	5.14	0.02
HOMA-IR[b]	1048	2.20	0.08	2.13	0.11	2.06	0.33	2.13	0.11	1050	2.07	0.06	2.20	0.09	1.88	0.26	2.16	0.09

[a]Analysis of subjects who were not under lipid treatment. Adjusted for age. [b]Analysis of subjects who were not on oral hypoglycemic agents or insulin. Adjusted for age. No significant differences were observed in any metabolic values among three different genotypes (CC, CA and AA) or between CC and CA/AA. Analysis of variance and the Tukey–Kramer *post hoc* test or the unpaired Student's *t*-test between two groups. Abbreviations: HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein.

suggests that preproghrelin 72Met carrier status may be protective against fat accumulation.[10] A limited number of observations have been made on the relationship between preproghrelin Leu72Met polymorphism and overweight/obesity in middle-aged subjects, and no report has been published to date on older subjects. In a Swedish middle-aged female obese cohort, no difference of 72Met allele frequency was observed between obese subjects and controls.[7] However, the self-reported age of onset of weight problems tended to be lower among 72Met allele carrier obese subjects than among those without this allele.

In the present study, we observed a significant effect of the preproghrelin variant on overweight/obesity only in middle-aged men, as the 72Met allele was more commonly observed among overweight/obese middle-aged men. We also demonstrated that body weight change from weight at 18 years of age is associated with Leu72Met variants, given that middle-aged men with the 72Met allele had a greater body weight change than Leu72 homologous subjects. Similar trends were also observed for BMI, waist circumference and WHR in middle-aged men, but not in older men or in women when our population was subdivided into three subgroups according to preproghrelin genotype. Consequently, the 72Met allele may contribute to body weight change from adolescence to middle age in men but not in women. We observed the absence of the effect of Leu72Met genotypes on the anthropometric measurements in older man. Although we do not know the exact reasons, the effects of aging or environmental influences may overcome the genetic influence on the anthropometric measurements. The limitation of our study is that the weight at 18 years was recalled by the participants in the present study, as the documented measurements of weight at 18 years of age were not available. Although several studies have observed that adults are able to recall their earlier weights fairly accurately,[15] it is possible that the reported weight might not be accurate or under-reported. In fact, it has been reported that overweight women underestimated their earlier weights and that lean men overestimated their earlier weight.[16]

Based on recent studies, it appears that ghrelin may play a role in the glucose and lipid metabolisms. However, only limited data are currently available with regard to the effect of ghrelin polymorphism on these metabolisms. It has been reported that Leu72Met polymorphism is associated with triglyceride or lipoprotein (a) levels.[10,17] In the present study, however, we observed no association between serum lipid levels, fasting glucose, insulin, HbA1c or HOMA-IR levels and preproghrelin Leu72Met genotypes.

In the present study of a community-dwelling Japanese middle-aged to elderly cohort, we demonstrated that the 72Met allele of the preproghrelin gene is a contributing factor for midlife weight change in men but not in women or elderly men. However, Leu72Met polymorphism was not found to be associated with the metabolic variables studied.

Acknowledgements

This work was supported by Research Grants for Longevity Sciences (H15-Shi-02) from the Ministry of Health, Labor, and Welfare of Japan.

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Pflugers Arch - Eur J Physiol (2006) 452: 363–369
DOI 10.1007/s00424-005-0036-3

TEMPERATURE REGULATION

Youxue Wang · Kazuhiro Kimura · Ken-ichi Inokuma · Masayuki Saito · Yasuhide Kontani · Yoshinori Kobayashi · Nozomu Mori · Hitoshi Yamashita

Potential contribution of vasoconstriction to suppression of heat loss and homeothermic regulation in UCP1-deficient mice

Received: 11 October 2005 / Accepted: 7 December 2005 / Published online: 5 January 2006

Abstract To investigate the thermoregulatory mechanism in mice lacking uncoupling protein 1 (UCP1) from the viewpoint of heat loss, we measured oxygen consumptions (VO2), skin-surface temperatures (Tskin, an index of heat release), blood flows in the tails, and rectal temperatures (Trectal) of mice housed in an animal room under the standard thermal condition of ~23°C. Compared with wild-type ($Ucp1^{+/+}$) mice, adult UCP1-deficient ($Ucp1^{-/-}$) mice tended to show a reduced VO2. Thermograhic analysis of the acute response of $Ucp1^{-/-}$ mice to a small change (a drop of 1–2°C) in the ambient temperature revealed a sustained fall in the Tskin of $Ucp1^{-/-}$ mice; but this fall was only transient in $Ucp1^{+/+}$ mice. Analysis of tail blood flow under anesthesia clearly showed a stronger vasoconstrictor response in $Ucp1^{-/-}$ mice than in $Ucp1^{+/+}$ mice. Administration of a vasodilator, evodiamine, transiently increased Tskin in $Ucp1^{+/+}$ and $Ucp1^{-/-}$ mice similarly; whereas the induction of vasodilation caused a greater and more prolonged reduction in Trectal in $Ucp1^{-/-}$ mice than in $Ucp1^{+/+}$ mice. These results indicate that $Ucp1^{-/-}$ mice highly, or at least partly, rely on vasoconstriction for heat conservation to compensate for their UCP1 deficiency and to maintain homeothermy under the condition of normal housing temperature.

Keywords Thermoregulation · Heat release · Vasoconstriction · Uncoupling protein 1 · Knockout mouse

Y. Wang · Y. Kontani · N. Mori · H. Yamashita (✉)
Department of Aging Intervention,
National Institute for Longevity Sciences,
36-3 Gengo,
Obu, 474-8522, Japan
e-mail: hyamashi@nils.go.jp
Fax: +81-562-468461

K. Kimura · K. Inokuma · M. Saito
Department of Biomedical Sciences,
Graduate School of Veterinary Medicine, Hokkaido University,
Sapporo, 060-0818, Japan

Y. Kobayashi
Faculty of Applied Life Sciences,
Niigata University of Pharmacy and Applied Sciences,
Niigata, 956-8603, Japan

Present address:
Y. Wang
Department of Physiology,
UT Southwestern Medical Center at Dallas,
5323 Harry Hines Boulevard,
Dallas, TX 75390, USA

Present address:
Y. Kontani
Department of Microbiology,
Kanazawa Medical University,
1-1 Uchinada,
Ishikawa, 920-0293, Japan

Present address:
N. Mori
Department of Neuroanatomy and Molecular Neurobiology,
Nagasaki University School of Medicine,
1-12-4 Sakamoto,
Nagasaki, 852-8523, Japan

Introduction

In homeothermic animals, thermoregulation is an essential function to sustain life, i.e., to maintain body temperature and the wide variety of biochemical reactions in the body. Animals sense the changes in ambient temperature at all times through primary sensory neurons located in the periphery of the skin that transduce the signals to the central nervous system to maintain body temperature at a constant level by regulating the balance of heat loss and production [8, 29, 31]. Several physiological mechanisms such as cutaneous vasoreaction or insulation are involved in the control of heat loss in animals. In particular, cutaneous vasodilation is the major mechanism for heat loss from the skin surface. Effective heat loss is achieved through various body parts, e.g., the hand in humans and the tail in rats and mice, both of which have high surface-to-volume ratios, absence of hair or fur, and high densities of arteriovenous anastomoses. In rats, approximately 25% of the basic metabolic heat can be dissipated from the tail [34].

At present, adaptive nonshivering thermogenesis is recognized to be an important means of heat production, which is used not only for thermoregulation in the cold but also for the dissipation of excess caloric energy [2, 9, 12, 20]. Brown adipose tissue (BAT) is a tissue specialized for thermogenesis, which allows hibernators or rodents to live in cold environments. The characteristic feature of BAT is the presence of a specific mitochondrial protein, uncoupling protein 1 (UCP1), which is located in the inner mitochondrial membrane and functions to generate heat by uncoupling oxidative phosphorylation [2, 9, 17, 26]. Due to its unique function, UCP1 has prompted many investigators to examine its relationship to cold tolerance [5] or obesity [9, 17]. Studies using UCP1-deficient ($Ucp1^{-/-}$) mice or BAT-ablated transgenic mice have afforded great advances in our understanding of the physiological roles of UCP1 in thermoregulation and energy metabolism [4, 7, 10, 14, 18, 21, 22]. More specifically, although $Ucp1^{-/-}$ mice can acclimate to 18°C, which may be greatly owing to improved shivering thermogenesis and other unknown mechanisms, the mutant mice cannot survive long-term in severe cold, e.g., 4°C [4, 7]. However, $Ucp1^{-/-}$ mice can live normally and have almost the same life span as wild-type ($Ucp1^{+/+}$) mice under the standard temperature condition (~23°C) used for raising mice in many animal facilities [16], even though this room temperature is apparently below thermoneutrality for mice (approximately 28~32°C).

How do $Ucp1^{-/-}$ mice maintain their body temperatures at the standard animal room temperature? Are there any mechanisms contributing to thermoregulation besides shivering thermogenesis in the physiological situation lacking UCP1 nonshivering thermogenesis? To address these questions, we investigated how $Ucp1^{-/-}$ mice regulate homeothermy without UCP1, especially in terms of heat loss.

Materials and methods

Animals Ucp^{tm1} knockout mice on a congenic C57BL/6J background were kindly provided by Dr. Leslie Kozak (Pennington Biomedical Research Center). Adult mice of N12–N15 generations were used in this study. The mice were bred and reared at 23±1°C under artificial lighting for 12 h per day, and provided a standard chow (CE-2, CLEA Japan, Japan) and tap water ad libitum in the animal facility of the National Institute for Longevity Sciences. The experiments in the present study were performed according to our institutional guidelines for animal care.

Indirect calorimetry Whole body oxygen consumption (VO2) was measured by using an O_2 analyzer (MM-102R; Muromachi Kikai, Tokyo, Japan). Male mice (6–12 months old, 28.0±1.1 g for both genotypes, n=7 for each genotype) were housed in an airflow chamber at a room temperature of 24°C with standard chow and tap water ad libitum for 48 h, and the air samples were taken every 3 min for analysis, except when taken once per hour for calibration. The basal VO2 in each mouse was determined by averaging the data obtained during light and dark phases during the last 24 h (228 data points per 12 h in each phase).

Temperature measurement and thermographic analysis The body temperature of the mice was measured between 1000 and 1400 hours in the animal room at ~23°C with an electronic thermistor equipped with a rectal probe (TD-300, Shibaura Electronics, Tokyo, Japan). Changes in the skin-surface temperature (Tskin) of the mice were recorded at 10-s intervals by use of an infrared thermographic device, Thermo-Viewer (JTG-5200, JEOL, Tokyo, Japan), as pre-

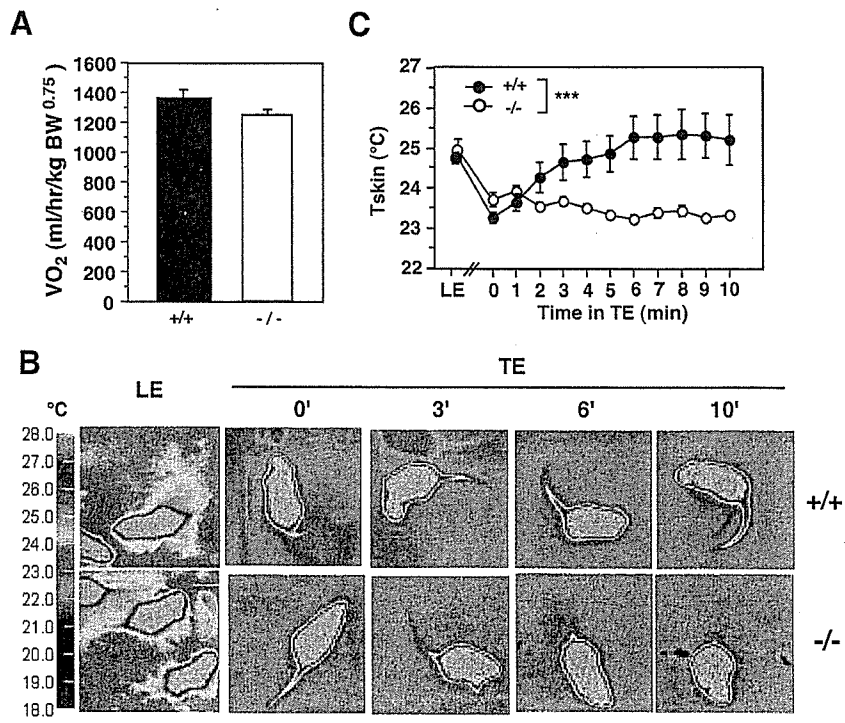

Fig. 1 Analyses of VO2 and heat release in $Ucp1^{-/-}$ mice. **a** VO2 of mice was measured for 48 h as described in the "Materials and methods". Data are expressed as mean±SE (n=7 for each genotype). **b**, **c** Regulation of heat release in response to a lowering of ambient temperature. Tskin of the unrestrained mouse was recorded thermographically for 10 min after the mice had been transferred from their regular cage (LE, 23.5–24.0°C) to a new cage without wooden chips (TE, 21.6–22.4°C). Time point just after the transfer was referred to as 0 min. $Ucp1^{+/+}$, n=17 and $Ucp1^{-/-}$, n=15 for the mice. Representative images of the analysis are presented. Data are expressed as mean±SE. *** P<0.001 vs $Ucp1^{-/-}$ groups

viously described [15], which is a good tool to noninvasively and successively evaluate heat release in conscious, unrestrained animals. We determined the Tskin in the area of the tail because it has no fur, thus facilitating accurate analysis, and because it is a crucial site for regulation of heat release in rodents [8, 27, 28]. After the Tskins of the mice in their regular cage [living environment (LE), 23.5–24.0°C] had been analyzed for several minutes, the mice were transferred individually to new cages without wooden chips [test environment (TE), 21.6–22.4°C]. This transfer gave mice a drop of approximately 1–2°C in the ambient temperature around them. Four cages were set in the field of the thermographic device, and the recording for 3–4 mice was done at the same time for 10 min after transferring the mice to the TE. The highest skin temperature in a fixed area of the tail was measured by using an image analyzing software (TG-5000CNTA, Japan Electron Optics Laboratory Datum, Tokyo, Japan).

In the experiments on the chemical induction of heat release, the mice were intraperitoneally administered evodiamine [3 mg/kg body weight (BW), Kisida Chemical, Japan], a natural vasodilator [3], or vehicle (10% dimethyl sulfoxide + 10% Tween80 + 80% saline). Compound solutions were kept at ~35°C and injected at a dose of 100 μl/g BW (3 mg/ml). The Tskin of mice was recorded for 25 min in a cage without wooden chips as described above. Similarly, the compound solutions were injected to examine the effect of heat release on the body temperature of the mice. The changes in the rectal temperature (Trectal) of mice were measured by using an electronic thermistor before (0 min) and after the injection (at 5, 30, 60, 120, and 180 min).

Measurement of blood flow Peripheral blood flow was assessed by using a laser Doppler perfusion monitor equipped with an optical probe (ALF21R, Advance, Tokyo, Japan) in an air-conditioned room of 22°C. Male mice (6–12 months old) were anesthetized with an intraperitoneal injection of urethane (1.3 mg/g body weight) and placed in the decumbent position on a heating plate prewarmed at 40°C before being monitored for core body temperature as described above. The mouse tail was placed in a custom-made holder such that it would not directly touch the heating plate; and the probe was positioned over the tail. When the Trectal reached approximately 37°C and the blood flow became stable (referred to as 0 min), the heater was turned off. After 30 min the heater was turned on again with a setting temperature of 45°C, which was changed to 36.5°C at 40 min.

Statistical analysis Data were expressed as the mean±SE. Significant differences among groups were assessed by analysis of variance (ANOVA) or repeated measure ANOVA with Fisher's protected least significant difference test.

Results

To examine the level of heat production in $Ucp1^{+/+}$ and $Ucp1^{-/-}$ mice, we measured whole body VO2 by using indirect calorimetry. As shown in Fig. 1a, the VO2 was not significantly different between the two genotypes, although it tended to be lower in $Ucp1^{-/-}$ mice than in $Ucp1^{+/+}$ mice at 24°C (p=0.0696).

We measured the skin-surface temperature (Tskin) of the tail, an index of heat release and vasoconstrictor response, by using an infrared thermographic device, because body

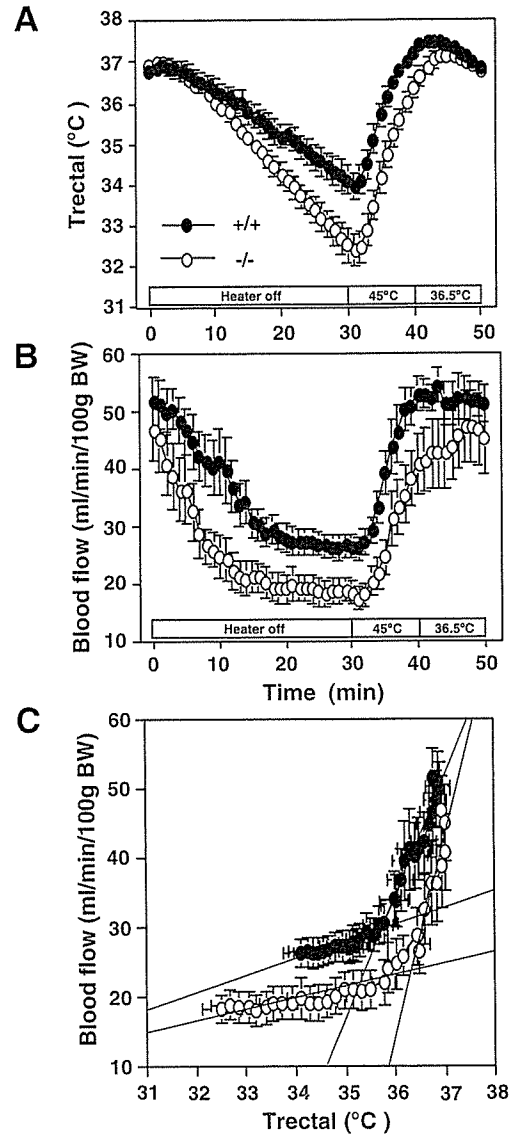

Fig. 2 Regulation of blood flow in the tail during the change in body temperature of mice. The Trectal (**a**) and tail blood flow (**b**) of anaesthetized mice were monitored at the same time under the condition of varying the temperature of the heating plate. After the Trectal reached approximately 37°C and the blood flow became stable (referred to as 0 min), the heater, prewarmed at 40°C, was turned off at 0 min, and turned on at 30 min with a setting of 45°C, and at 40 min with a setting of 36.5°C. The heater temperature at 30 min was approximately 26°C. Statistical differences between $Ucp1^{+/+}$ and $Ucp1^{-/-}$ mice at each time point were detected at 18–42 min in Trectal and at 7–37 min in blood flow (p<0.05). **c** Relation between Trectal and blood flow. The data in first 30 min were plotted. Data are expressed as mean±SE ($Ucp1^{+/+}$, n=10 and $Ucp1^{-/-}$, n=13). The changes in Trectal and blood flow were significantly different between genotypes (p<0.001).

temperature is controlled by the balance between heat loss and production. As shown in Fig. 1b,c the Tskin of the mice in their regular home cage (LE, 23.5–24.0°C) was not significantly different between the genotypes. To evaluate in terms of the regulation of heat loss the acute response to a small change in the ambient temperature, the mice were then transferred from their LE to the TE (21.6–22.4°C). After the transfer, the Tskin in the *Ucp1*$^{+/+}$ mice decreased immediately (0 min) and then increased with time (Figs. 1b,c). In the *Ucp1*$^{-/-}$ mice, however, the Tskin did not increase after the initial fall. The mice in each genotype were quite active and did not show obvious thermodefensive actions such as huddling or curling up during the measurements in the TE.

Based on the results shown in Fig. 1, we assumed that *Ucp1*$^{-/-}$ mice effectively suppressed heat release to maintain homeothermy. To test this hypothesis, we examined the blood flow of the tail during the change in body temperature of the mice under anesthesia (Fig. 2). After the heating plate had been turned off, the Trectal of the anesthetized mice was retained at ~37°C for a while and then began to decrease linearly with time, but the velocity of the decline was significantly greater in *Ucp1*$^{-/-}$ mice than in *Ucp1*$^{+/+}$ mice (0.174 and 0.107°C/min, respectively, Fig. 2a). The tail blood flow was reduced 47 and 20% in the respective *Ucp1*$^{-/-}$ and *Ucp1*$^{+/+}$ mice in the first 10 min ($p<0.05$, Fig. 2b). The reduction in blood flow reached a plateau faster in *Ucp1*$^{-/-}$ mice (at 13 min) than in *Ucp1*$^{+/+}$ mice (at 17 min), at which time the blood flow was about 45 and 56% of the steady-state level (time 0) in the *Ucp1*$^{-/-}$ and *Ucp1*$^{+/+}$ mice, respectively. When the heating plate was rewarmed, the blood flow was recovered in parallel with the increase in Trectal in both genotypes.

Figure 2c shows a 2-phase relationship between Trectal and blood flow of the mice during the first 30 min. The blood flow decreased greatly to a certain level at the beginning of the reduction in Trectal (referred to as the fast phase) and then dramatically changed to a phase in which it decreased gradually despite the great reduction in Trectal (referred to as the slow phase). When linear curves for each phase were made, the rates of reduction in blood flow per Trectal change were 17.7 and 29.0 ml min^{-1} 100 g^{-1} BW °C^{-1} in the fast phase and 2.4 and 1.7 ml min^{-1} 100 g^{-1} BW °C^{-1} in the slow phase in *Ucp1*$^{+/+}$ and *Ucp1*$^{-/-}$ mice, respectively.

To further verify the hypothesis that the inhibition of heat loss is a mechanism compensating for the lack of UCP1 thermogenesis, we examined the effect of heat loss due to vasodilation on the body temperature in the *Ucp1*$^{-/-}$ mice. A natural vasodilator, evodiamine induced a marked increase in the Tskin of the *Ucp1*$^{-/-}$ mice, as it did in the *Ucp1*$^{+/+}$ mice (Fig. 3a). The temperature transiently increased by approximately 5°C after the injection of evodiamine, peaked at approximately 5 min, and then decreased to the level before the injection. After the injection of evodiamine, the mice became inactive in the prone position for a while and then huddled. Although the pattern of heat release in response to evodiamine administration was similar between the two genotypes, the changes in core body temperature were quite different between them (Fig. 3b). The Trectal in *Ucp1*$^{+/+}$ mice was transiently decreased by 1°C at 5 min after evodiamine administration, but the decrease was reversed by 30 min. In the case of *Ucp1*$^{-/-}$ mice, the decrease in Trectal was greater and sustained even at 30 min after evodiamine administration. The Trectal in *Ucp1*$^{+/+}$ mice, but not that in *Ucp1*$^{-/-}$ mice, was slightly but significantly increased by approximately 0.5°C by the vehicle injection ($p<0.01$).

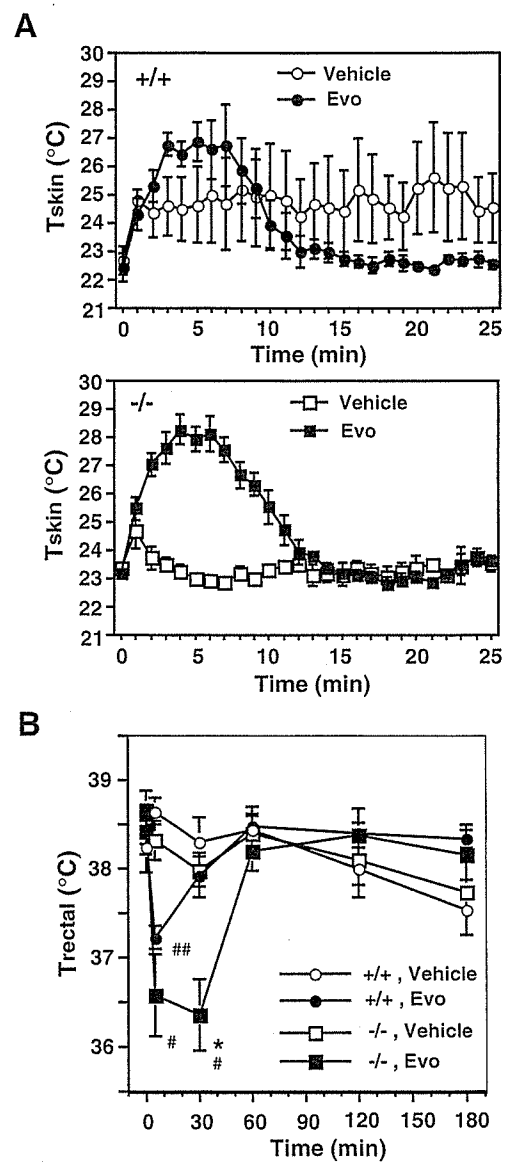

Fig. 3 Effects of evodiamine on heat release and body temperature in *Ucp1*$^{-/-}$ mice. **a** Induction of heat release by evodiamine (Evo). The Tskin of mice was recorded for 25 min after the intraperitoneal administration of vehicle or Evo (3 mg/kg BW). Data are expressed as mean±SE ($n=5$, except for the Evo group in *Ucp1*$^{+/+}$ mice, $n=4$). The patterns of Tskin change in vehicle and Evo groups were significantly different in both genotypes ($p<0.05$ and $p<0.001$ in *Ucp1*$^{+/+}$ and *Ucp1*$^{-/-}$ mice, respectively). **b** Change in Trectal of mice administered with vehicle or Evo. The Trectal was measured at the indicated time point. * $p<0.05$ vs *Ucp1*$^{+/+}$ mice in the same condition. # $p<0.05$ and ## $p<0.01$ vs vehicle condition in each genotype at the same time point

Discussion

The function of BAT of heat production through UCP1 is a crucial element of thermoregulation in rodents, which is affected profoundly by the ambient temperature and diet. In fact, UCP1 deficiency in mice impairs tolerance against a cold temperature of ~5°C [4]. However, *Ucp1*$^{-/-}$ mice can live normally at a room temperature of ~23°C [16]. In addition, Liu et al. [18] recently reported that *Ucp1*$^{-/-}$ mice showed no significant difference in VO2 but rather had a higher body temperature at 20°C compared with *Ucp1*$^{+/+}$ mice, suggesting compensatory mechanisms including heat conservation for thermoregulation at the ambient temperature. These findings were unexpected, because Klaus et al. [14] demonstrated that BAT ablation, causing a marked reduction in UCP1 amount, attenuated heat production, as judged from the measurement of VO2, at ambient temperatures between 20 and 37°C in the adult mutant mice, which showed a lower Trectal at 24°C when compared with control mice [14].

In the present study, we also failed to detect a significant difference in VO2 between the two genotypes at 24°C; however, a trend toward a reduction in VO2 was observed in *Ucp1*$^{-/-}$ mice compared to *Ucp1*$^{+/+}$ mice (Fig. 1a). These results basically support the previous findings [7, 18] that *Ucp1*$^{-/-}$ mice possess the ability to generate a level of heat to maintain their body temperature at standard room temperatures (i.e., 20–25°C). But, at the same time, the VO2 data suggest a possible reduction in overall heat production in *Ucp1*$^{-/-}$ mice, and even though it was, at most, a 10% reduction, the reduced heat production may affect the strategy of thermoregulation to decrease heat loss. This subtle decrease in VO2 may also be a latent factor to induce obesity, because we recently reported that *Ucp1*$^{-/-}$ mice develop diet-induced obesity with age in spite of their resistance against it at a young age [16].

We measured Tskin by using infrared thermography, which is a good tool to noninvasively and successively assess heat loss from the body in conscious, unrestrained animals. The importance of this methodology in thermoregulation research has been recently highlighted by Rudaya et al. [28]. They also showed that thermoregulatory responses in mice are greatly affected by the experimental conditions such as ambient temperature and procedures. In our experimental setup, we first examined the Tskin in mice in their regular cage (at ~24°C) because they lived in this thermal condition throughout their lives. As was shown in Fig. 1b,c we could not detect any significant difference in Tskin between genotypes in the home cage of mice (LE). This result may suggest that the mice had adapted to the thermal condition and improved thermal comfort in the LE irrespective of the existence of UCP1, although the ambient temperature was far below thermoneutrality (~30°C). We then examined the acute responses of the mice to a change in their thermal environment. For this purpose, we used a procedure in which the mice were exposed to a lower environmental temperature (TE) than that in their home cage. As expected, the Tskin in mice decreased quickly just after the exposure to TE, suggesting a typical response to reduce skin circulation and heat release, whereas the regulation of heat release after the initial response was completely different between the *Ucp1*$^{+/+}$ and *Ucp1*$^{-/-}$ mice. We do not deny that this experimental procedure includes several stresses such as isolation or new cages besides the expected thermal stress. The sensitivities to those stresses may also vary among mouse strains. Nevertheless, the remarkable difference in the time course of the change in Tskin between the two genotypes on a C57BL/6J background clearly shows a difference in the thermoregulatory response in our experimental setup. Thus, the results of thermographic analysis suggest that the sustained repression of heat loss from the body surface might be required for thermoregulation in *Ucp1*$^{-/-}$ mice in the case of decreasing ambient temperature. This suggestion is supported by the analysis of vasoreaction, in which *Ucp1*$^{-/-}$ mice were more dependent on vasoconstriction to prevent heat loss and to maintain body temperature when compared to *Ucp1*$^{+/+}$ mice (Fig. 2). The contribution of suppression of heat loss to maintain body temperature was recently reported in fasted rats with reduced production of metabolic heat [24].

Nevertheless, the ability of vasoconstriction to prevent the decrease in body temperature was limited because the *Ucp1*$^{-/-}$ mice had decreases in body temperature which were more rapid than those in the *Ucp1*$^{+/+}$ mice, leading to hypothermia under the condition of a gradual decrease in heater temperature, in spite of the stronger vasoconstrictor response of the *Ucp1*$^{-/-}$ mice (Fig. 2). The limited capacity of vasoconstriction for keeping homeothermy was obvious even in the *Ucp1*$^{+/+}$ mice from the analysis in Fig. 2c. Namely, the regulation of blood flow contributed to maintenance of body temperature only within the narrow range close to the normal body temperature (~37°C). In this fast phase, a stronger suppression (about 1.6-fold) of blood flow was required in the mutant mice than in the *Ucp1*$^{+/+}$ mice to keep the same body temperature. Meanwhile, when body temperature decreased beyond a certain threshold (35.7 and 36.3°C in *Ucp1*$^{+/+}$ and *Ucp1*$^{-/-}$ mice, respectively, in Fig. 2c), further suppression of blood flow was restricted and the body temperature continued to decrease in the slow phase. These results strongly suggest that the absence of UCP1 contributed to the faster and greater reduction in the body temperature in the mutant mice than in the *Ucp1*$^{+/+}$ mice.

Anesthetization of the mice was necessary for accurate measurements of tail blood flow. This experimental condition also appeared to be important to evaluate the difference in vasoreaction under a condition excluding the influence of stresses with the environmental change in the thermographic analysis using conscious mice. However, we cannot rule out the effect of anesthetization on the thermal response in the mice under urethane anesthesia, because such treatment is known to affect thermoregulation, reducing the thresholds for vasoconstriction and shivering thermogenesis [30]. Particularly, the latter appears to become more important in thermoregulation in *Ucp1*$^{-/-}$ mice than in *Ucp1*$^{+/+}$ mice when the animals are exposed to cold [7], which was supported by the fact that the body

temperatures of *Ucp1*$^{-/-}$ mice decreased faster than those of *Ucp1*$^{+/+}$ mice, while both were under anesthesia.

Vasoconstriction is mainly mediated by the action of norepinephrine through sympathetic neural stimulation, which is enhanced by cold stimuli [6, 23]. The importance of vasoconstriction in preventing heat loss has been verified by the study of mice deficient in dopamine β-hydroxylase, in which the animals, lacking norepinephrine and epinephrine, were unable to maintain their body temperatures in the cold due to the failure of peripheral vasoconstriction [32]. The thermogenic function (activity and amount of UCP1) and capacity (increase in number of brown adipocytes) of BAT are also under the control of norepinephrine via a β-adrenergic pathway [2, 19]. So, the augmented vasoconstrictor response in *Ucp1*$^{-/-}$ mice might be attributed to increased norepinephrine release and/or responsiveness to this neurotransmitter, probably as a response to the animals sensing the deficit in UCP1-dependent thermogenesis.

Vasodilation is also critical as the final step to release extra heat for thermoregulation. This response is controlled through temperature- and capsaicin-sensitive peripheral sensory neurons [11, 25]. When the heating plate was rewarmed (Fig. 2), the blood flow was recovered quickly in parallel with the increase in Trectal in both genotypes, and the extra heat was released through the increase in the peripheral blood flow, confirming that the control of vasoreaction is a crucial part of homeothermic regulation.

To further analyze the influence of vasodilation on homeothermic regulation in the absence of UCP1 thermogenesis in conscious, unrestrained animals, we used evodiamine, a compound in the fruit of *Evodia rutaecarpa*, to promote heat release from the skin surface (Fig. 3a). This compound has a vasodilative effect like that of capsaicin and has been prescribed in Chinese medicine as a treatment for feeling cold [3, 13]. Kobayashi et al. previously reported the effective dose of evodiamine for increasing the Tskin in normal mice without hypothermia [15]. In the present study, the induction of vasodilation by the same dose of evodiamine (3 mg/kg BW) caused a greater and more prolonged reduction in body temperature in *Ucp1*$^{-/-}$ mice than in *Ucp1*$^{+/+}$ mice, indicating that the suppression of heat loss greatly contributes to homeothermy in *Ucp1*$^{-/-}$ mice. We do not deny the contribution of muscular shivering thermogenesis to thermoregulation in *Ucp1*$^{-/-}$ mice, as its alternative function in *Ucp1*$^{-/-}$ mice was reported by Golozoubova et al. [7]. Nevertheless, the shivering thermogenesis might be insufficient to compensate for the lack of UCP1 nonshivering thermogenesis in homeothermic regulation after acute heat loss induced by evodiamine.

The skin temperatures of *Ucp1*$^{+/+}$ mice, but not of *Ucp1*$^{-/-}$ mice, were increased even by the vehicle injection (Fig. 3b). This result was similar to that for a β3-adrenergic receptor agonist, BRL37344, the effect of which was blunted in *Ucp1*$^{-/-}$ mice (data not shown). Because norepinephrine can be released in response to various stresses such as handling or injection [1], the increase in heat release in the vehicle control of *Ucp1*$^{+/+}$ mice might have been induced by the stress of the injection through norepinephrine-activated UCP1-dependent thermogenesis.

The amount of body fat may also contribute to heat conservation [5, 33]. Particularly, the thickness of subcutaneous fat affects the skin-to-ambient temperature thermal gradient and seems to be an important factor for the thermoinsulative property of this fat. However, this factor may not explain the distinct feature of defending against heat loss in *Ucp1*$^{-/-}$ mice because there was no significant difference in the thickness of subcutaneous fat or of skin in the abdominal regions, nor in body weight, between the genotypes of the age-matched mice (our unpublished data).

Although animals always face fluctuations in the ambient temperature even in an air-conditioned facility, mice deficient in UCP1, the major protein for thermogeneration, can live out their natural lives by maintaining homeothermy [16]. The present findings that rapid repression of heat release from the tail by a vasoconstrictive response and easy loss of body temperature after forced vasodilation in *Ucp1*$^{-/-}$ mice strongly suggest that inhibition of heat loss by repressing heat release from the skin surface is at least a part of the compensatory mechanism for maintaining homeothermy in *Ucp1*$^{-/-}$ mice.

Acknowledgement The authors thank Dr. L. P. Kozak for the UCP1-deficient mice. This work was supported by a Research Grant for Longevity Sciences (15C-8) from the Ministry of Health, Labor, and Welfare to H. Y.

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Am J Physiol Endocrinol Metab 290: E1014–E1021, 2006.
First published December 20, 2005; doi:10.1152/ajpendo.00105.2005.

Indispensable role of mitochondrial UCP1 for antiobesity effect of β_3-adrenergic stimulation

Ken-ichi Inokuma,[1] Yuko Okamatsu-Ogura,[1] Asako Omachi,[1] Yukiko Matsushita,[1] Kazuhiro Kimura,[1] Hitoshi Yamashita,[2] and Masayuki Saito[1]

[1]*Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo;* [2]*Department of Molecular Genetics, National Institute for Longevity Sciences, Obu, Japan*

Submitted 10 March 2005; accepted in final form 16 December 2005

Inokuma, Ken-ichi, Yuko Okamatsu-Ogura, Asako Omachi, Yukiko Matsushita, Kazuhiro Kimura, Hitoshi Yamashita, and Masayuki Saito. Indispensable role of mitochondrial UCP1 for antiobesity effect of β_3-adrenergic stimulation. *Am J Physiol Endocrinol Metab* 290: E1014–E1021, 2006. First published December 20, 2005; doi:10.1152/ajpendo.00105.2005.—Mitochondrial uncoupling protein-1 (UCP1) has been thought to be a key molecule for thermogenesis during cold exposure and spontaneous hyperphagia and thereby in the autonomic regulation of energy expenditure and adiposity. However, UCP1 knockout (KO) mice were reported to be cold intolerant but unexpectedly did not get obese even after hyperphagia, implying that UCP1 may not be involved in the regulation of adiposity. Treatment of obese animals with β_3-adrenergic agonists is known to increase lipid mobilization, induce UCP1, and, finally, reduce body fat content. To obtain direct evidence for the role of UCP1 in the anti-obesity effect of β_3-adrenergic stimulation, in the present study, UCP1-KO and wild-type (WT) mice were fed on cafeteria diets for 8 wk and then given a β_3-adrenergic agonist, CL-316,243 (CL), or saline for 2 wk. A single injection of CL increased whole body oxygen consumption and brown fat temperature in WT mice but not in KO mice, and it elicited almost the same plasma free fatty acid response in WT and KO mice. WT and KO mice increased similarly their body and white fat pad weights on cafeteria diets compared with those on laboratory chow. Daily treatment with CL resulted in a marked reduction of white fat pad weight and the size of adipocytes in WT mice, but not in KO mice. Compared with WT mice, KO mice expressed increased levels of UCP2 in brown fat but decreased levels in white fat and comparable levels of UCP3. It was concluded that the anti-obesity effect of β_3-adrenergic stimulation is largely attributable to UCP1, but less to UCP2 and UCP3, and thereby to UCP1-dependent degradation of fatty acids released from white adipose tissue.

uncoupling protein-1; adiposity; CL-316,243; energy expenditure; hyperphagia

UNCOUPLING PROTEIN (UCP) IS A MOLECULE, as its name suggests, that uncouples mitochondrial oxidative phosphorylation by bypassing the electrochemical gradient across the inner membrane from the F1-ATPase and thereby dissipates energy as heat. Among several isoforms of the UCP family so far reported in mammals, UCP1 is the only one whose physiological importance has been firmly established; that is, UCP1 is present exclusively in brown adipose tissue (BAT), an organ specified for nonshivering thermogenesis during cold acclimation, arousal from hibernation, and recovery from anesthetic hypothermia (2). UCP1 has also been proposed to be involved in diet-induced thermogenesis, as well as cold-induced nonshivering thermogenesis, and play a significant role in the control of energy expenditure and whole body energy balance. This is supported by the observations, for example, that spontaneous overfeeding of highly palatable diets and/or high-fat diets gives rise to increased energy expenditure (oxygen consumption) in association with BAT hyperplasia and increased UCP1 contents in the same way as seen after cold acclimation (1, 2, 11, 20). It is also known that the thermogenic activity and UCP1 expression of BAT are decreased in most genetic and hypothalamic obese animals (18, 19, 21). Thus it seems likely that UCP1 is one of the key molecules for adaptive thermogenesis and energy expenditure in response to changes in energy intake, and thereby its dysfunction contributes to the development and maintenance of obesity.

However, the above-mentioned view was challenged by the findings of Enerback et al. (5) and Liu et al. (13) that transgenic mice with complete absence of UCP1 are not obese, but rather lean compared with wild-type control (WT) mice, both on normal and on high-fat diets, although they are apparently cold intolerant. This unexpected finding may suggest the existence of some critical molecules, other than, or in addition to, UCP1, that dissipate excess amounts of energy. Other UCP isoforms, such as UCP2 and UCP3, which are expressed ubiquitously in many tissues and abundantly in skeletal muscle, respectively, may be possible candidates because UCP2 expression is upregulated in BAT of UCP1-deficient mice (5). This possibility, however, has not been evidenced yet. Thus the precise role and significance of energy expenditure by UCP1 in body fat regulation are still to be debated.

UCP1 thermogenesis in BAT is under direct control of sympathetic nerves abundantly entering into this tissue; that is, norepinephrine released from the sympathetic nerve endings stimulates the β-adrenergic receptor (AR)-adenylate cyclase-protein kinase A signaling pathway and activates hormone-sensitive lipase. The released fatty acids activate UCP1 and are oxidized as a major substrate for thermogenesis. Because the same signaling pathway is also present in white adipose tissue (WAT), where UCP1 is absent, the activation of this pathway is expected to lead to UCP1-mediated energy dissipation of fatty acids released from WATs. In fact, it has been demonstrated that various agonists specific to the β_3-AR expressed predominantly in white and brown adipocytes effectively activate the pathway noted above, and chronic treatment of obese animals with β_3-AR agonists, as expected, reduces body fat content (10, 12, 16). Thus the anti-obesity effect of β_3-AR

Address for reprint requests and other correspondence: M. Saito, Dept. of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan (e-mail: saito@vetmed.hokudai.ac.jp).

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agonists has been believed to be due to the activation of UCP1. However, to our knowledge, there has been no direct evidence for this idea because almost all previous reports (10, 12) have shown only a parallel relationship between UCP1 activation and reduced adiposity but not the relation of cause and effect. Moreover, the observation of obesity resistance of UCP1-deficient mice noted above seems to not support the critical role of UCP1 in the long-term regulation of adiposity. Accordingly, to determine whether UCP1 is indispensable for the anti-obesity effect of β_3-AR agonists, in the present study we examined both short- and long-term effects of a selective β_3-AR agonist, CL-316,243 (CL), on the thermogenic and lipomobilizing activities and adiposity of UCP1 knockout (KO) mice, comparing them with those of WT control mice. Our results clearly indicate that the fat-reducing effect of β_3-AR stimulation is largely attributable to the activation of UCP1 thermogenesis.

MATERIALS AND METHODS

Animals. UCP1-KO ($UCP1^{-/-}$) mice on a congenic C57BL/6J background were generated by backcross mating of heterozygous ($UCP1^{+/-}$) mice on a mixed 129/SvPas and C57BL/6J background with C57BL/6J mice 15 times [mice were kindly given by Dr. L. Kozak (Pennington Biomedical Research Center, Baton Rouge, LA)]. All WT ($UCP1^{+/+}$) mice were C57BL/6J. They were housed in plastic cages placed in an air-conditioned room at 26°C with a 12:12-h light-dark cycle (lights on at 0700–1900) and given free access to laboratory chow (Oriental Yeast, Tokyo, Japan) and tap water. The experimental procedures and care of animals were approved by the Animal Care and Use Committee of Hokkaido University.

Acute responses of thermogenesis and lipolysis to CL. BAT thermogenesis in response to a single injection of the β_3-AR agonist CL (American Cyanamid, Pearl River, NY) was assessed by measuring the temperature changes in BAT and the rectum, as described previously (22). Briefly, male mice (10–30 wk old) fasted overnight were anesthetized with pentobarbital sodium (50 mg/kg ip), a small incision was made above the scapula, and the interscapular brown fat pads were partially separated from the muscle below, with the vasculature and nerve supplies to the pads being left intact. Then, mice were placed on a heat plate, and a plastic-coated thermistor with a diameter of 1 mm was placed under the fat pads. Another thermistor was also inserted into the rectum, and the plate was heated gently. After the rectal temperature reached a steady level at about 37°C, CL (0.1 mg/kg) or saline was injected intraperitoneally, and the temperature changes were monitored for 20 min.

To assess lipolytic response to CL in WAT, CL (0.1 mg/kg) or saline was injected intraperitoneally into overnight-fasted conscious mice, and blood (20 μl) was taken from the tail vein 0–4 h after the injection. Plasma free fatty acid concentrations were measured enzymatically using a kit (NEFA C test; WAKO Pure Chemical, Tokyo, Japan).

Whole body oxygen consumption was measured for 2 h after intraperitoneal injection of CL (0.1 mg/kg) or saline into conscious mice by the use of an open-circuit-type metabolic chamber (MK-5000; Muromachi Kikai, Tokyo, Japan) in a room kept at 26°C.

Diet and chronic treatment with CL. When mice became 10–12 wk old, they were divided into three groups consisting of four males and four females in each group. One group was fed on laboratory chow as previously described, whereas the other two groups were kept on cafeteria feeding for 10 wk, during which they were allowed free access to, in addition to laboratory chow, two kinds of snacks with various tastes (ChocoCrisp; Nisshin Cisco and Mire-Fry; Watayoshi Seika, Nagoya, Japan) that contained 68.1–70.5 g carbohydrate, 18.8–19.8 g fat (mixture of rapeseed, sunflower, and coconut oil), 7.7–7.8 g protein, and 481–483 kcal in 100 g. Laboratory chow contained 54 g carbohydrate, 5.1 g fat, 23.8 g protein, and 357 kcal in 100 g. During the last 2 wk, the cafeteria-feeding groups were subcutaneously given either CL (0.1 mg/kg) or saline once a day at 1300–1400. The laboratory chow-fed mice were similarly injected with saline. Body weight and the amount of food intake were measured every day. For calculation of total energy intake, the amount of actual intake of individual foods (laboratory chow and snacks) was measured after those lying scattered in the cages were corrected for. At *week 10*, all mice were killed by cervical dislocation at 1000–1300, and fat pads of various regions (interscapular BAT and inguinal, perigonadal, retroperitoneal, and mesenteric adipose tissues) and some other tissues were quickly removed and weighed. Tissue specimens were transferred into RNALater (Invitrogen, Carlsbad, CA) for RNA analysis, 10% buffered formalin for conventional histological examinations, or liquid nitrogen for Western blot analysis.

UCP mRNA analysis. Total RNA was extracted with the use of RNALater according to the manufacturer's protocol, and mRNA levels of UCP1, UCP2, and UCP3 were measured semiquantitatively by real-time RT-PCR using respective cDNA fragment as a standard and expressed as relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Briefly, 2 μg of total RNA were reverse transcribed with an oligo(dT) 15-adaptor primer and MMLV reverse transcriptase (Invitrogen). Real-time PCR was performed on a fluorescence thermal cycler (LightCycler system; Roche Diagnostics, Mannheim, Germany), using SYBR Green I as a double-strand DNA-specific dye according to the manufacturer's protocol. Primers used were 5′-GTG AAG GTC AGA ATG CAA GC-3′ and 5′-AGG GCC CCC TTC ATG AGG TC-3′ for mouse UCP1, 5′-GGC TGG TGG TGG TCG GAG AT-3′ and 5′-CCG AAG GCA GAA GTG AAG TG-3′ for mouse UCP2, 5′-GAG CGG ACC ACT CCA GCG TC-3′ and 5′-TGA GAC TCC AGC AAC TTC TC-3′ for mouse UCP3, and 5′-GAA GGT CGG TGT GAA CGG ATT-3′ and 5′-GAA GAC ACC AGT AGA CTC CAC GAC ATA-3′ for mouse GAPDH.

Histological examinations. Sections of formalin-fixed tissue specimens were stained with hematoxylin-eosin and examined light microscopically. The average diameter of adipocytes of each mouse was calculated from those of 100–200 cells at three different sections.

Data analysis. All values were presented as means ± SE, unless otherwise specified, and analyzed by analysis of variance with post hoc testing by the Scheffé's multiple range test.

RESULTS

Acute responses to CL of lipomobilization, BAT thermogenesis, and oxygen consumption. To confirm the impaired responses of thermogenic activity of BAT of UCP1-KO mice, we first monitored temperature changes in the interscapular BAT and the rectum after a single injection of CL. Although the basal temperatures of the BAT and rectum did not differ between UCP1-KO and WT mice, BAT temperature was slightly lower than rectal temperature in all mice. In WT mice, CL injection elicited a rapid rise in BAT temperature in couples of minute followed by a gradual rise in rectal temperature (Fig. 1, *A* and *B*). In contrast, in UCP1-KO mice, the temperature responses were much less. Saline injection elicited no temperature change in either type of mice. Being consistent with these results, CL increased the whole body oxygen consumption by 46% in WT mice, whereas it did not change in UCP1-KO mice (Fig. 1*C*). To assess lipolytic response in WAT, changes in plasma free fatty acid concentration were also monitored after CL injection. As shown in Fig. 1*D*, CL produced remarkable and sustained rises in the fatty acid concentrations for ≥4 h. There was no notable difference in the fatty acid responses between UCP1-KO and WT mice. It was

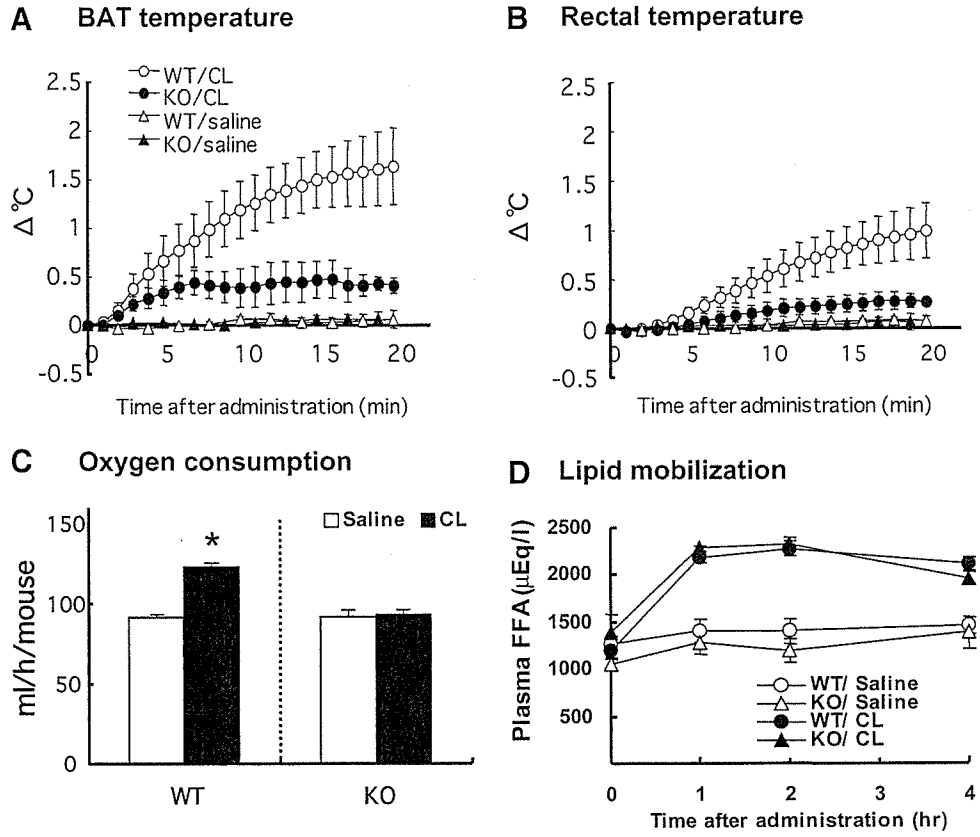

Fig. 1. Acute responses of brown adipose tissue (BAT) thermogenesis, lipolysis, and energy expenditure to CL-316,243 (CL). Wild-type (WT) and uncoupling protein (UCP)1 knockout (KO) male mice were anesthetized, and temperature changes of interscapular BAT (*A*) and rectum (*B*) were monitored after ip injection of CL (0.1 mg/kg) or saline. Values are means ± SE for 4 mice. Difference between values of WT and UCP1-KO mice was significant ($P < 0.05$) at 10 min and thereafter. *C*: whole body oxygen consumption was measured for 2 h after ip injection of CL (0.1 mg/kg) or saline to free-moving male mice. Values are means ± SE for 6 mice. *$P < 0.05$ vs. saline. *D*: to assess lipolytic response of white adipose tissue (WAT) to CL, male mice were fasted overnight and injected ip with saline or CL (0.1 mg/kg). Changes in plasma free fatty acid concentration were monitored for 4 h. Values are means ± SE for 4 mice. Difference between values of CL- and saline-injected mice was significant ($P < 0.05$) at all time points except 0 h.

thus confirmed that UCP1-KO mice are incapable of increasing BAT thermogenesis and energy expenditure in response to acute β_3-AR stimulation, although they can respond normally in lipid mobilization from WAT.

Effects of cafeteria feeding and CL treatment on body and tissue weights. Mice were kept in a cafeteria-feeding condition for 10 wk, during which they were allowed free access to palatable foods, in addition to laboratory chows, for 10 wk. Female WT mice kept under such a cafeteria-feeding condition, compared with those kept on laboratory chow, took 43% more energy due to a large intake of snacks despite decreased intake of laboratory chow (Fig. 2*C*). In parallel, these mice gained their body weight more rapidly, and after 8 wk they weighed more (26.4 ± 1.5 vs. 23.1 ± 0.7 g). Similar to WT mice, female UCP1-KO mice were also hyperphagic (+37%) and weighed slightly more (24.8 ± 0.8 vs. 22.4 ± 0.5 g) when kept under the cafeteria-feeding condition. Because quite similar effects of cafeteria feeding were also found in male mice, the data obtained from male and female mice were combined and are summarized in Fig. 2. After the 8-wk period, cafeteria-fed mice were injected with either CL or saline every day for 2 wk while they were kept under the same feeding condition. As shown in Fig. 2, *A* and *B*, the CL treatment stopped the weight gain in WT mice but not in UCP1-KO mice, which gained more weight, similarly to those treated with saline. The total energy intake during the 2-wk period was not affected by CL treatment (Fig. 2*C*).

At *week 10*, all mice were killed and their individual tissues were weighed. There was no significant difference in the weights of liver and some other organs such as skeletal muscle, brain, heart, and kidney among the three groups of both WT and UCP1-KO mice. In contrast, the weight of fat pads, particularly those of WAT, was markedly influenced by cafeteria feeding and CL treatment; that is, WAT of cafeteria-fed mice was apparently heavier (+99% in WT and +75% in UCP1-KO mice) than that of mice fed on laboratory chow. WATs of the four regions of UCP1-KO mice were similar to those of WT mice, except for perigonadal WAT, which was smaller. The effects of cafeteria feeding were almost abolished by the CL treatment in WT mice but not in UCP1-KO mice (Fig. 3*A*). In WT mice, the weight-reducing effect of CL treatment was similarly observed in all WAT regions (Fig. 3*B*). The weight of interscapular BAT was not changed by cafeteria feeding or CL treatment in WT mice. The BAT weight of UCP1-KO mice was heavier than that of WT mice and increased by cafeteria feeding, but little affected by CL treatment.

Histology of WAT and BAT. Figure 4 shows the histology of BAT and WAT. Interscapular BAT consisted of multilocular adipocytes in both WT and UCP1-KO mice but with apparently larger lipid droplets in UCP1-KO mice, particularly in those kept under the cafeteria feeding condition (Fig. 4*A*). In perigonadal WAT, almost all adipocytes were unilocular (Fig. 4*C*), and cafeteria feeding increased their size similarly in WT (48 ± 4 vs. 36 ± 3 μm in diameter, $P < 0.05$) and UCP1-KO mice (45 ± 6 vs. 34 ± 2 μm, $P < 0.05$). When treated with CL, the adipocytes became smaller, to 35 ± 3 μm ($P < 0.05$), in WT mice but not in UCP1-KO mice (42 ± 2 μm). In inguinal WAT of WT mice, most adipocytes were unilocular, and multilocular cells were observed to be abundant after CL treatment (Fig. 4*B*). In inguinal WAT of UCP1-KO mice, however, abundant multilocular adipocytes were observed regardless of whether they were treated with CL or not.