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Brief Genetic Analysis

No Association between rs7566605 Variant and Being Overweight in Japanese

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Abstract

KUZUYA, MASAFUMI, FUJIKO ANDO, AKIHISA IGUCHI, AND HIROSHI SHIMOKATA. No association between rs7566605 variant and being overweight in Japanese. *Obesity.* 2007;15:2531–2534.

It has recently been demonstrated that a common single-nucleotide polymorphism (rs7566605) upstream of the transcription start site for the insulin-induced gene 2 is associated with obesity in several European/European origin or African-American cohorts. We tested whether this variant is also linked to overweight among Asian populations. Our sample included 2233 randomly selected, community-dwelling, middle-age and older Japanese people (men, 1128; women, 1105; age, 40 to 79 years; C allele frequency, 0.32). We observed that there were no differences in BMI levels [men, 22.9 ± 0.3 (mean ± standard error) vs. 22.9 ± 0.1, $p = 0.820$; women, 22.8 ± 0.3 vs. 22.9 ± 0.1, $p = 0.792$], waist circumferences and hip circumferences, waist-to-hip ratio, and fat mass between rs7566605 GG/GC and CC genotypes in both genders. In addition, logistic regression analysis, using age and sex as covariates, revealed no association of the single-nucleotide polymorphism with overweight (BMI ≥25) between rs7566605 genotypes in the Japanese cohort (CC vs. CG/GG, odds ratio = 1.18; 95% confidence interval = 0.84 to 1.65, $p = 0.333$; CC vs. GG, odds ratio = 1.19, 95% confidence interval = 0.84 to 1.69, $p = 0.325$). No significant associations were observed between polymorphism and glucose or insulin levels. These results suggested no association of the rs7566605 variant with overweight in Japanese people.

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Key words: glucose metabolism, genotype, BMI, body weight, insulin resistance

Recently, Herbert et al. (1) demonstrated that a common genetic single-nucleotide polymorphism (rs7566605) upstream of the transcription start site for insulin-induced gene 2 was associated with obesity in 694 individuals of the National Heart, Lung, and Blood Institute-Framingham Heart Study. Analysis suggests that rs7566605 CC homozygotes (C allele frequency, 0.37) have higher BMI levels than individuals with GC or GG genotypes, regardless of sex or age. This finding was replicated in four of five populations studied. A meta-analysis of all four case-control samples showed that CC homozygosity was also significantly associated with obesity (BMI ≥30 kg/m^2), with an odds ratio (OR)[1] of 1.22 [95% confidence interval (CI), 1.05 to 1.42]. However, more recent studies do not support the association of the rs7566605 polymorphism with obesity for the different samples (2–4). There may be many reasons that the association is not seen in these studies, including those related to study design, underlying genetic heterogeneity of populations, and different environmental exposures. However, most of the samples studied were of European/European origin or African Americans. We tested whether this variant is also linked to obesity/overweight among Asian populations. Our sample consisted of 2233 randomly selected, community dwelling, middle-age and older Japanese people.

The genotype frequencies for rs7566605 polymorphism were: GG, 0.465; GC, 0.432; and CC, 0.104 (C allele frequency, 0.32), which are similar frequencies to those reported by Herbert et al. (1) (Table 1). These frequencies are consistent with those expected under Hardy-Weinberg equilibrium (1 *df*). There were no significant differences in the genotype distributions of rs7566605 polymorphism between men and women or among the different age groups (Table 1). Table 2 shows the mean (standard error) of BMI, other anthropometric variables, and glucose metabolic variables tested in the GG/GC and CC genotypes. There were

[1] Nonstandard abbreviations: OR, odds ratio; CI, confidence interval; NILS, National Institute for Longevity Sciences; LSA, Longitudinal Study of Aging.

Table 1. Distribution of rs7566605 genotypes

		CC		GC		GG		GC/GG	
	n	*n*	%	*n*	%	*n*	%	*n*	%
Total	2233	233	10.4	962	43.1	1038	46.5	2000	89.6
Men*	1128	121	10.7	470	41.7	537	47.6	1007	89.3
Women	1105	112	10.1	492	44.5	501	45.3	993	89.9
Age (yrs)†									
40 to 49	563	61	10.8	224	39.8	278	49.4	502	89.2
50 to 59	556	56	10.1	265	47.7	235	42.3	500	89.9
60 to 69	561	64	11.4	241	43.0	256	45.6	497	88.6
70 to 79	553	52	9.4	232	42.0	269	48.6	501	90.6

* CC, GC, GG, men vs. women, $\chi^2 = 1.863$, $p = 0.394$; CC, GC/GG, men vs. women, $\chi^2 = 0.209$, $p = 0.648$.
† CC, GC, GG, age groups, $\chi^2 = 9.306$, $p = 0.157$; CC, GC/GG, age groups, $\chi^2 = 1.373$, $p = 0.712$.

no differences in BMI levels between these genotypes. In addition, no significant differences in waist and hip circumferences, waist-to-hip-ratio, and fat mass were observed between these genotypes in either gender (Table 2). No significant association was observed between these genotypes and fasting glucose, insulin, hemoglobin A_{1c}, or homeostasis model assessment for insulin resistance levels in men or women (Table 2). The rs7566605 genotypes showed similar allele frequencies in diabetic individuals and in non-diabetic controls (data not shown).

In our cohort, only a small number of participants had a BMI ≥30 kg/m^2 (0.97% in men, 3.37% in women). When logistic regression was performed to calculate the OR for the CC genotype compared with the CG/GG genotypes or the GG genotype, defining overweight as a BMI ≥25 kg/m^2 (23.6% in men, 22.0% in women), using age and sex as covariates, the CC genotype showed no association of the single-nucleotide polymorphism with overweight (vs. CG/GG, OR = 1.18, 95% CI, 0.84 to 1.65, $p = 0.333$; vs. GG, OR = 1.19, 95% CI, 0.84 to 1.69, $p = 0.325$). The CC/CG

Table 2. Anthropometric and glucose metabolic variables according to rs7566605 genotypes

	Men						Women					
		CC		GG/GC				CC		GG/GC		
	n	Mean	SE	Mean	SE	*p*	*n*	Mean	SE	Mean	SE	*p*
Weight (kg)	1127	62.0	0.8	62.1	0.3	0.804	1104	52.8	0.8	52.4	0.3	0.612
Height (cm)	1127	164.3	0.5	164.6	0.2	0.695	1104	151.9	0.5	151.2	0.2	0.176
BMI (kg/m^2)	1127	22.9	0.3	22.9	0.1	0.820	1104	22.8	0.3	22.9	0.1	0.792
Waist circumference (cm)	1127	82.0	0.8	82.4	0.3	0.585	1104	75.2	0.9	75.1	0.3	0.969
Hip circumference (cm)	1127	90.9	0.4	91.1	0.2	0.622	1104	90.6	0.5	90.6	0.2	0.985
Waist-to-hip ratio	1127	0.9	0.01	0.9	0.002	0.641	1104	0.8	0.01	0.8	0.002	0.877
Fat mass (kg)	1125	21.0	0.4	21.4	0.1	0.335	1096	31.4	0.5	31.5	0.2	0.864
Glucose (mM)*	1056	5.8	0.1	5.7	0.03	0.233	1049	5.4	0.1	5.5	0.03	0.304
Insulin (μU/mL)*	1071	8.0	0.5	8.2	0.2	0.691	1069	7.8	0.5	8.4	0.2	0.210
Hemoglobin A_{1c} (%)*	1055	5.3	0.1	5.2	0.02	0.373	1048	5.1	0.05	5.2	0.02	0.252
HOMA-IR*	1055	2.2	0.2	2.2	0.1	0.959	1048	1.9	0.2	2.1	0.1	0.190

SE, standard error; HOMA-IR, homeostasis model assessment of insulin resistance.
* Analysis of subjects who were not on oral hypoglycemic agents or insulin. Data were adjusted for age.

genotype also showed no significant association of the single-nucleotide polymorphism with overweight under a recessive model (vs. GG, OR = 1.05, 95% CI, 0.86 to 1.28, $p = 0.614$).

These results suggested no association of the rs7566605 variant with overweight in Japanese people. An ethnic difference may have contributed to the lack of this association. Another possibility is that the lower BMI levels and fewer obese individuals in our cohort may have affected our results. It is possible that due to the low number of obese individuals in our population, we lacked sufficient power to attempt replication of the previously reported association between rs7566605 and obesity in this study. In fact, it has been reported that excluding the upper quartile from the analysis in the Kooperative Gesundheitsforschung in der Region Augsburg (KORA) population in the study by Herbert et al. (1) eliminated the evidence for association of rs7566605 CC genotype with obesity, indicating that an association with obesity is strongest in those who are more obese. However, similar genotype frequency for this variant between our cohort and others may indicate that environmental influences can overcome the genetic influence on the anthropometric measurement.

Research Methods and Procedures

The present study consisted of a cross-sectional analysis of 1105 women and 1128 men who participated in the first wave of examinations in the National Institute for Longevity Sciences (NILS)-Longitudinal Study of Aging (LSA) from April of 1998 to March of 2000. The subjects of the NILS-LSA were male and female residents, 40 to 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 men and women was to be the same to test gender difference. Age at baseline was to be 40 to 79 years, and the number of participants in each decade (40s, 50s, 60s, 70s) was 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 (5,6). 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 for the entire procedure was obtained from each participant. The study was approved by the Ethics Committee of the NILS.

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 BMI was calculated as weight (kilograms) divided by height squared (meters squared). Waist circumference and waist-to-hip ratio 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 midpoint between the anterior superior iliac crest and the lowest rib-to-hip circumference. Whole-body fat mass, assessed by DXA (QDR-4500; Hologic, Madison, WI), was used as an index for determining body composition.

Biochemical Assays of Blood

An antecubital blood sample was drawn from each subject after an overnight fast. Fasting plasma glucose was assayed by the glucose oxidase method (7). Plasma insulin was measured by radioimmunoassay (8). Coefficients of variation of glucose and insulin were 16.3% and 64.3%, respectively. The homeostasis model assessment for insulin resistance was calculated as fasting serum insulin (microunits per milliliter) × fasting plasma glucose (millimolar)/22.5 (9).

Determination of rs7566605 Genotypes

Genotypes were determined using a fluorescence-based allele-specific DNA primer assay system (Toyobo Tsuruga Gene Analysis, Tsuruga, Japan). The polymorphic regions of rs7566605 were amplified by polymerase chain reaction with allele-specific sense primers labeled at the 5′ end with either fluorescein isothiocyanate (5′-TCATTGCAATAGCCACTGCCAAGTAC-3′) or Texas red (5′-GGATATTTGATCGTGGTCCTTTA-3′) as allele-specific hybridization probe and with an antisense primer labeled at the 5′-end with biotin (5′-AAAAACTGAAAACCACCCTGGTACAGAC-3′). The reaction mixtures (25 μL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mM deoxynucleoside triphosphate, 2.0 mM $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo Co., Ltd.) in polymerase buffer. The amplification protocol consisted of initial denaturation at 95 °C for 5 minutes, followed by 45 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C for 30 seconds, and extension at 72 °C for 30 seconds; a final extension was conducted at 72 °C for 2 minutes. Our genotyping error rate was ~0.1%.

Data Analysis

Quantitative data adjusted for age were compared between the two groups by 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. Logistic regression was performed to calculate the OR for the CC allele genotype compared with CG/GG genotypes or the GG genotype, defining overweight as BMI ≥25 kg/m^2, using age and sex as covariates. In this study, the significant difference in BMI

by genotype should be >0.76 kg/m^2 in men and >0.92 kg/m^2 in women with a power ($1 - \beta$) of 0.8 and an α of 0.05. In the analyses to examine the association between genotypes and glucose metabolisms, participants who were being treated with oral hypoglycemic agents or insulin were excluded. The general linear model was applied to control for age. In the model, each quantitative variable was the dependent variable, and age and genotype were the independent variables. Least square means of the dependent variable by genotype were compared and tested by Student's t test. A p value <0.05 was considered to be statistically significant. The data were analyzed with SAS, release 8.2 (SAS Institute, Inc., Cary, NC).

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Evodiamine Improves Diet-Induced Obesity in a Uncoupling Protein-1-Independent Manner: Involvement of Antiadipogenic Mechanism and Extracellularly Regulated Kinase/Mitogen-Activated Protein Kinase Signaling

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Evodiamine is an alkaloidal compound with antiobesity effects that have been thought to be due to uncoupling protein-1 (UCP1) thermogenesis similar to the effects of capsaicin, but the underlying mechanisms are poorly understood. To clarify the mechanisms, we first examined whether the antiobesity effect of evodiamine could be attributed to the involvement of UCP1. When UCP1-knockout mice were fed a high-fat diet with 0.03% evodiamine (wt/wt) for 2 months, the increases in body weight, adiposity, and the serum levels of leptin and insulin were reduced in a manner indistinguishable from control mice fed a high-fat diet with evodiamine, suggesting that evodiamine triggered a UCP1-independent mechanism to prevent diet-induced obesity. By using preadipocyte cultures, we found that evodiamine, but not capsaicin, increased phosphorylation of ERK/MAPK, reduced the expression of transcription factors such as peroxisome proliferator-activated receptor-γ, and strongly inhibited adipocyte differentiation. Evodiamine treatment also reduced insulin-stimulated phosphorylation of Akt, a crucial regulator of adipocyte differentiation; and the reduction of phosphorylated-Akt and augmentation of phosphorylated ERK were reversed by blockade of the MAPK kinase/MAPK signaling pathway, restoring adipogenesis in the cultures. The changes in ERK and Akt phosphorylation levels were also observed in white adipose tissues of UCP1-knockout mice fed the evodiamine diet. These findings suggest that evodiamine has a potential to prevent the development of diet-induced obesity in part by inhibiting adipocyte differentiation through ERK activation and its negative cross talk with the insulin signaling pathway. (*Endocrinology* 149: 358–366, 2008)

OBESITY, THE STATE of excess fat deposition in the body, is a serious health problem in industrialized societies because it is associated profoundly with type 2 diabetes mellitus, coronary heart disease, atherosclerosis, and certain cancers (1, 2). Because obesity develops as the result of energy imbalance when energy intake exceeds energy expenditure, intervention to reduce caloric intake through hormonal regulation and/or to increase energy expenditure by thermogenic function such as uncoupling protein 1 (UCP1) would be reasonable ways for preventing or curing obesity (3–5). It has been recently reported that α-lipoic acid has these potentials because it causes weight loss in rodents by reducing food intake and enhancing energy expenditure through a hypothalamic AMP-activated protein kinase pathway (6).

The regulation of adipogenesis also appears to be a potential strategy for the treatment of obesity because the excessive growth of adipose tissue in obesity has been suggested to result from adipocyte hypertrophy and the recruitment of new adipocytes from precursor cells (4, 7). Adipogenesis is a complex process that is highly regulated by positive and negative stimuli, including molecules involved in the insulin signaling pathway and various transcription factors (8, 9). ERK/MAPK signaling is a pathway activated by insulin. To date, many studies including one showing the pivotal role of peroxisome proliferator-activated receptor (PPAR)-γ, 8) have brought about great advances in our understanding of the molecular mechanism of adipogenesis. Although contradictory results on the role of ERK1/2 (p44/42 MAPK) in adipogenesis have been obtained, recent results indicate that ERK activation is necessary to initiate the process of differentiation of preadipocytes into adipocytes and that, thereafter, this signal pathway needs to be shut off for adipoctye differentiation to proceed

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* T.W. and Y.W. contributed equally to this work.

Abbreviations: aP2, Adipocyte fatty acid-binding protein; AR, adrenergic receptor; BAT, brown adipose tissue; C/EBP, CCAAT/enhancer-binding protein; CS, calf serum; HF, high fat; IGF-IR, IGF-I receptor; IPGTT, ip glucose tolerance test; IR, insulin receptor; IRS, insulin receptor substrate; KO, knockout; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein; WAT, white adipose tissue.

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(10–13). Despite the high potential for antiobesity intervention, however, safe and effective agents inhibiting adipocyte differentiation, thereby preventing obesity, are not yet available.

Evodiamine, a major alkaloidal compound in the fruit of *Evodia fructus (Evodia rutaecarpa* Bentham, Rutaceae) was previously reported to exhibit capsaicin-like antiobesity effects (14). The major mechanism eliciting the effect was postulated to be enhancement of energy dissipation by UCP1 thermogenesis, probably through β3-adrenergic stimulation in brown adipose tissue (BAT). Capsaicin, the pungent main principle of red pepper, has also been reported to decrease body weight by reducing food intake in rats (15), although the molecular basis on this antiobesity effect of capsaicin is still obscure. If evodiamine has a high potential for preventing obesity, this compound may be suitable for dietary supplementation because it has no perceptible taste, unlike capsaicin. However, the mechanisms underlying the antiobesity effects of evodiamine are still not clear. In this report, we demonstrate that evodiamine inhibited adipocyte differentiation through stimulation of an ERK/MAPK pathway and that dietary supplementation with this nonpungent compound could ameliorate diet-induced obesity in animals lacking UCP1 thermogenesis. This work may lead to the development of drugs and therapeutic strategies for treatment of obesity in adult humans who are virtually UCP1 deficient.

Materials and Methods

Experimental animals

Ucp^{tm1} knockout (KO) mice on a C57BL/6J background (16) were kindly provided by Dr. Leslie Kozak (Pennington Biomedical Research Center, Baton Rouge, LA), and N13-N15 generations were used in the experiments. The mice were maintained according to our institutional guidelines for animal care under artificial lighting for 12 h/d and provided a standard chow (11.6% kcal from fat; Diet CE-2; CLEA Japan, Inc., Tokyo, Japan) and tap water *ad libitum* in our animal facility at 23 ± 1 C. In the experiments on the effects of evodiamine, UCP1-KO and the control (wild-type and hetero-type) littermates mice were fed the standard chow until they were 4 months old and then were fed a high-fat diet (HF: 41.9% kcal from fat, Diet B15040; CLEA Japan) (17) with or without 0.03% evodiamine (wt/wt; Kishida Chemical, Osaka, Japan) for 2 months. The 6-month-old mice were sampled to determine the effects of evodiamine on body weight, adiposity, blood biochemical parameters, histology of tissues, and/or gene expression.

Cell culture

3T3-L1 cells, which were provided kindly by Dr. Masayoshi Imagawa (Nagoya City University, Nagoya, Japan), were grown in DMEM (Invitrogen, Grand Island, NY) containing 10% calf serum (CS; ICN Biomedicals, Aurora, OH). The adipocyte differentiation was performed as described (18). Briefly, 2 d after confluence, the medium was changed to DMEM containing 10% fetal bovine serum (ICN Biomedicals), 10 μg /ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. Dexamethasone and 3-isobutyl-1-methylxanthine were withdrawn after 2 d of exposure, and insulin was withdrawn after 4 d. To determine the effect of evodiamine, we induced 2-d postconfluent preadipocytes to differentiate in the presence of evodiamine or capsaicin (Wako Pure Chemical, Osaka, Japan) for 4 d and then in its absence for 6 d. After 10 d of differentiation, the cells were stained with oil Red O (Muto Pure Chemicals, Tokyo, Japan). Similarly, we determined the effects of evodiamine on the protein and mRNA expression and triglyceride content of the cultured cells.

For the preparation of primary cultures of adipocyte precursor cells, small pieces of epididymal white adipose tissue (WAT) of C57BL/6J mice were incubated at 37 C for 30 min in PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.2% collagenase (Sigma, St. Louis, MO), 5 mM glucose, and 1.5% BSA (Sigma). The mixture was then passed through a 70-μm nylon filter (Falcon, Becton Dickinson Labware, Franklin Lake, NJ) and centrifuged at 130 × *g* for 3 min. After the upper (lipid) layer had been removed, the lower layer and pellets were suspended and passed through a 40-μm nylon filter (Falcon). After the filtrate had been mixed with an equal volume of DMEM supplemented with 10% CS, the mixture was centrifuged at 170 × *g* for 6 min, and the cell pellet including the stromal vascular fraction was recovered for use as the primary culture. The cells were inoculated into 6-well plates (7×10^5 cells/well) and cultured in 10% CS/DMEM. The conditions for adipocyte differentiation were the same as those in the experiment using 3T3-L1 cells.

Human hepatoma HepG2 cells were obtained from the European Collection of Cell Cultures (Wiltshire, UK). Cells were cultured in 10% fetal bovine serum/DMEM containing 5.5 mM D-glucose as described previously (19). After HepG2 cells reached approximately 70% confluence in 60-mm-diameter dishes, the cells were maintained in serum-free medium overnight and then incubated in DMEM containing 24.75 mM D-glucose for an additional 24 h. To determine the effect of evodiamine on ERK phosphorylation, the cells were treated with or without evodiamine.

Biochemical analysis

Blood samples were collected from a tail vein and used immediately to determine the glucose level by use of a glucometer (NovoAssist Plus, Novo Nordisk, Tokyo, Japan). The following other parameters were measured by using serum and commercial assay kits: insulin (ultrasensitive insulin ELISA; Mercodia, Winston-Salem NC, or immunoassay kit; Shibayagi, Gunma, Japan) and leptin (Enzyme Immunoassay kit, Cayman, Ann Arbor, MI). The concentrations of protein and triglyceride in the tissue lysate and cultured cells were measured by using a BCA protein assay (Pierce Biotechnology, Rockford, IL) and Triglyceride E test (Wako Pure Chemical), respectively. An ip glucose tolerance test (IPGTT) using 1.5 mg of glucose per gram body weight was performed after 17 h of starvation. The blood glucose level was measured by the glucometer before glucose injection (0 min) and at 30, 60, and 120 min after that. Hepatic lipids were extracted with CHCl3/MeOH (2:1) according to the method of Folch *et al.* (20) and were dissolved in 2-propanol. Triglyceride or cholesterol concentrations in the lipids were determined by using commercially available kits: triglyceride G-Test Wako (Wako Pure Chemical) or Determiner TC555 (KYOWA MEDEX Co., Ltd., Tokyo, Japan).

Northern blot analysis

Total RNA was prepared from tissues and cultured cells with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Northern blot analysis was performed by using total RNA (WAT: 20 μg, 3T3-L1 cells: 10 μg), as described earlier (17). Blots were hybridized successively with probes (labeled with [^{32}P]dCTP) for the mRNAs of UCP2, β3-adrenergic receptor (AR), PPARγ, leptin, adipocyte fatty acid-binding protein (aP2), resistin, and 18S rRNA. In the analysis of β3-AR, three transcripts of 2.1, 2.8, and 3.6 kb were detected in WAT, as reported (21). Like probes for UCP2, β3AR, aP2, and leptin mRNAs and 18S rRNA (16, 17), probes for PPARγ and resistin mRNAs were produced by the RT-PCR technique. The sequences used were the following: PPARγ, positions 464-1945 of the mouse sequence (GenBank accession no. U01841), and resistin, positions 38–558 of the mouse sequence (GenBank accession no. AF323080). The PCR products were sequenced after subcloning into the pCRII vector (Invitrogen). Hybridization signals were quantified with Bioimage (FUJIFILM; Fuji, Tokyo, Japan).

Histological analysis

Tissues were fixed immediately in 10% formaldehyde in neutral buffer solution (Kishida Chemical) and embedded in paraffin. Tissue sections of 3 μm were cut and then stained with hematoxylin and eosin.

Immunoblotting and immunoprecipitation

Total cell lysates were prepared and analyzed as described previously (22). Briefly, cells in 100-mm dishes were washed with ice-cold PBS

containing 1 mM Na_3VO_4 and lysed with a lysis buffer (pH 7.2) consisting of 50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, and 0.25% protease inhibitor mixture solution (Sigma). The proteins of cell lysates were separated by 4–20% SDS-PAGE and electrotransferred onto a polyvinylidene difluoride membrane. Immunoblotting and immunoprecipitation were performed by using cell lysates (30 and 500 μg, respectively) and specific antibodies against CCAAT/enhancer-binding protein (C/EBP)-β, PPARγ, insulin receptor (IR)-β, IGF-I receptor β(IGF-IRβ), phosphatidylinositol 3-kinase (PI3K) p85 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-tyrosine (4G10; Upstate, Charlottesville, VA), insulin receptor substrate (IRS)-1, p44/42 MAPK, phospho-p44/42 MAPK, serine/threonine kinase Akt, and phospho-Akt (Cell Signaling Technology, Danvers, MA). The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

Data were expressed as the mean ± SE. Significant differences between groups were assessed by ANOVA or Student's *t* test.

Results

Effects of evodiamine on the development of diet-induced obesity in UCP1-KO mice

We provided UCP1-KO and the control mice with a HF diet with or without evodiamine for 2 months. Body weight gain and adiposity index were significantly lower in the mice with evodiamine (+Evo) than in the mice without it (−Evo) in both groups (Fig. 1, A and B), even though there was no significant difference in food intake between the +Evo and −Evo groups (0.43 ± 0.02 and 0.43 ± 0.02 kcal/d·g body weight in the control mice, 0.45 ± 0.01 and 0.48 ± 0.01 kcal/d·g body weight in the KO mice, respectively). The serum leptin levels in the +Evo group were reduced to 27 and 43% of the −Evo group in the control and KO mice, respectively (Fig. 1C). Although the nonfasting glucose level in the mice was not changed by the evodiamine diet (Fig. 1D), the insulin levels in mice treated with evodiamine were decreased to about one third of those without it in both groups (Fig. 1E). Moreover, the evodiamine diet improved the impaired glucose tolerance in UCP1-KO mice fed the HF diet, bringing it close to that in the mice fed the standard chow diet (Fig. 1F).

In addition to the data on adiposity (Fig. 1B), histological analysis supported the effectiveness of evodiamine in reducing fat accumulation in WAT and BAT, as well as fatty liver, in the mice fed the HF diet in both groups (Fig. 2). Liver mass in the −Evo group and +Evo group was 1.535 ± 0.113 and 1.286 ± 0.056 g in the control mice and 1.540 ± 0.119 and 1.391 ± 0.100 g in the KO mice, respectively. When the accumulation of hepatic lipids was evaluated, the triglyceride content tended to be reduced in the +Evo group, compared with that in the −Evo group (16.4 ± 2.9 and 57.5 ± 22.2

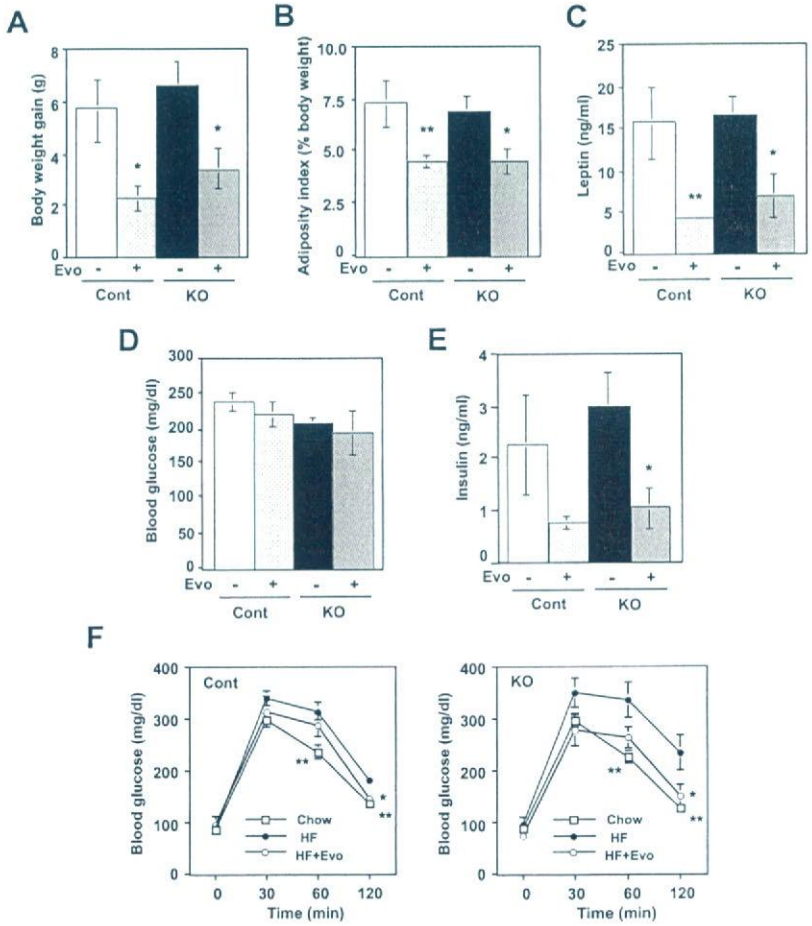

FIG. 1. Effects of evodiamine on body weight gain (A), adiposity (B), leptin (C), nonfasting levels of blood glucose (D) and insulin (E), and glucose tolerance (F) in the mice fed HF diet. Four-month-old control (Cont) and KO mice were fed the HF diet with or without evodiamine [Evo, 0.03% (wt/wt)] for 2 months. Data are expressed as the mean ± SE. The Cont/−Evo group contains four wild-type and one hetero-type mice. The Cont/+Evo group contains four wild-type and two hetero-type mice. The KO/−Evo and KO/+Evo groups contain six mice each. *, $P < 0.05$ and **, $P < 0.01$ *vs.* −Evo diet in the same group (ANOVA with Fisher's protected least significant difference test). F, IPGTT in Cont and KO mice after Evo feeding for 7 wk. The data in mice fed HF+Evo diet (*open circle*) were compared with those of age-matched mice fed the standard chow (*open square*) or HF diet (*closed circle*). Data are expressed as mean ± SE (chow: n = 9 and 8, HF: n = 8 and 9, HF+Evo: n = 6 and 6 for Cont and KO mice, respectively). *, $P < 0.05$ and **, $P < 0.01$ *vs.* HF group.

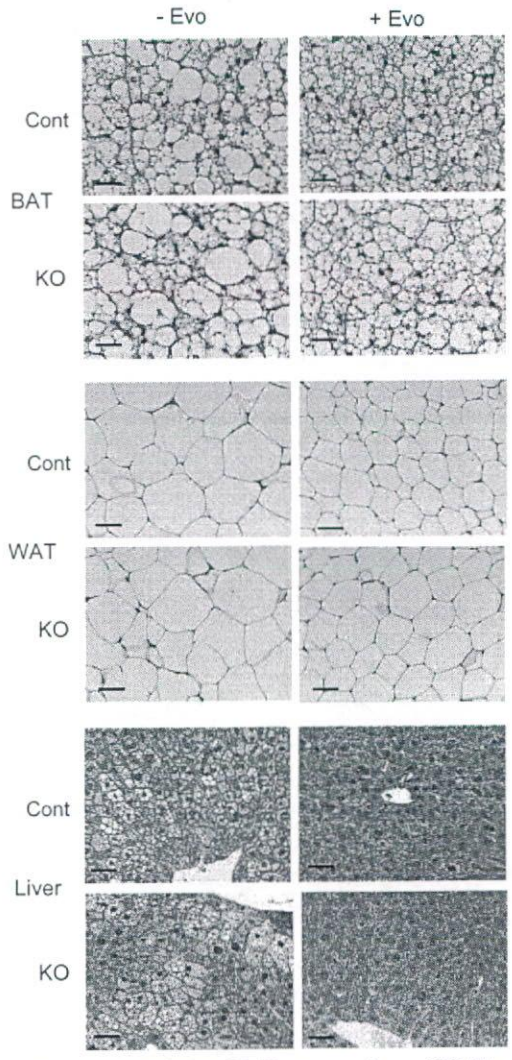

FIG. 2. Histological analysis of BAT, retroperitoneal WAT, and liver in the mice fed the HF diet with or without evodiamine (Evo). Tissue sections of the control (Cont) and KO mice were stained with hematoxylin and eosin. *Bars*, 25 μm for BAT and 50 μm for WAT and liver.

mg/g liver in the control mice, 25.1 ± 9.3 and 51.3 ± 10.8 mg/g liver in the KO mice, respectively). There was no difference in the total cholesterol content in the liver between the +Evo and −Evo groups (3.98 ± 0.37 and 3.42 ± 0.38 mg/g liver in the control mice, 3.75 ± 0.58 and 4.05 ± 0.47 mg/g liver in the KO mice, respectively).

In the analysis of gene expression in WAT, similar effects of evodiamine in the control and KO mice were observed (Fig. 3). Namely, reductions in the mRNA levels of leptin (control, 53%; KO, 26%), UCP 2 (control, 29%; KO, 22%), and PPARγ (control, 17%; KO, 26%) were found in the +Evo group, compared with those levels in the −Evo group, whereas the mRNA level of β3-AR increased in the +Evo group (WT: 201%, KO: 135%; Fig. 3), consistent with the effects of increased adiposity on the expression of these genes (17). There was no difference in the mRNA level of UCP1 in the BAT between the +Evo and −Evo groups in the control mice (data not shown). We also could not detect a significant

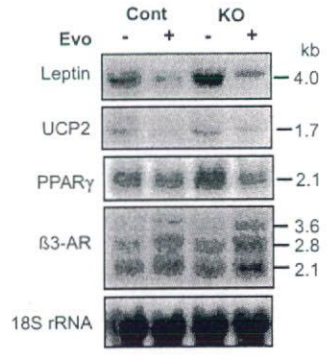

FIG. 3. Effects of evodiamine on the gene expression for leptin, UCP2, PPARγ, and β3-AR in WAT of the mice. Northern blot analyses were performed by using 20 μg of total RNA from WAT of the control and KO mice fed the HF diet with or without evodiamine (Evo). Three transcripts of 2.1, 2.8, and 3.6 kb were detected in the analysis for β3-AR. Hybridization signals were quantified and normalized by 18S rRNA levels. Representative images are shown.

difference in the mRNA levels of UCP2 and UCP3 in the BAT and muscles between the mice with or without evodiamine in either group (data not shown).

Evodiamine inhibits adipocyte differentiation in 3T3-L1 cells

The phenotypes of evodiamine *in vivo* could be mediated by changes in energy expenditure and lipolysis or through its effects on adipogenesis. Accordingly, we examined the effects of evodiamine on adipocyte differentiation *in vitro*. When the differentiation of 3T3-L1 preadipocytes was assessed quantitatively in terms of triglyceride contents, evodiamine dose-dependently inhibited the differentiation of the preadipocytes incubated in differentiation medium containing insulin, dexamethasone, and 1-isobutyl-3-methylxanthine (Fig. 4A). The addition of 1 μM evodiamine to the differentiation medium almost completely inhibited the adipocyte differentiation as evidenced by oil Red O staining of lipids. This inhibitory effect of evodiamine was reduced when it was added 2 d after the start of stimulation of differentiation, and the alkaloid did not suppress differentiation at all when it was added after d 4 (data not shown). Not only did evodiamine inhibit fat accumulation in adipocytes, but also the greatly reduced expression of aP2, leptin, and resistin indicated that adipogenesis in 3T3-L1 cells was strongly suppressed (Fig. 4B).

We then analyzed the induction of transcription factors regulating adipogenesis in the cells (Fig. 4C). In the absence of evodiamine, C/EBPβ, a crucial regulator in an early step of the transcriptional cascade in adipogenesis, and PPARγ were expressed in a time-dependent manner as the 3T3-L1 cells proceeded toward adipocyte differentiation, as previously described (23, 24). Treatment of postconfluent cultures with evodiamine severely suppressed the early induction of C/EBPβ and then blocked the induction of PPARγ (Fig. 4C), indicating that evodiamine acted to inhibit the early transcriptional steps of adipocyte differentiation. When the intracellular signaling molecules involved in adipocyte differentiation were examined, an increased level of phosphorylated ERK1/2 was found in

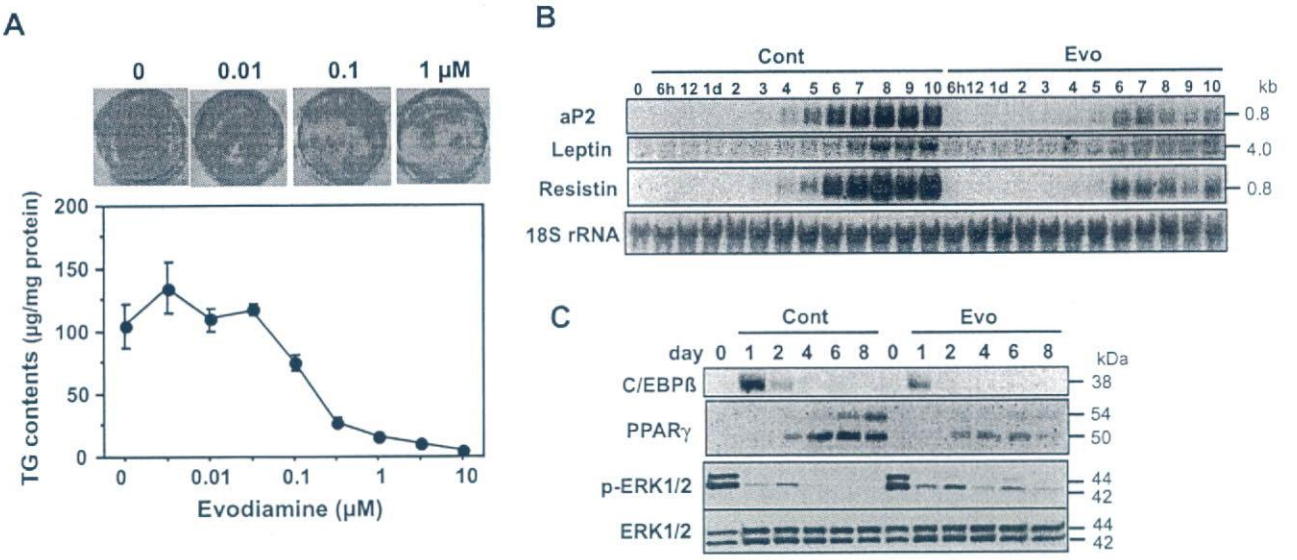

FIG. 4. Effects of evodiamine on adipocyte differentiation in 3T3-L1 cells. A, Dose-dependent inhibition of adipocyte differentiation by evodiamine. 3T3-L1 cells were cultured in the differentiation medium with the indicated concentration of evodiamine for 4 d and then without it for 6 d. On d 10, the lipid accumulation in the cells was evaluated by oil Red O staining (macroscopic images, *upper panel*) or determined in terms of triglyceride (TG) content (*lower panel*). Data are expressed as the mean ± SE (n = 4). B, Effects of evodiamine on the induction of adipocyte-characteristic genes. Northern blot analysis for aP2, leptin, resistin, and 18S rRNA was performed by using 10 μg of total RNA isolated from the cells. C, Effects of evodiamine on the activation of ERK and expression of transcription factors during adipogenesis. Western blot analysis for C/EBPβ, PPARγ, phospho- and total ERK was performed by using cell lysates. In B and C, 3T3-L1 cells were treated with 1 μM evodiamine for 4 d and then without it for 4–6 d (Evo) or without evodiamine through the differentiation culture (Cont). The cells were harvested at the indicated time point and used for Northern or Western blot analysis. Day 0, Time point before the stimulation of differentiation.

the cells treated with evodiamine, compared with that in the control cells (Fig. 4C). In the control cells, the very high level of ERK phosphorylation in growth medium before differentiation stimulation (d 0) was reduced greatly within the first 2 d, and it disappeared thereafter. On the other hand, a steady level of ERK phosphorylation was detected in Evo-treated cells in the first 2 d, and phosphorylated ERK was detectable by d 8. Evodiamine did not stimulate ERK phosphorylation in the 3T3-L1 mature adipocytes (data not shown).

Evodiamine inhibits adipocyte differentiation by the sustained activation of ERK in 3T3-L1 cells

To further assess the involvement of ERK signaling in the effects of evodiamine on adipocyte differentiation, we examined the acute effects of insulin and evodiamine on the stimulation of ERK phosphorylation. As previously reported (25), a transient increase in ERK phosphorylation that peaked after 5 min was observed in 3T3-L1 cells after insulin stimulation (Fig. 5A). Evodiamine addition led to a modest but considerable stimulation of ERK phosphorylation that lasted over a 1-h period (Fig. 5B), indicating the differences in mode of action between insulin and evodiamine. When the evodiamine effect was checked in a longer time course, ERK phosphorylation lasted 18 h after evodiamine stimulation (data not shown). Cotreatment of evodiamine with PD98059, a specific inhibitor of MAPK kinase (an upstream kinase for ERK), reduced ERK phosphorylation (Fig. 5B) and restored adipocyte differentiation (Figs. 5C). Interestingly, capsaicin had no effect on ERK phosphorylation (Fig. 5D) or adipocyte differentiation (Fig. 5E). In addition, the effect of evodiamine on ERK phosphorylation was detected in a nonadipogenic cell line, human hepatoma HepG2 cells. Similar to the results in 3T3-L1 preadipocytes, ERK phosphorylation greatly increased 1 h after evodiamine stimulation, and its increased level was detectable even after 24 h (Fig. 5F). As expected, the increase in ERK phosphorylation was blocked by cotreatment of evodiamine with PD98059.

Effects of evodiamine on insulin signaling pathway

We examined whether evodiamine would affect insulin signaling through the PI3K/Akt pathway during adipocyte differentiation because this pathway is important in transducing the proadipogenic effects of insulin through IR and/or IGF-IR (26, 27). As shown in Fig. 6A, evodiamine did not affect the tyrosine phosphorylation of either IR or IGF-IR in 3T3-L1 preadipocytes. Likewise, the tyrosine phosphorylation of IRS-1 and its binding with the PI3K p85 subunit in the preadipocytes were not changed by the evodiamine treatment (Fig. 6B). Evodiamine also did not affect PI3K activity (data not shown). However, we found that evodiamine strongly reduced the insulin-induced phosphorylation of Akt Ser473, a regulatory site of Akt activity, in the 3T3-L1 preadipocytes, which contrasted with the steady level of ERK phosphorylation (Fig. 7A). Reduced phosphorylation of Akt Ser303 was also detected in the evodiamine-treated cells (data not shown). When the cells were pretreated with PD98059, this inhibitory effect of evodiamine was strongly blocked, and Akt phosphorylation was restored by about 70% of level in the insulin treatment. The stimulation of ERK phosphorylation and inhibition of insulin-stimulated Akt phosphorylation were also observed in primary cultures of

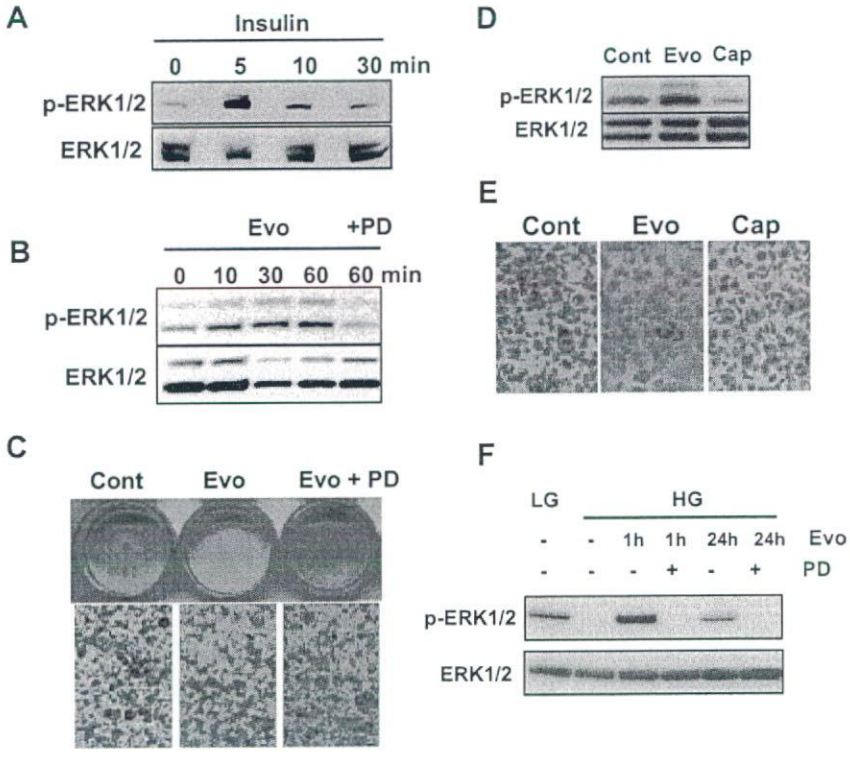

FIG. 5. Evodiamine inhibits adipocyte differentiation by stimulating the ERK pathway. A and B, Time course of ERK activation by insulin (A) and evodiamine (B). Two-day postconfluent 3T3-L1 cells were serum deprived for 4 h and then treated with 20 nM insulin or 10 μM evodiamine in the absence or presence of 10 μM PD98059 (+PD) for the indicated times, and the lysates were analyzed for activated ERK. C, PD98059 inhibits the evodiamine effect and restores adipogenesis. The cells were cultured in the differentiation medium with 1 μM evodiamine in the absence or presence of 10 μM PD by using the same protocol as in Fig. 4A. The lipid accumulation in the cells was evaluated by oil Red O staining. D and E, Capsaicin neither stimulates ERK phosphorylation (D) nor inhibits adipocyte differentiation (E). Cells were treated with 20 μM evodiamine (Evo), capsaicin (Cap), or an equal volume of dimethylsulfoxide control (Cont) for 30 min (D) or cultured in the differentiation medium with 1 μM evodiamine or capsaicin by using the same protocol as in Fig. 4A (E). F, Effect of evodiamine on ERK phosphorylation in HepG2 cells. Cells (~70% confluent) were quiesced in serum-free low-glucose (LG) medium (5.5 mM D-glucose) overnight and then stimulated in high-glucose (HG) medium (24.75 mM D-glucose) with or without evodiamine (Evo) for the indicated periods. One and 10 μM Evo were used for 24 h treatment and for 1 h treatment, respectively, in the absence or presence of 10 μM PD98059 (+PD), and the cell lysates were analyzed for activated ERK by Western blot analysis. Data shown are representatives of three independent experiments.

adipocyte precursor cells (Fig. 7B). Moreover, the effects of evodiamine on ERK and Akt signaling were determined in the WAT derived from the mice in the diet study (Fig. 8A). Compared with those for the −Evo group, the phosphorylation levels of ERK and Akt were significantly higher (2-fold) and lower (about half), respectively, in the WAT of the +Evo group in KO mice. In the control mice, the phosphorylation level of Akt in the +Evo group was reduced to 28% of the −Evo group, whereas the effect on ERK phosphorylation was not clear. The effects of evodiamine on ERK and Akt signaling were also confirmed in the WAT of mice administered with the compound. An injection of evodiamine significantly up-regulated ERK phosphorylation (1.7-fold) and down-regulated Akt phosphorylation (about a fourth) in the WAT of mice, compared with those for the control mice (Fig. 8B).

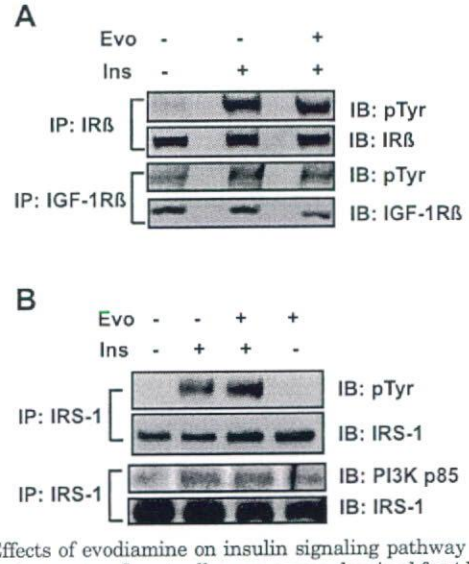

FIG. 6. Effects of evodiamine on insulin signaling pathway in 3T3-L1 cells. Two-day postconfluent cells were serum deprived for 4 h and then treated with 20 μM evodiamine (Evo) for 1 h and with 20 nM insulin (Ins) for the last 10 min. Tyrosine phosphorylation of IRβ or IGF-IRβ (A) and tyrosine phosphorylation of IRS-1 or its binding with PI3K (B) were determined by immunoprecipitation (IP) and immunoblot (IB) analysis. Immunoprecipitation experiments were performed by using the cell lysates and antibodies specific for each molecule. Data shown are representative of three independent experiments.

Discussion

It was previously reported that evodiamine showed an antiobesity effect, which was thought to depend on the enhancement of UCP1 thermogenesis through β3-adrenergic stimulation in BAT (14). To clarify whether the antiobesity effect of evodiamine depended on energy dissipation mediated by UCP1 thermogenesis, we first examined the effects of evodiamine on the development of diet-induced obesity in UCP1-KO mice in the present study. Interestingly, we found that evodiamine showed a potent effect of preventing the increases in body weight and adiposity even in the UCP1-KO mice fed the HF diet, which was indistinguishable from the effects of this compound in the control mice. The data on blood parameters suggested the improvement of leptin resistance and insulin sensitivity in the mice fed the evodiamine diet. In addition, IPGTT data supported the improvement of glucose metabolism in the mice treated with the evodiamine diet, in which the phenotypes in fed glucose and insulin levels were similar to those in PPARγ$^{+/-}$ mice (24). These results were unexpected because the diet-induced obe-

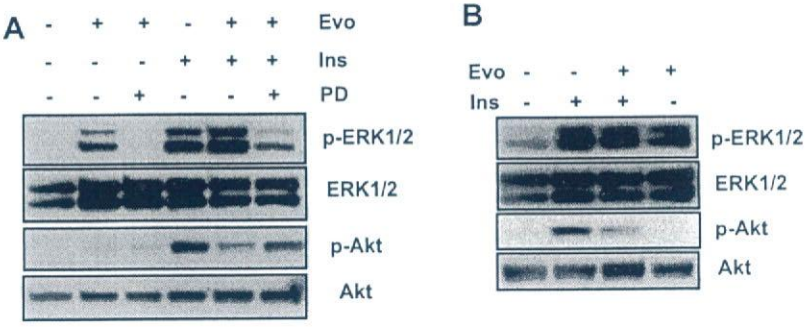

FIG. 7. Evodiamine negatively regulates insulin-stimulated Akt activation through an ERK pathway in preadipocytes. A, 3T3-L1 preadipocytes were serum deprived for 4 h and then treated with 20 μM evodiamine (Evo) for 1 h and with 20 nM insulin (Ins) for the last 10 min. PD98059 (PD; 20 μM) was added 1 h before the evodiamine treatment. Western blot analyses for ERK and Akt were performed by using cell lysates. B, Adipocyte precursor cells isolated from the WAT of mice were cultured as described in *Materials and Methods*. The cells were serum deprived for 4 h and then treated with 20 μM evodiamine for 1 h and with 20 nM insulin for the last 10 min. Representative images of three independent experiments are shown.

sity in UCP1-KO mice was not prevented by treatment with CL316,243, a strong β3-adrenergic agonist, whereas this compound effectively stimulated UCP1 thermogenesis and prevented the diet-induced obesity in wild-type mice (28). An injection of CL316,243 is known to increase oxygen consumption acutely in wild-type mice but not UCP1-KO mice (16, 28), whereas evodiamine did not appear to change oxygen consumption for the periods of 24 h after its injection in both of wild-type and UCP1-KO mice (our unpublished data). We also could not detect any change in UCP1 mRNA level in BAT of the control mice fed the evodiamine diet. These results suggest that energy dissipation mediated by UCP1 does not play a role in the antiobesity effect of evodiamine in mice, raising the possibility that this compound could be related more directly to the inhibition of fat deposition.

Therefore, we next investigated the direct effects of evodiamine on adipocyte differentiation. Our results suggest that reduced diet-induced obesity by evodiamine in the diet could be due to a reduction in the recruitment of new adipocytes from precursor cells. Such recruitment has been suggested to play a role in the expansion of adipose tissue in obesity (4, 7). In addition to the serial induction of transcriptional regulators, modulation of intracellular signaling molecules is essential for adipocyte differentiation. In the early steps of the differentiation, the regulation of MAPK activity is critical for initiating the entry of preadipocytes into the differentiation process (10). After its transient activation of ERK, its activity is down-regulated to enable the differentiation program to proceed because the sustained activation of the ERK signaling pathway inhibits adipocyte differentiation *in vitro* (13). Indeed, we found that evodiamine had a strong inhibitory effect for adipogenesis of the cells via sustained activation of the ERK/MAPK signaling pathway in 3T3-L1 and primary preadipocytes.

A similar ERK-activating effect was recently reported in 3T3-L1 preadipocytes treated with a quite high dose (500 μM) of α-lipoic acid (25). Because ERK stimulates the phosphorylation of PPARγ, causing a reduction in its transcriptional activity (29), the inactivation of PPARγ activity mediated by ERK together with reduced PPARγ mRNA levels could compound the inhibitory effect of evodiamine on adipogenesis. Because C/EBPβ is an important factor to initiate mitotic clonal expansion, which is a crucial step to enter the late stages of adipogenesis (23), the marked reduction of C/EBPβ in 3T3-L1 preadipocytes treated with evodiamine might affect the initiation of mitotic clonal expansion. The p38MAPK pathway has also been reported recently to play a role in

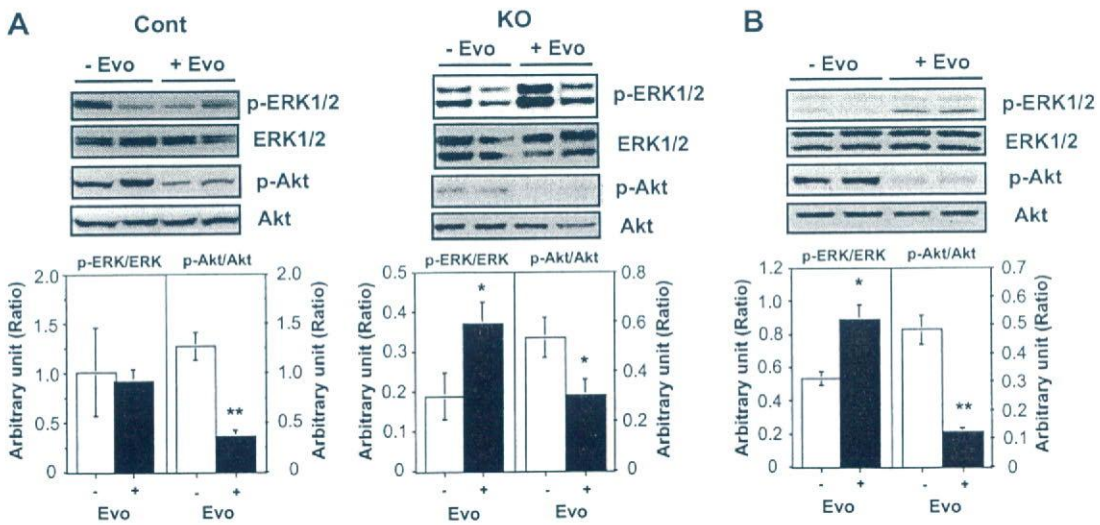

FIG. 8. Effects of evodiamine on the phosphorylation of ERK and Akt *in vivo*. Phosphorylation levels of ERK and Akt in the WAT of mice fed the HF diet with or without evodiamine (Evo) in the diet study (A) and in the WAT of wild-type mice treated with evodiamine (3 mg/kg, ip) or vehicle for 24 h (B). Western blot analyses for ERK and Akt were performed by using tissue lysates (50 μg protein) of epididymal WAT from the mice. Data are expressed as the mean ± SE (n = 4). *, $P < 0.05$ and **, $P < 0.01$ *vs.* −Evo group (Student's *t* test).

adipogenesis via regulation of C/EBPβ and PPARγ transcriptional activities (30). Of note, unlike evodiamine, capsaicin neither stimulated ERK phosphorylation nor inhibited adipogenesis, even though these compounds show similar actions *in vivo* on vasorelaxation and hypothermia (14, 31, 32). Considering the results on the signal analysis of mouse WAT, it is likely that evodiamine contributed to the suppression of diet-induced obesity in mice by inhibiting adipogenesis, although other mechanisms could be involved in the antiobesity effects of evodiamine.

We also found an effect of evodiamine on ERK phosphorylation in HepG2 cells. Similar to 3T3-L1 preadipocytes, evodiamine significantly stimulated ERK phosphorylation in the nonadipogenic cells. Kosone *et al.* recently suggested an involvement of ERK in a reducing effect of hepatocyte growth factor on lipid accumulation in HepG2 cells through induction of several genes related to lipid metabolism (33). Because the fatty liver observed in HF diet-induced obesity was improved considerably in the mice fed the evodiamine diet, it would be of interests to know the effect of evodiamine on lipid metabolism in hepatocytes.

In addition to ERK/MAPK signaling, PI3K/Akt is an important intracellular signal cascade in the regulation of many cellular activities including growth, glucose metabolism, and adipogenesis (34–36). Insulin stimulates tyrosine phosphorylation of the IR and/or IGF-IR, which promotes the activation of Akt via phosphorylation of PI3K. Differentiation of 3T3-L1 preadipocytes is stimulated strongly by the expression of a constitutively active form of Akt (37). On the other hand, adipogenesis is blocked in cultured cells or mice lacking Akt (26, 36). In the present study, the upstream signals within the insulin/IGF-I pathway were not affected by evodiamine in 3T3-L1 cells. However, we found that insulin-stimulated Akt phosphorylation was inhibited strongly in the preadipocytes treated with evodiamine in contrast to the stimulation of ERK phosphorylation. In addition, the Akt inhibition was restored by a MAPK kinase inhibitor, which profoundly blocked ERK phosphorylation in the cells, suggesting a connection between PI3K/Akt and ERK/MAPK pathways in the preadipocytes. Taken together, evodiamine may inhibit adipogenesis by suppressing insulin-stimulated Akt phosphorylation through the activation of ERK signaling. Similar effects of evodiamine on Akt and ERK phosphorylation were detected in the WAT from the UCP1-KO mice in the diet study. Although we could not detect a significant effect of evodiamine on ERK phosphorylation in the WAT from the control mice, the effects of evodiamine on ERK and Akt signaling *in vivo* were supported from the evidence that an injection of evodiamine to wild-type mice considerably stimulated ERK phosphorylation and reduced Akt phosphorylation in the WAT. Because Akt has important roles in growth (34), the decrease in Akt phosphorylation by evodiamine might inhibit mitotic clonal expansion in preadipocytes. Takada *et al.* (38) recently reported that evodiamine inhibits Akt activation in tumor cells.

We presently do not know how ERK regulates Akt phosphorylation in preadipocytes. Because the Akt activity is regulated negatively by several phosphatases such as the phosphatidylinositol 3′ lipid phosphatase (39) or protein phosphatase type 2A (40), we examined the effect of evodiamine on the phosphorylation level of these phosphatases. However, evodiamine did not affect the phosphorylation levels of phosphatidylinositol 3′ lipid phosphatase and protein phosphatase type 2A in the presence of insulin (Wang, T., unpublished data). Therefore, the contribution of these phosphatases to Akt inactivation in 3T3-L1 preadipocytes stimulated with evodiamine appears to be low, so other molecules may be involved in the negative cross talk of ERK signaling for the regulation of Akt activity in adipocyte differentiation.

In summary, our results indicate that evodiamine has the previously unrecognized action of inhibiting adipogenesis by a mechanism in which the stimulation of ERK/MAPK signaling down-regulates the expression of adipocyte transcription factors and insulin-induced Akt signaling. Because evodiamine clearly showed an antiobesity effect in UCP1-deficient mice, this compound may offer a new approach to circumvent the development of diet-induced obesity, especially in animals lacking UCP1 thermogenesis including adult humans; however, further details of the inhibitory mechanism and the effects on insulin sensitivity remain to be clarified.

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Erratum

In the article "Dihydrotestosterone Differentially Modulates the Mitogen-Activated Protein Kinase and the Phosphoinositide 3-Kinase/Akt Pathways through the Nuclear and Novel Membrane Androgen Receptor in C6 Cells" by Joshua W. Gatson, Paramjit Kaur, and Meharvan Singh (*Endocrinology* **147:** 2028-2034), the authors note the following error in their paper. In the legend to Fig. 10, the sentence "The fluorescence histogram, depicting increasing fluorescence intensity on the x-axis and cell number on the y-axis, shows significant labeling of cells with DHT-BSA-FITC" should read "The fluorescence histogram, depicting increasing fluorescence intensity on the x-axis and cell number on the y-axis, shows significant labeling of cells with testosterone-BSA-FITC". Similarly, the description of the compound within the *Methods* and *Results* sections, both pertaining to Fig. 10, should read 'testosterone-BSA-FITC' rather than 'DHT-BSA-FITC'. This change does not alter the interpretation of data shown in Fig. 10. *The authors apologize for the error.*

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特集：高齢者の肥満と痩せ・栄養

各論

高齢者の肥満・痩せと老年疾患との関係
1．高齢者の肥満と高血圧症・動脈硬化

下方　浩史

株式会社　ライフ・サイエンス

Geriat. Med. 46(5)：445～448, 2008

KEY WORD
●肥満 ●動脈硬化 ●高血圧症 ●脳卒中
●冠動脈疾患

高齢者の肥満・痩せと老年疾患との関係
1．高齢者の肥満と高血圧症・動脈硬化

下方 浩史

SUMMARY

■高齢者でも肥満は，高血圧症，冠動脈疾患，脳卒中などの動脈硬化性疾患の危険因子である．
■高齢者では肥満と動脈硬化との関連には，肥満以外の要因や個人差の影響が大きい．
■今後，高齢者における肥満と動脈硬化との関連について，欧米とは生活習慣が大きく異なるわが国での疫学的な検討によるエビデンスの集積が必要である．

はじめに

メタボリックシンドロームの概念にみるように肥満は，高血圧症，高脂血症，糖尿病・糖代謝異常と密接に関連しており，これらの危険因子が重積することにより，全身の動脈硬化が進行していくことになる．さらに冠動脈の硬化が狭心症や心筋梗塞に，脳血管や頸動脈の硬化が脳血管障害に進展し，それぞれに特有の臨床症状を呈していく．しかし高齢者での肥満と動脈硬化との関連には，肥満以外の要因や個人差の影響が大きく，はっきりしないことが多い．ここでは，主に高齢者を対象に，肥満と高血圧症，冠動脈疾患，脳血管障害との関連について述べていく．

肥満と高血圧症

肥満者に高血圧症が多く，また心臓病患者が多いことはよく知られている．肥満は過剰な脂肪組織をもつ状態であり，例えば総脂肪量が10 kg増加すれば，10 kgの荷物を持って1日中生活をしているのと同じことであり，循環動態や心機能に大きな負担を与える．

図1は，筆者らによる肥満度(BMI)と血圧との検討の結果である[1]．対象は14～94歳までの男女64,152人で，性別にみたBMIと収縮期血圧，拡張期血圧との関係を示している．対象のうち，降圧薬服用者を除き，年齢，喫煙量，飲酒量で調整した上で各BMIでの平均値を求めている．男女で，BMIの上昇に対して，収縮期血圧，拡張期血圧がともに直線的に上昇することがわかる．Framingham Studyの検討では，標準体重を20%超えると，高血圧症の罹患率は8倍増えるという[2]．また同じくFramingham Studyでは，BMIが25未満に対して25以上30未満では高血圧症のリスクは男性1.46倍，女性1.75倍，BMIが30以上では男性で2.21倍，女性2.75倍であり，これは喫煙や糖尿病，高コレステロール血症の有無で調整してもあまり変化がなかった[3]．

減量の血圧に対する影響については，8万人の16年間の追跡調査では，体重の変化がほとんどなかった人に比べて5 kg以上10 kg未満の体重減少で高血圧症の発症は15%少なくなり，10 kg以上の体重減少で26%少なくなっていたという報告がある．逆に5 kg以上10 kg未満の体重増加で高血圧症の発症は1.74倍に，25 kg以上の体重増加で5.21倍になっていた[4]．

高齢者では若年者と同じように減量によって血圧は下がるかどうかは，まだはっきりと結論づけられていない．筆者らは大規模集団の縦断

■しもかた ひろし(国立長寿医療センター研究所疫学研究部長)

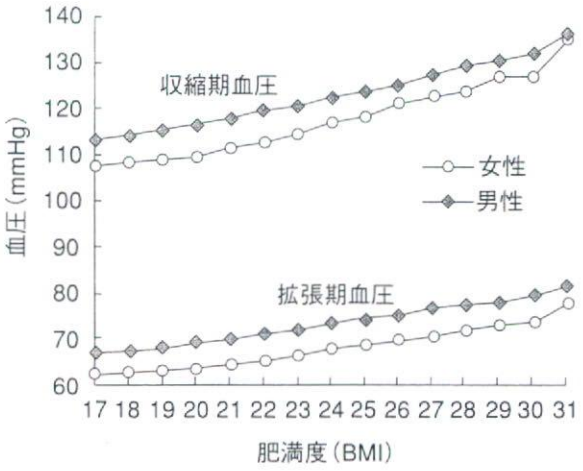

図1　性別にみた肥満度と血圧

14～94歳までの男女64,152人での検討．降圧薬服用者を除き，年齢，喫煙量，飲酒量で調整を行っている（文献1より引用）．

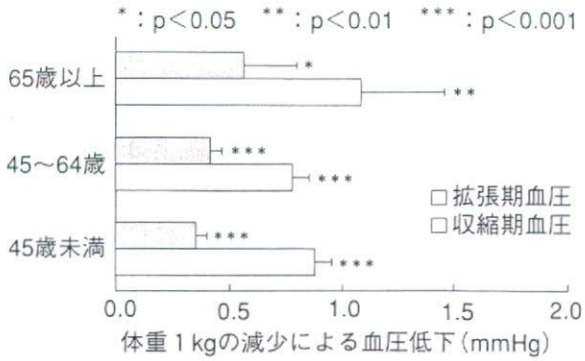

図2　年齢階級別にみた体重1kg減少による血圧の変化

18～88歳までの男女6,425人での検討．降圧薬服用者を除き，性別，血圧初期値，喫煙量，飲酒量，BMIで調整を行っている（文献1より引用）．

データを用いて，年齢階級別に体重変動と血圧の変化の関係について検討してみた[1]．対象は，人間ドックを連続2年間受診した19～88歳の男女6,425名の集団である．1年間の体重1kg減少による血圧の変化量を，性別，BMI，喫煙，飲酒，血圧の観察開始時の値別で調整後，一般線型モデル分析より年齢階級別（45歳未満，45歳以上65歳未満，65歳以上）に推定した．その結果，高齢者でも**図2**に示したように体重減少によって血圧は有意に低下した．10kgの体重減少により収縮期血圧は約10mmHg，拡張期血圧は約5mmHg低下すると期待できる．反対に体重が増加すれば，血圧は高齢者でも若年者，中年者同様に高くなることもわかった．米国での介入研究では，60～80歳の肥満高齢男女に対して平均2年半で3.5～4.5kgの減量を行ったところ，高血圧症の発症リスクは30％下がった[5]．

このように肥満と高血圧症の間には密接な関係がある．しかし，高血圧症患者のすべてが肥満を呈しているわけではないし，また肥満だからといっても必ずしも高血圧症にはならない．血圧には，肥満以外の多くの因子が関与していることも忘れてはならない．特に高齢者では，加齢に伴う様々な変化が影響を与えており，個人差も大きい．高齢者の高血圧症で，減量を治療法として積極的に勧めることには注意が必要である．急激な過度の減量は高齢者では，予備力の急激な低下をもたらし，生命予後を悪くする場合も多い．また減量は骨量を減少させ，骨粗鬆症や骨折のリスクを高めることにも留意しなければならない[6]．高度な肥満があって，これが原因と思われる高血圧症であり，感染症やほかの重篤な消耗性疾患の合併がない場合に限って，数年間かけて3～4kgのゆっくりとした減量を行うべきである．

肥満と冠動脈疾患

肥満が冠動脈疾患の重要な危険因子であることは広く知られている．例えば，31万人を対象として7年間の追跡が行われたAsia-Pacific Cohort Collaboration Studyでは，BMIが2低下すると虚血性心疾患のリスクは11％低下した[7]．Framingham StudyではBMIが25未満に対して25以上30未満では，虚血性心疾患のリスクは男性1.40倍，女性1.32倍，BMIが30以上では男性で1.65倍，女性1.83倍であり，これは喫煙や糖尿病，高コレステロール血症の有無で調整してもあまり変化がなかった[3]．

高齢者での検討はそれほど多くはない．ちょうど70歳を迎えた高齢者を，その後15年間追

跡したスウェーデンでの調査結果では，高齢者でもBMIの上昇が冠動脈疾患の発症に男女ともに有意に関連しており，これは喫煙や糖尿病の有無，血圧，コレステロール値を調整しても同じであった．またウエスト周囲長も同様に冠動脈疾患の発症に関連しており，これはBMIと独立して冠動脈疾患の発症に関連していた[8]．55～69歳までの女性3万人の12年間の追跡調査では，BMIは30以上で初めて冠動脈疾患のリスクが高くなっていたが，内臓肥満の指標であるウエスト・ヒップ比(WHR)は大きくなるほど冠動脈疾患のリスクが高くなっていた[9]．

心疾患を既に有する者での肥満と心臓病死との関連は強くはない．5,000人の心筋梗塞あるいは脳卒中の既往を有する者での5年間の追跡では，肥満によって心臓病による死亡の増加はみられず，むしろBMIが22未満の痩せた者で，総死亡と心臓病死のリスクが高くなっていた[10]．American Academy of Family Physiciansによる高齢者の冠動脈疾患二次予防法でも，高脂血症の治療，運動，禁煙が勧められており，減量については推奨されていない[11]．

肥満と脳血管障害

Asia-Pacific Cohort Collaboration Studyでは，BMIが2低下すると脳梗塞リスクは12%，脳出血のリスクは8%低下していた[7]．Framingham StudyではBMIが25未満に対して25以上30未満では，脳血管障害のリスクは男性1.21倍，女性1.20倍，BMIが30以上では男性で1.46倍，女性1.64倍であり，これは喫煙や糖尿病，高コレステロール血症の有無で調整してもあまり変化がなかった[3]．

高齢者での調査はやはりそれほど多くはない．スウェーデンの70歳高齢者の15年間の追跡調査でウエスト周囲長が99 cm以上，BMIが28以上で男性の脳卒中のリスクが高くなっていたが，女性では肥満は脳卒中のリスクにはなっていなかった[12]．ハワイ在住日系人の男性高齢者での22年間の追跡による解析でも，対象者をBMIで3群に分けて比較したところ，平均20.3の群に比べて26.6の群では脳梗塞のリスクは2.1倍になっていた[13]．男性の高齢者では肥満は脳卒中のリスクになっている可能性は高いが，女性でははっきりしていない．今後の疫学的な検討が必要である．

おわりに

高齢者でも肥満は高血圧症や動脈硬化の重要な危険因子となっている可能性があるが，研究はそれほど多くはない．低栄養状態にある高齢者も多く，減量は高齢者には時として生命予後を悪化させる場合も多い．また，特に高齢女性では骨粗鬆症のリスク増加も考慮せねばならない．高齢者の減量は，どうしても必要と考えられる場合に慎重に行っていく必要がある．日本では，BMIが30を超えるような肥満者は欧米ほどに多くなく，欧米でのエビデンスをそのまま適用できないことも考えられる．高齢者では脊椎の変化で身長の見かけ上の低下があり，このためBMIが高くなることにも注意しなければならない[14]．例えば，150 cmの身長の人が50 kgであればBMIは22.2とほぼ理想的な値であるが，見かけ上の身長が9 cm低くなればBMIは25を超えてしまう．超高齢社会を迎え，高齢者や超高齢者への減量効果のエビデンスを，欧米とは生活習慣が大きく異なるわが国でも，今後，多数集積していく必要がある．

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長期縦断疫学で分かったこと

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