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Weight Loss Reduces Plasma Endothelin-1 Concentration in Obese Men

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Obesity is associated with endothelial dysfunction that may contribute to the development of diabetes, hypertension, and atherosclerosis. Endothelin-1 (ET-1), which is produced mostly by vascular endothelial cells, has potent vasoconstrictor and proliferative activity in vascular smooth muscle cells and, therefore, has been implicated in regulation of vascular tonus and the progression of atherosclerosis, suggesting that ET-1 may be important in endothelial dysfunction. We studied whether diet-induced weight loss (i.e., lifestyle modification) affects plasma ET-1 concentration in obese individuals. We measured plasma ET-1 concentration in seven obese men (age: 48 ± 4 years old, body mass index: 27.7 ± 0.5 kg/m^2) before and after a 3-month, diet-induced weight reduction program (i.e., lifestyle modification program). Caloric restriction reduced body weight from 78 ± 3 to 68 ± 2 kg ($P < 0.001$) and resulted in 12.1 ± 1.2% reduction in body mass index (24.3 ± 0.3 kg/m^2, $P < 0.0001$). After the weight reduction program, systolic and diastolic blood pressure significantly decreased (128 ± 7 vs. 115 ± 4 mm Hg, $P < 0.05$ and 88 ± 4 vs. 77 ± 2 mm Hg, $P < 0.01$, respectively). The plasma level of ET-1 significantly decreased after the program (5.1 ± 0.4 vs. 4.0 ± 0.3 pg/ml, $P < 0.05$). The percentage systolic blood pressure reduction and percentage plasma ET-1 concentration reduction was in a linear relationship ($r = 0.86$, $P < 0.05$). Furthermore, the relationship between percentage weight reduction and percentage plasma ET-1 concentration reduction was linear ($r = 0.87$, $P < 0.05$). We conclude that weight loss by low-calorie diet (i.e., lifestyle modification) reduces plasma ET-1 concentration in obese individuals. This reduction may contribute to the improvement of obesity-induced endothelial dysfunction. Exp Biol Med 231:1044–1047, 2006

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Key words: endothelin-1; obesity; endothelial dysfunction; diet

Introduction

Obesity is strongly associated with endothelial dysfunction that may play a role in the development of diabetes, hypertension, and atherosclerosis (1, 2). Endothelial dysfunction is a common abnormality in obesity. Damage to the endothelium is an important risk factor for cardiovascular diseases because it leads to structural changes, such as thickening of the intima and media of vessel walls. Clinical and animal studies have confirmed a strong relationship between obesity and cardiovascular disease such as diabetes, hypertension, and atherosclerosis (2–4). However, mechanisms linking obesity with endothelial dysfunction have not yet been fully clarified.

Vascular endothelial cells play a major role in maintaining cardiovascular homeostasis in health and diseases (5, 6). Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells (6, 7). ET-1 has potent vasoconstrictor effect on vascular smooth muscle cells (8). It has also been reported that systemic administration of an endothelin receptor antagonist significantly decreased systemic blood pressure and peripheral vascular resistance in healthy humans, strongly suggesting that endogenous generated ET-1 contributes to basal vascular tonus in humans (9). Furthermore, ET-1 is a pro-mitogen, potentiating the response of other growth factors; therefore, ET-1 has been implicated in the progression of atherosclerosis (6, 10, 11). Thus, ET-1 has been implicated in regulation of vascular tonus and progression of atherosclerosis, suggesting that ET-1 may be important in endothelial dysfunction.

Obesity is strongly associated with endothelial dysfunction. However, the mechanisms underlying obesity-induced endothelial dysfunction are unclear. Endogenous ET-1 may play an important role in endothelial dysfunction because ET-1 has been implicated in regulation of vascular tonus and progression of atherosclerosis. The purpose of the

Table 1. Body Weight, BMI, Blood Pressure, and Plasma Glucose Level in Obese Men Before and After 3-Month Diet-Induced Weight Reduction Program[a]

	Before	After	P
Body weight, kg	78 ± 3	68 ± 2	$P < 0.001$
BMI, kg/m^2	27.7 ± 0.5	24.3 ± 0.3	$P < 0.0001$
Blood pressure, mm Hg			
Systolic	128 ± 7	115 ± 4	$P < 0.05$
Diastolic	88 ± 4	77 ± 2	$P < 0.01$
Plasma glucose, mg/dl	100.0 ± 5.1	94.3 ± 3.0	$P < 0.10$

BMI, body mass index. Values are means ± SE.

resent study was to examine whether diet-induced weight oss affects plasma ET-1 concentration in obese individuals. t is of great interest and importance to study whether veight loss causes a decrease in plasma ET-1 concentration n obese humans. We hypothesized that weight loss can educe plasma ET-1 concentration in obese individuals, and hat this reduction contributes to the improvement of besity-induced endothelial dysfunction. In the present tudy, we measured plasma ET-1 concentration in obese ien before and after a 3-month, diet-induced weight eduction program.

Materials and Methods

Subjects. Seven obese men participated in the study 48 ± 4 years old, height: 167.6 ± 2.0 cm, body mass ndex [BMI]: 27.7 ± 0.5 kg/m^2). None of the participants vas taking medication on a regular basis at the time of the tudy. The obese subjects performed a 3-month, low-calorie iet intervention study.

The study was approved by the Ethical Committee of ne Institute of Health and Sport Sciences, the University of Tsukuba. This study conformed with the principles outlined n the Helsinki Declaration, and all subjects gave their vritten informed consent before inclusion in the study.

Experimental Design. All obese men were studied efore and after 3 months of diet-induced weight reduction rogram (i.e., lifestyle modification program). Body weight, MI, systolic blood pressure, diastolic blood pressure, enous plasma glucose concentration, and venous plasma T-1 concentration were measured before and after a 3- ionth, diet-induced weight reduction program in the obese ien. Before they were tested, subjects fasted for 12 hrs. lood pressure was measured in duplicate, with subjects in ie upright sitting position. All measurements were erformed at a constant room temperature (25°C).

Dietary Protocol. All subjects were instructed to ke meals per day consisting on average of 420 kcal of otein, 840 kcal of carbohydrate, and 420 kcal of fat (total: 680 kcal/day). Subjects kept daily food diaries during the month intervention period and learned about proper daily utrition (well-balanced protein, carbohydrates, fat, various amino acids, vitamins, and minerals) through weekly lectures and counseling by skilled dieticians.

Measurement of Plasma ET-1 Concentration by Sandwich-Enzyme Immunoassay. Each blood sample was placed in a chilled tube containing aprotinin (300 KIU/ml) and EDTA (2 mg/ml), and was then centrifuged at 2000 *g* for 15 min at 4°C. The plasma was stored at −80°C until use. Plasma (1 ml) was acidified with 3 ml of 4% acetic acid, and immunoreactive ET-1 was extracted with a Sep-Pak C18 cartridge (Waters, Milford, MA), as previously described in our article (12). The elutes were reconstituted with 0.25 ml of assay buffer and were subjected to sandwich-enzyme immunoassay. The sandwich-enzyme immunoassay for ET-1 was carried out as previously described using immobilized mouse monoclonal antibody AwETN40, which recognizes the NH_2-terminal portion of ET-1, and peroxidase-labeled rabbit anti-ET-1 COOH-terminal peptide (15–25) Fab' (12). The Fab' fragment of this rabbit antibody was used as an enzyme-labeled detector antibody after being coupled with horseradish peroxidase. The coefficient of variation of the ET-1 assay for intraassay variation was 11% and coefficient of variation for interassay variation was 13% (13). We previously reported that the lowest detection limit of this assay was 0.4 pg/ml for ET-1 (14). The plasma ET-1 levels in the present study were far beyond the lowest limit of detection with this assay (0.4 pg/ml) in all subjects.

Statistics. Values are means ± SE. To evaluate differences in the levels before and after weight reduction program in obese men, Student's *t* test for paired values was used. $P < 0.05$ was accepted as significant.

Results

All seven obese men completed the 3-month, low-calorie diet intervention study. Table 1 shows the body weight, BMI, blood pressure, and plasma glucose concentration in the obese men before and after 3 months of the weight reduction program. Body weight markedly decreased after the weight reduction program (Table 1). BMI also remarkably decreased after the program (Table 1). After the weight reduction program, systolic blood pressure and diastolic blood pressure significantly decreased (Table 1). There was no significant change in plasma glucose concentration before and after the program (Table 1). The plasma level of ET-1 significantly decreased after the program (5.1 ± 0.4 vs. 4.0 ± 0.3 pg/ml, $P < 0.05$; Fig. 1). Figure 2 shows the relationship between the percentage plasma ET-1 concentration reduction and the percentage systolic blood pressure reduction or percentage weight reduction. The percentage systolic blood pressure reduction and percentage plasma ET-1 concentration reduction was in a linear relationship ($r = 0.86$, $P < 0.05$; Fig. 2A). Furthermore, the relationship between percentage weight reduction and percentage plasma ET-1 concentration reduction was linear ($r = 0.87$, $P < 0.05$; Fig. 2B).

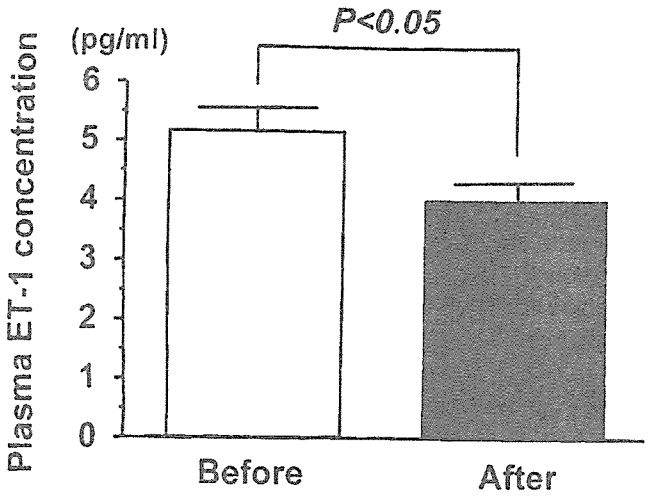

Figure 1. Plasma concentration of ET-1 before and after 3 months of weight reduction program in obese men ($n=7$). Values are means ± SE.

Discussion

In the present study, we measured plasma ET-1 concentration in obese men before and after a 3-month, diet-induced weight reduction program. After the program, because of which the body weight and BMI markedly decreased, the plasma ET-1 concentration significantly decreased. We also demonstrated that the low-calorie diet in obese men significantly decreased systolic and diastolic blood pressure, suggesting the improvement of endothelial dysfunction. Furthermore, there was a significant positive correlation between the percentage plasma ET-1 concentration reduction and percentage systolic blood pressure or body weight reduction in obese men. Therefore, we suggest that the reduction of ET-1 by weight loss may contribute to the improvement of obesity-induced endothelial dysfunction because ET-1 has been implicated in regulation of vascular tonus and progression of atherosclerosis.

Obesity is strongly associated with cardiovascular disease (1–4). Obese individuals are at increased risk for diabetes, hypertension, atherosclerosis, and other cardiovascular diseases. Obesity is also associated with endothelial dysfunction that may play a role in the development of hypertension and atherosclerosis (1). Endothelial dysfunction is a common abnormality in obesity. Vascular endothelial cells produce ET-1, which is a potent vasoconstrictor peptide and has potent proliferating activity in vascular smooth muscle cells (6, 7, 9–11). Thus ET-1 has been implicated in regulation of vascular tonus and progression of atherosclerosis, suggesting that ET-1 may be important in the endothelial dysfunction. Cardillo and colleagues (15) have shown recently that blockade of endothelin A receptor induces significant vasodilation in overweight and obese humans. Therefore, increased vascular production of ET-1 in hypertensive patients with increased body mass has been suggested as a potential mechanism for endothelial dysfunction. In the present study,

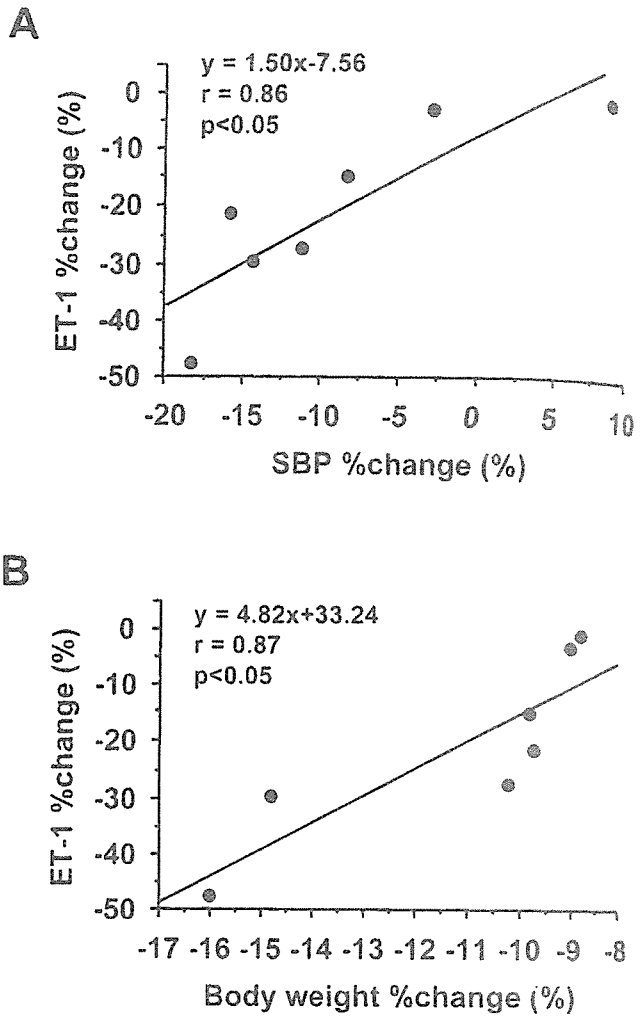

Figure 2. The relationships between the percentage plasma ET-1 concentration reduction and the percentage systolic blood pressure reduction (A) and the percentage weight reduction (B) after 3 months of weight reduction program in obese men.

the plasma ET-1 concentration significantly decreased after the weight reduction program. The present study also demonstrated that blood pressure reduced after the program. We considered that the reduction of ET-1 may participate in the mechanism underlying the improvement of endothelial dysfunction by modest weight loss. Therefore, it is possible that modest weight loss can improve endothelial dysfunction in obese humans through reduction of ET-1 production.

Several reports indicate that weight loss and lifestyle modifications can improve endothelial function. It has been shown that 6 months of weight reduction and exercise improve endothelial function and reduce selective markers of endothelial activation and coagulation in obese individuals (16). Thus lifestyle modification in the form of caloric restriction and moderate intensity physical exercise in obese subjects may be of importance for improvement of endothelial dysfunction. A recent study demonstrated that weight reduction with a very low-calorie diet improves flow-mediated vasodilation in obese subjects (17). It has

been reported that, after 2 weeks of low-calorie intake, a significant improvement in flow-mediated dilatation was observed in obese hypertensive patients (18). Furthermore, healthy premenopausal obese women after 1 year of a weight reduction progam were able to reduce body weight (10%) with an improvement in vascular responses to L-arginine (19, 20). The present study demonstrated that the plasma ET-1 concentration significantly decreased after the weight loss program with reduction of blood pressure. Therefore, we suggest that weight loss (i.e., lifestyle modification) reduces plasma ET-1 concentration in obese men, and this reduction may participate in improvement of endothelial dysfunction, thereby contributing to beneficial effects on the cardiovascular system (i.e., prevention of progression of hypertension and atherosclerosis by endogenous ET-1).

The plasma ET-1 concentration in healthy humans was 1.0–1.5 pg/ml (12), and significantly increased with aging (i.e., plasma ET-1 concentration was markedly higher in healthy older humans than in healthy young or middle-aged humans) (12). Our laboratory previously reported that plasma ET-1 concentration is increased in some human diseases (14, 21) (e.g., chronic heart failure [22] and acute myocardial infarction [21]). In the present study, the plasma ET-1 concentration in obese humans was 5.1 ± 0.4 pg/ml, and was clearly higher in obese humans than in healthy humans. Therefore, the increased plasma ET-1 concentration may be associated with obesity-induced diabetes, hypertension, atherosclerosis, and other cardiovascular diseases.

In conclusion, we demonstrated that weight reduction in obese men significantly decreased plasma ET-1 concentration. Because ET-1 has potent vasoconstrictor and proliferative activity in vascular smooth muscle cells and has been implicated in the regulation of vascular tonus and the progression of atherosclerosis, we propose that the decrease in production of ET-1 by weight loss may be partly involved in the improvement of endothelial dysfunction in obese individuals.

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CARDIOVASCULAR DISEASES

Lower HDL-cholesterol among healthy middle-aged Japanese-Brazilians in São Paulo compared to Natives and Japanese-Brazilians in Japan

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Abstract. Blood lipid levels are determined by a combination of genetic and environmental factors. Higher than average values of high-density lipoprotein cholesterol (HDL-cholesterol) have been observed in people of Japanese ethnicity. The aim of this study was to investigate whether Japanese immigrants to Brazil and subsequent generations maintain the protective benefits associated with higher levels of HDL-cholesterol, and to examine the potential associations between HDL-cholesterol and a variety of other blood lipids, anthropometric and lifestyle factors. Healthy men and women aged 35 years and older who were Native Japanese (n = 198) or Japanese-Brazilians (JB) living in São Paulo, Brazil (n = 198) and in some Japanese cities (n = 246) were investigated. Anthropometric variables, blood lipids including HDL-cholesterol, and lifestyle factors were assessed. Serum HDL-cholesterol was observed to be lower for JB in São Paulo (both women and men) compared with Natives and JB in Japan. Among the groups, triglycerides, waist circumference, LDL-cholesterol, meat intake, stress, and smoking were observed to be independently negatively associated with HDL-cholesterol, whereas total cholesterol, fish intake, and physical activity were positively associated. Lower levels of HDL-cholesterol among both men and women of JB in São Paulo compared with both other groups were confirmed even after lifestyle adjustments. Our findings highlight the significantly lower levels of HDL-cholesterol among Japanese-Brazilians living in São Paulo city compared to Japanese-Brazilians and Native Japanese residing in Japan. Although several lifestyle factors were found to be significantly associated with HDL-cholesterol, they cannot adequately explain the role of the Brazilian cultural environment on HDL-cholesterol levels.

Key words: Cardiovascular disease, HDL-cholesterol, Lifestyle, Japanese immigrants, Japanese-Brazilian

Introduction

Epidemiological and clinical studies provide evidence that blood serum concentrations of high-density lipoprotein cholesterol (HDL-cholesterol) are independently associated with increased risks for coronary heart disease (CHD) [1–3]. Levels of HDL-cholesterol in the Japanese population are higher than those reported in Caucasian populations[4, 5]. This favorable lipid profile may be one of the explanations for the lower morbidity of CHD in Japanese populations [6]. Also, there is a growing consensus in support of high HDL-cholesterol as an additional therapeutic target to promote longevity [7]. Japanese have the longest life expectancy of the world, which may be associated with their lower mortality from CHD. Lipid profiles play an important role in overall health and protection from cardiovascular pathology [8]. The protective effect of HDL-cholesterol has been attributed to its role in reverse cholesterol transport, its effect on endothelial cells, and its antioxidant activity [9]. Other lipid abnormalities tend to accompany low HDL-cholesterol, such as increased triglycerides (TG) and low-density lipoprotein cholesterol (LDL-cholesterol) [2]. In many individuals, these characteristics occur as part of the metabolic syndrome, a constellation of risk factors for diabetes and CHD that also includes abdominal obesity, elevated fasting blood glucose levels, and hypertension [10].

Significant differences in serum lipid levels have been reported between various ethnic groups and nationalities which are likely a reflection of both environmental and genetic factors [11–13]. Environmental factors affecting serum HDL-cholesterol concentrations include physical activity, obesity, composition of diet, use of tobacco and alcohol, stress, and medication [1, 14–20]. Previous studies have shown unfavorable lipid profiles to be associated with increased prevalence of CHD among Japanese immigrants to Hawaii [21] and California [22] compared to Native Japanese. Likewise, lower levels of HDL-cholesterol were observed in Japanese-Americans in Seattle compared to Native Japanese, and it is suggested that the adoption of a westernized lifestyle may be harmful to people of Japanese ancestry who may have a greater propensity for the development of various metabolic abnormalities such as diabetes and hypercholesterolemia [13].

The Japanese immigration to Brazil has concentrated in and around São Paulo city. It has become the largest Japanese community outside of Japan. Previous studies of Japanese-Brazilians living in there found that several changes in lifestyle were related to an increased risk of developing chronic diseases [23]. The study reported lower levels of HDL-cholesterol in Japanese-Brazilians in Brazil compared to Natives. Furthermore, since twenty years ago a movement of back-immigration to Japan has been initiated, and nowadays a population of nearly 300 thousand Japanese-Brazilians is estimated to be living in Japan. However, there is little information about HDL-cholesterol and its association with other lipids and lifestyle factors among Japanese-Brazilians in Brazil, or among those who returned to live in Japan. With this in mind, this study investigated whether Japanese-Brazilians retained the increased HDL-cholesterol profile compared with Native Japanese, and examined the association between HDL-cholesterol levels and other lipids and lifestyle factors. This investigation clarifies our understanding of the impact of immigration on an important risk factor for CHD, a significant public health concern.

Methods

Study population

This cross-sectional study was undertaken in 2004 and 2005 to examine the serum lipid profiles and lifestyle factors of people aged 35 years and over in urban areas of Japan and Brazil. This study involved Native Japanese and Japanese-Brazilians (JB), with both parents of Japanese ancestry, who were raised in Brazil. The participants had no history of heart failure, stroke, angina pectoris, myocardial infarction, diabetes, or cardiac surgery, and were not under medication for dyslipidemia, heart failure or diabetes. Also, those with blood glucose levels above 126 mg/dl were excluded from this analysis. Consequently, two hundred fifty-two participants were excluded from the analysis (parents were not of Japanese ancestry in 85 cases, 17 cases displayed difficulties with blood assessment, 150 participants did not meet the above inclusion criteria regarding disease and medication). In total, 198 Natives Japanese (110 women and 88 men) and 444 Japanese-Brazilians (217 women and 227 men), ranging in age from 35 to 79 (52.9 ± 10.3) years were included in the study.

Native Japanese

Japanese natives were recruited through newspapers and local newsletters in three community centers located in Chiba (Sodegaura), Ibaraki (Tsukuba), and Mie (Shima) prefectures. Data were collected on weekends in October and November of 2005 and 198 participants (110 women and 88 men) were included in this group. None of the participants had lived in abroad for more than 3 months during their lifetime.

Japanese-Brazilians in Japan

Brazilians of Japanese ancestry residing in three different provinces in Japan (Oizumi in Gunma, Kamisato in Saitama, and Minokamo in Gifu) were recruited through Nippon-Brazilian community centers, Brazilian schools, restaurants, stores, newspapers, magazines, and local television stations. Data were collected during weekends in October and November of 2004. A total of 246 participants were included in this group (97 women and 149 men). The majority of JB in Japan were from the second generation (*nisei*) of Japanese descendents (73.2%, 68 women and 112 men), in addition, 2.5% (1 women and 5 men) from the first generation (*issei*), and 24.4% (28 women and 32 men) from the third generation (*sansei*). At the time of data collection, all participants in this group were living in Japan for a length of greater than 1 year, with 59.3% (56 women and 90 men) for a period of greater than 5 years.

Japanese-Brazilians in São Paulo

Data were collected in ten Nippon-Brazilian associations and cultural centers located in São Paulo city during March and April of 2005. A total of 198 participants were included in this group (120 women and 78 men). The majority of JB in São Paulo were *nisei* (61.6%, 49 men and 73 women), with 15.7% (13 men and 18 women) and 22.7% (17 men and 28 women) *issei* and *sansei*, respectively.

Protocol

The overall protocol was approved in Brazil by the Ethical Committee of the School of Medicine, University of São Paulo, and in Japan by the Human Investigation Review Committee at the School of Comprehensive Human Sciences, University of

Tsukuba. A statement of informed consent (available bilingually in Japanese and Portuguese) was obtained from all participants prior to initiation of the data collection. Data collection was carried out for all groups during mild seasons (spring or autumn), in order to avoid the influence of climatic factors on the variables measured in this study. The survey consisted of both quantitative clinical examinations and qualitative information assessed through self-administered questionnaires checked by trained researchers.

Data collection was conducted in the morning, following an at least 12 h fasting period in which no food or medication was taken by the participants. A sample of venous blood (approximately 10 cc) was drawn from each participant and collected in vacuum tubes. The blood samples in Japan were stored at 4°C until they were delivered to the laboratory for analysis (Kotobiken Medical Laboratories, Inc., Tsukuba, Japan). All analyses were conducted within 72 h of the blood collection. For samples collected in Brazil, blood tubes were immediately centrifuged to obtain plasma and serum, and frozen at −80°C. After the data collection in Brazil, frozen blood samples (approximately 5 cc) were transferred to Japan to be analyzed by the same procedure in the same laboratory. Serum total cholesterol (TC) was measured by the cholesterol oxidase HDAOS method (Wako Pure Chemical Industries, Ltd.), HDL-cholesterol using a modified enzymatic method (Kyowa Medex Co., Ltd.), and TG by a GPO-HDAOS method, a glycerol blanking method assay (Daiichi Pure Chemicals Co., Ltd.). The LDL-cholesterol was calculated according to the method of Friedewald et al [24]. The total/HDL-cholesterol ratio was calculated as TC divided by HDL-cholesterol (in mg/dl). A homogeneous method based on an innovative detergent technology (A&T Corporation) was used for the assessment of fasting plasma glucose.

Weight to the nearest 0.1 kg was measured using a digital scale balance (TBF-551, Tanita, Tokyo, Japan) and height to the nearest 0.1 cm using a wall-mounted stadiometer (YG-200, Yagami, Nagoya, Japan). Body mass index (BMI) was calculated as weight (kg) by squared height (m^2). Waist circumference (WC) was measured at the umbilical level (cm). A questionnaire on history of disease, medication and lifestyle, including personal data about immigration history for Japanese-Brazilians, was completed by each participant. Participants were classified into the three groups (Native, JB in Japan, JB in São Paulo) by residence and according to their history of immigration. A questionnaire examining smoking history was used to determine whether or not the participant was a current smoker and the number of cigarette smoked per day. For consumption of alcoholic beverages (wine, beer, liquor, Japanese *sake*, Brazilian *cachaça*, vodka, and others), fish (fillet, sticks, sushi, and others) and meat (hamburger, steak, roast beef, meatloaf, and others), participants were asked to report their usually weekly intake frequency. Physical activity in a typical week was assessed in four domains: (1) occupational (whether a participant's job was physically demanding); (2) household (cleaning, gardening, and cooking); (3) transportation (bicycle or walking as transportation for moving around the city); and (4) sports/recreation (regular physical exercise or leisure-time activity). Each domain was assessed separately, and those who reported moderate or high levels of physical demand were classified as "active", whereas those who reported none or low physical demand were classified as "low active". A physical activity index was established according to information from all four domains, and those reporting moderate or high physical demand in all domains were considered "more active". The assessment of stressful events in life was based on a previous study [25] which reported several stress items in life associated with increased prevalence of CHD. The stress variables included: concern about a family member (serious illness, death or other serious concern), divorce or separation, change in residence, concern about work (feelings of insecurity at work or forced to change job), serious financial trouble, legal problems, and having difficulties in sleeping. For the present study, those who reported "never experienced" or "experienced temporally" were grouped as "no stress present", whereas those that had experienced the stressful event item for "some periods", "several periods", or "permanent stress" during the past 5 years were categorized as "presence of stress". In addition to evaluating stress levels for each of the seven variables identified above, an index of overall stress was computed based on a participant's report of the "presence of stress" for at least one of the stressful life events. Lastly, hours of sleep in a typical day were also recorded.

Statistical analysis

Chi-square tests were used for the analyses of categorical variables, and post hoc tests were applied for the comparison between each pair of groups. Analyses of variance (ANOVA) and covariance (ANCOVA) were used in comparisons of metabolic risks among groups. Stepwise multiple regression analyses were performed to examine potential independent associations of waist circumference, blood lipids and lifestyle factors by gender, and their relative importance as determinants of HDL-cholesterol. Analyses of covariance were assessed as shown in Figure 1, adjusted by age (year), and the following selected lifestyle factors that were shown to significantly vary across groups: smoking (never and former = 0, current = 1), alcohol only for women (abstainer or former = 0, current = 1), fish and meat consumption (≤ 5 times/week = 0, >5 times/week = 1), physical activity index only for men (low active = 0, more

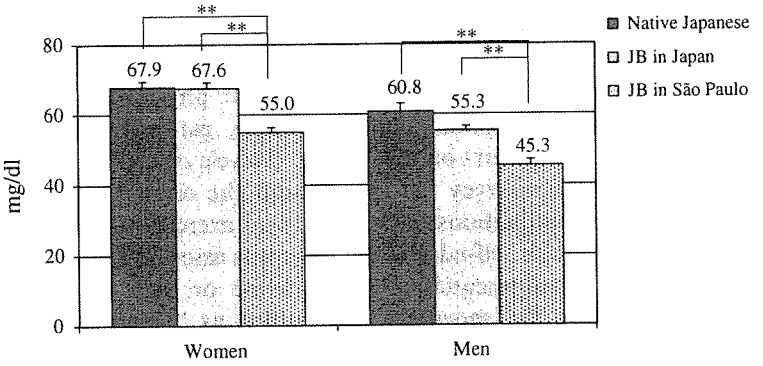

Figure 1. Mean and SE values of HDL-cholesterol adjusted for age and selected lifestyle factors. JB, Japanese-Brazilian. ** $p < 0.001$.

active = 1), and stress index (no stress presence = 0, stress presence = 1). All statistical analyses were performed using SPSS 13.0 for Windows and statistical significance was set at $p < 0.05$.

Results

Table 1 shows physical characteristics and lipid profiles of the participants. As significant differences between groups were observed in age, group differences were examined by ANCOVA with age as covariate. BMI was not significantly different among groups, ranging between 23.8 and 25.0 kg/m^2 in women, and between 24.3 and 25.3 kg/m^2 in men. Waist circumference in men from the JB in São Paulo group was significantly higher, followed by JB in Japan, and Native; whereas no significant difference was observed among women. The analyses for both men and women confirmed significantly lower means of serum HDL-cholesterol among JB in São Paulo (44.0 mg/dl) compared to Native (63.4 mg/dl) and JB in Japan (55.7 mg/dl). For men, JB in Japan HDL-cholesterol was also significantly lower than for Natives. Women showed slightly higher values than men, and women JB in São Paulo averaged values of 55.1 mg/dl, whereas Natives and JB in Japan showed 68.3 and 67.0 mg/dl, respectively. Among the other lipids investigated in men and women, the mean TC/HDL-cholesterol ratio and TG were also significantly higher for JB in São Paulo, however, JB in Japan showed higher TC and LDL-cholesterol compared to the other two groups.

Among lifestyle factors (Table 2), compared to Native Japanese, cigarette smoking was more frequently observed among participants in the JB in Japan group (21.6 and 32.96% in women and men, respectively), and JB in São Paulo (13.3 and 51.3% in women and men, respectively). Current alcoholic beverage consumption was more frequently observed among Native Japanese women (30%) and no differences were observed among men. Fish consumption was higher among Native Japanese women (23.6%) and men (16.3%); whereas consumption of meat was higher among JB in São Paulo (19.2 and 23.1% in women and men, respectively), and JB in Japan (18.6% and 24.8% in women and men, respectively). For women, JB in Japan reported more physically demanding occupational activities and lower sports/recreation activities, whereas Natives Japanese and JB in São Paulo reported higher household and sport/recreation activities. For men, JB in Japan reported higher physically demanding occupational activities, and higher household and transportation activities. Whereas Native Japanese and JB in Japan reported higher sports/recreation activities. The overall physical activity index was not significantly different among women (75.5% of Native Japanese reported to be active in all domains, 86.6% of the JB in Japan, and 75% of the JB in São Paulo). However, among men, JB in Japan were found to be significantly more active (77.9%), compared to JB in São Paulo (34.6%), and Native Japanese (36.7%). Native Japanese women and men self-reported lower presence of stress among all items investigated compared to JB in São Paulo and Japan. Therefore, the overall stress index was higher for JB in São Paulo (71.7% in women and 78.2% in men) and JB in Japan (68.0 and 61.7% in women and men, respectively) compared to Native Japanese (50.9 and 38.8% in women and men, respectively).

The stepwise regression model (Table 3) confirmed the independent association of lipids, anthropometric and lifestyle factors with HDL-cholesterol levels. For women, TG, LDL-cholesterol, meat intake and the stress index (trend) were negatively associated, whereas TC and fish intake were positively associated. For men, TG, waist circumference, LDL-cholesterol, the stress index and smoking (trend) were negatively associated, while TC and physical activity index were positively associated. Further analyses, stratifying subjects by Japanese ancestry, confirmed a significant negative association of living in São Paulo with elevated HDL-cholesterol, and this association remained even after adjustment for selected lifestyle factors (Figure 1). Men of JB in Japan also showed a

Table 1. Characteristic of the participants and lipid profile

	Native Japanese		JB in Japan		JB in São Paulo		*P*
	Mean ± SD	Adjusted* mean (95% CI)	Mean ± SD	Adjusted* mean (95% CI)	Mean ± SD	Adjusted* mean (95% CI)	
Participant, *n*							
Women	110		97		120		
Men	88		149		78		
Age, year							
Women	57.6 ± 9.6		46.9†‡ ± 7.3		54.1† ± 10.1		< 0.001
Men	57.1 ± 12.4		48.3†‡ ± 6.6		56.7 ± 10.2		< 0.001
Waist circumference, cm							
Women	83.4 ± 8.6	82.8 (81.2 to 84.5)	81.7 ± 9.4	82.5 (80.7 to 84.4)	82.1 ± 8.1	82.0 (80.4 to 83.5)	0.741
Men	83.2 ± 8.1	83.4 (81.6 to 85.3)	86.8 ± 8.9	86.5†‡ (85.0 to 87.9)	90.4 ± 8.5	90.7† (88.7 to 92.6)	< 0.001
Body mass index, kg/m^2							
Women	24.6 ± 4.8	24.2 (23.4 to 25.0)	24.4 ± 4.5	25.0 (24.2 to 25.9)	23.8 ± 3.2	23.8 (23.1 to 24.6)	0.128
Men	24.2 ± 3.3	24.3 (23.6 to 25.0)	24.9 ± 3.3	24.7 (24.1 to 25.3)	25.2 ± 3.3	25.3 (24.6 to 26.1)	0.142
Total cholesterol, mg/dl							
Women	216.1 ± 29.3	211.1 (204.7 to 217.5)	208.2 ± 38.2	215.6‡ (208.5 to 222.7)	203.2 ± 34.3	202.1 (196.2 to 207.9)	0.011
Men	202.5 ± 34.7	203.6 (196.1 to 211.1)	213.7 ± 34.5	212.5‡ (206.5 to 218.4)	196.5 ± 34.1	197.6 (189.7 to 205.4)	0.014
HDL-cholesterol, mg/dl							
Women	69.4 ± 15.3	68.3 (65.4 to 71.2)	65.5 ± 17.1	67.0‡ (63.8 to 70.3)	55.4 ± 12.4	55.1† (52.4 to 57.7)	< 0.001
Men	63.9 ± 18.7	63.4 (60.2 to 66.6)	55.2 ± 14.0	55.7†‡ (53.4 to 58.0)	44.4 ± 10.7	44.0† (40.6 to 47.4)	< 0.001
Total/HDL-cholesterol ratio							
Women	3.23 ± 0.72	3.17 (2.98 to 3.36)	3.35 ± 0.93	3.43‡ (3.22 to 3.64)	3.87 ± 1.17	3.86† (3.68 to 4.03)	< 0.001
Men	3.35 ± 0.88	3.41 (3.15 to 3.66)	4.15 ± 1.34	4.08†‡ (3.88 to 4.28)	4.63 ± 1.17	4.69† (4.42 to 4.96)	< 0.001
LDL-cholesterol, mg/dl							
Women	130.3 ± 27.0	127.4 (121.9 to 133.0)	125.2 ± 32.6	129.3†‡ (123.1 to 135.5)	118.1 ± 27.3	117.5† (112.4 to 122.6)	0.005
Men	113.4 ± 31.7	113.7 (105.9 to 121.5)	134.5 ± 33.4	134.2†‡ (128.7 to 139.7)	114.2 ± 28.1	114.6 (107.2 to 121.9)	< 0.001
Triglycerides, mg/dl							
Women	85.9 ± 36.3	81.2 (70.1 to 92.3)	88.1 ± 45.9	94.9 (82.6 to 107.3)	111.1 ± 75.7	110.1† (99.9 to 120.3)	0.001
Men	106.0 ± 69.7	108.4 (89.6 to 127.3)	127.1 ± 94.7	124.5 (109.7 to 139.4)	151.0 ± 89.7	153.3† (133.5 to 173.0)	0.004

JB, Japanese-Brazilian. *Age-adjusted.† Differ from Native Japanese, ‡Differ from JB in São Paulo.

Table 2. Lifestyle factors among groups by gender

	Women					Men				
	Native Japanese	JB in Japan	JB in São Paulo	χ^2	post hoc	Native Japanese	JB in Japan	JB in São Paulo	χ^2	post hoc
Participant, *n*	110	97	120			88	149	78		
Cigarette smoke, %										
Never	90.0	66.0	84.0	26.68**	†‡	46.1	40.3	39.7	24.64**	†‡§
Former	7.3	12.4	2.5			35.5	26.8	9.0		
Current	2.7	21.6	13.3			18.4	32.9	51.3		
Amount per day, mean SD	16.0 ± 6.6	12.8 ± 6.2	12.4 ± 6.1	NS		16.4 ± 9.5	15.0 ± 8.5	17.4 ± 11.4	NS	
Alcoholic beverage, %										
Abstainer	67.3	80.4	83.3	9.83*	†‡	30.6	45.6	48.7	9.43	
Former	2.7	2.1	2.5			8.2	3.4	0.0		
Current	30.0	17.5	14.2			61.2	51.0	51.3		
Fish intake > 5 days/week, %	23.6	7.2	5.0	21.89**	†‡	16.3	8.1	3.8	6.17*	†
Meat intake > 5 days/week, %	1.8	18.6	19.2	18.65**	†‡	0.0	24.8	23.1	14.93*	†‡
Physical activity[a], %										
Occupation	43.8	83.2	23.6	72.36**	†‡§	35.7	91.7	14.5	136.46**	†‡§
Household	98.2	93.8	93.3	3.37		28.3	67.1	29.5	39.23**	‡§
Transportation	37.3	40.2	40.0	0.25		20.8	43.0	20.5	15.44*	‡§
Sports/ recreation	52.7	19.6	53.3	31.14**	‡§	44.9	24.2	59.0	27.75**	‡§
Overall physical activity index [b]	75.5	86.6	75.0	5.26		36.7	77.9	34.6	50.81**	‡§
Stressful events in life[c], %										
Concern about family member	22.7	49.0	55.0	26.93**	†‡	6.1	38.6	52.6	28.38**	†‡§
Divorce or separation	0.9	8.3	2.5	8.72*	‡	2.0	1.4	0.0	1.37	
Change in residence	1.8	14.6	1.7	21.42*	‡§	2.0	6.9	1.3	4.59	
Concern about work	22.7	34.4	20.8	5.81*	‡§	18.4	27.6	28.2	1.86	
Serious financial trouble	20.9	19.8	26.7	1.74		12.2	19.3	28.2	5.00	
Legal problems	1.8	2.1	0.8	0.64		0.0	0.7	3.8	4.38	
Sleeping difficulties	20.9	33.3	36.7	7.30*	†‡	6.1	25.5	28.2	9.65*	‡§
Overall stress index [d]	50.9	68.0	71.7	11.85*	†‡	38.8	61.7	78.2	19.97**	†‡§
Sleeping (hour/day), mean ± SD	6.7 ± 0.9	6.5 ± 1.0	6.6 ± 1.1	NS		6.9 ± 1.1	6.6 ± 1.0	6.7 ± 1.1	NS	

[†]Native Japanese different from JB in São Paulo, [‡]Native Japanese different from JB in Japan, [§]JB in São Paulo different from JB in Japan. JB, Japanese-Brazilian. [a]Active, [b] Active in all items above, [c]Presence of stress, [d]Presence of stress in at least one of the above items. NS, non-significant. * $p < 0.05$, ** $p < 0.001$.

Table 3. Stepwise multiple regression for HDL-cholesterol as the dependent variable, and other blood lipids, waist circumference, and lifestyle factors as the independent variables

	Standardized Coefficients	t	*p*
Women			
Triglycerides	−0.746	−33.724	<0.001
Total cholesterol	1.875	39.462	<0.001
LDL-cholesterol	−1.568	−34.040	<0.001
Fish intake	0.058	2.822	0.005
Meat intake	−0.045	−2.158	0.032
Stress index	−0.041	−1.959	0.051
Men			
Triglycerides	−0.799	−25.464	<0.001
Waist circumference	−0.107	−3.865	<0.001
Total cholesterol	1.842	27.479	<0.001
LDL-cholesterol	−1.724	−26.047	<0.001
Physical activity index	0.088	3.493	0.001
Stress index	−0.064	−2.533	0.012
Smoking	−0.048	−1.854	0.065

Independent variables included in the model: age, waist circumference, total cholesterol, LDL-cholesterol, triglycerides, smoking, alcohol, fish and meat intake, physical activity index, stress index, and sleeping hours.

significantly negative association with HDL-cholesterol; however, after selected lifestyle factor adjustments, the association disappeared. This finding was confirmed, as shown in Figure 1, with analysis of covariance adjusted for selected lifestyle factors.

Discussion

Regardless of immigration status, our study confirmed the presence of increased levels of HDL-cholesterol among healthy middle-aged men and women of Japanese descent, with average values above the cutoff point of 40 mg/dl proposed as a risk factor in metabolic syndrome. However, this study did find significant differences in serum HDL-cholesterol among people of Japanese ancestry living in São Paulo city and in Japan, suggesting that a variety of immigration factors including the cultural environment may play a role in influencing an individual's blood lipid profile. We have found that Japanese-Brazilians in São Paulo have significantly lower levels of HDL-cholesterol compared to Native Japanese and Japanese-Brazilians in Japan. Our findings support the hypothesis that the traditionally favorable lipid profiles of people of Japanese ethnicity may be negatively affected by immigration and exposure to a western cultural environment [13, 21–23].

The group of Japanese ancestry with lower HDL-cholesterol (JB in São Paulo) also showed higher waist circumference, TG and total/HDL-cholesterol ratio levels than the other two groups, but was not higher for BMI, TC and LDL-cholesterol. For women and men, HDL-cholesterol showed a significant negative association with TG and LDL-cholesterol, and positive association with TC. Conventionally, LDL-cholesterol is the first target of dyslipidemia therapy, [26] but a clear association between decreased HDL-cholesterol and increased CHD risk is also strongly supported by the Framingham Study, [27, 28] which confirms not only the apparent protective effect of elevated HDL-cholesterol, but the clear risk associated with low levels of HDL-cholesterol. As previously mentioned, Japanese people have the longest life expectancy among developed countries. Indeed, until recently, Okinawa (the most southern area of Japan) was known as the world's foremost prefecture for longevity. Previous studies have related more favorable HDL-cholesterol profiles of Okinawans to significantly lower atherogenic indices than those from people living on the mainland of Japan [29]. On the other hand, changes in lifestyle appear to promote increases in the incidence of CHD among Okinawans, which also seem to be associated with decreased HDL-cholesterol and increased TG levels [30]. One study of Okinawans in Brazil reported overall lower levels of HDL-cholesterol which was associated with a 1/3 decrease in the number of centenarians compared to Japan. According to the author of that study, changes in lifestyle (more calories and fewer fish and seafood products) are among the possible explanations for this finding [31].

In the present study, because all groups are originally from a similar Japanese genetic background, lifestyle changes are among the mechanisms proposed as possible explanations for the lower level of HDL-cholesterol among Japanese-Brazilians in São Paulo. In a stepwise multiple regression analysis including lifestyle, consumption of fish and meat, and stress were the factors most strongly associated with HDL-cholesterol among women. Likewise, physical activity, stress and smoking were found to be important factors associated with HDL-cholesterol among men. Although different mechanisms by which smoking decreases HDL-cholesterol have been reported by previous studies, these results confirm our findings that HDL-cholesterol has a negative association trend with smoking among men [32, 33]. For instance, a recent study on smoking habits and serum lipids in a large Japanese cohort confirmed the association of smoking with lower HDL-cholesterol in both genders at any age [34]. The hypolipidemic effect of dietary fish and fish oils has been associated with protection against CHD [19, 35, 36]. Results of a study with middle-aged Japanese-Americans in Hawaii and Natives Japanese showed a positive association of fish consumption with serum HDL-cholesterol, which may have been associated with reduced mortality due to CHD among the Native Japanese [21]. Our study identified significant associations between HDL-cho-

lesterol and consumption of fish (positive) and meat (negative) among women. Favorable effect of physical activity on increased levels of HDL-cholesterol has been observed [14, 18, 32, 37]. Previous studies accounted this mechanism by the increase lipoprotein lipase activity, concomitant rapid turnover of triglycerides-rich lipoproteins, [16] fat clearance, and increase reverse cholesterol transport from increase plasma lecithin cholesterol acyl trasnferase and cholesterol ester transfer protein activities [18]. Cross-sectional studies of physically active people have shown higher levels of HDL-cholesterol, but intervention studies in which non-athletes are encouraged to begin to exercise have often shown only moderate changes [38–40]. In our study, men with lower levels of physical activity were more likely to have lower levels of HDL-cholesterol than those who were more active. Another important factor associated with HDL-cholesterol was the higher presence of stress in life for Japanese-Brazilian (both men and women). Work stress and other forms of chronic stress have been found to be related with an unfavorable lipid profile [17, 20].

After adjusting for selected lifestyle factors in the covariance analysis, Japanese-Brazilian in São Paulo maintained a significant lower level of HDL-cholesterol. However, Japanese-Brazilian men living in Japan were no longer significantly different from Native Japanese after adjusted for selected lifestyle factors. For Japanese-Brazilian in Japan, smoking habits appear to be an important risk factor for decreased levels of HDL-cholesterol in men.

The present study has some limitations. First, as a population-based design, this study does not allow us to draw inferences about causal pathways. Second, significant differences in age were observed among groups. Despite the fact that participants were volunteers recruited using identical age inclusion criteria across all groups, it was not possible to precisely equalize the mean ages for each of the three groups. Due to cultural and logistic differences between countries there may have been small differences in exactly how participants were contacted. However, it is unclear to what extent this may have contributed to the age differences observed. An additional limitation to consider was that lifestyle factors were evaluated by non-standard methods. It is possible that our measurement instruments were not sensitive enough to assess significant associations between lifestyle factors and HDL-cholesterol.

In summary, we found substantial variation in serum HDL-cholesterol among people of Japanese ancestry, which we believe illustrates the heterogeneity of people of Japanese ancestry living under different cultural environments. Japanese-Brazilians in São Paulo showed lower levels of HDL-cholesterol, and, given the well-understood association of HDL-cholesterol and CHD, we would predict, a higher risk of developing CHD among Japanese-Brazilians in São Paulo. Maintaining high levels of HDL-cholesterol may also impact the risk of developing other morbidities for coronary disease, which is also important from a clinical and public health perspective. Also, our findings support the notion that the consumption of fish and maintaining a physically active lifestyle are important factors for increasing HDL-cholesterol levels. Furthermore, smoking, consumption of meat, and stress all negatively affected HDL-cholesterol levels. However, further investigations are necessary to elucidate the effects of cultural environment factors in Brazil on the lipid profiles of Japanese immigrants and subsequent generations.

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Functional Single-Nucleotide Polymorphisms in the Secretogranin III (*SCG3*) Gene that Form Secretory Granules with Appetite-Related Neuropeptides Are Associated with Obesity

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Context: Genetic factors are important for the development of obesity. However, the genetic background of obesity still remains unclear.

Objective: Our objective was to search for obesity-related genes using a large number of gene-based single-nucleotide polymorphisms (SNPs).

Design and Setting: We conducted case-control association analyses using 94 obese patients and 658 controls with 62,663 SNPs selected from the SNP database. SNPs that possessed $P \leq 0.02$ were further analyzed using 796 obese and 711 control subjects. One SNP (rs3764220) in the secretogranin III (*SCG3*) gene showed the lowest P value ($P = 0.0000019$). We sequenced an approximately 300-kb genomic region around rs3764220 and discovered SNPs for haplotype analyses. *SCG3* was the only gene within a haplotype block that contained rs3764220. The functions of *SCG3* were studied.

Patients: Obese subjects (body mass index ≥ 30 kg/m^2, n = 890) and control subjects (general population; n = 658, body mass index $\leq$ 25kg/m^2; n = 711) were recruited for this study.

Results: Twelve SNPs in the *SCG3* gene including rs3764220 were in almost complete linkage disequilibrium and significantly associated with an obesity phenotype. Two SNPs (rs16964465, rs16964476) affected the transcriptional activity of *SCG3*, and subjects with the minor allele seemed to be resistant to obesity (odds ratio, 9.23; 95% confidence interval, 2.77–30.80; $\chi^2 = 19.2$; $P = 0.0000067$). *SCG3* mRNA and immunoreactivity were detected in the paraventricular nucleus, lateral hypothalamic area, and arcuate nucleus, and the protein coexisted with orexin, melanin-concentrating hormone, neuropeptide Y, and proopiomelanocortin. *SCG3* formed a granule-like structure together with these neuropeptides.

Conclusions: Genetic variations in the *SCG3* gene may influence the risk of obesity through possible regulation of hypothalamic neuropeptide secretion. **(*J Clin Endocrinol Metab* 92: 1145–1154, 2007)**

OBESITY HAS BECOME one of the major issues in public health, medicine, and the economy (1). In particular, visceral obesity is considered to be important due to its relation to various complications such as diabetes mellitus, dyslipidemia, and hypertension. A combination of these dysfunctions is now defined as the metabolic syndrome (2), which significantly increases the risk of cardiovascular disease. Adipose tissue secretes various adipokines, and an increase in adipose tissue mass affects

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Abbreviations: ARC, Arcuate nucleus; BMI, body mass index; CHG, chromogranin; CI, confidence interval; IMS, Institute of Medical Science; JST, Japan Science and Technology; LD, linkage disequilibrium; LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus; SFA, sc fat area; SNP, single-nucleotide polymorphism; VFA, visceral fat area.

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the level of adipokines, resulting in the development of dyslipidemia, hypertension, and insulin resistance (3, 4).

Both genetic and environmental factors contribute to the development of obesity. In epidemiological studies, heritability of body weight is estimated to be approximately 70% (5, 6). Genetic studies in mice suggested that mutations in several genes, such as leptin, proopiomelanocortin (*POMC*), and melanocortin-4 receptor, were implicated in a monogenic form of inherited obesity, whereas mutations in such genes were also reported in human subjects with obesity (6, 7). However, the most prevalent *MC4R* gene mutations have been found in only 3–5% of obese patients with a body mass index (BMI) of more than 40 kg/m^2. In general, the vast majority of obesity is considered to be caused by a polygenic disorder, and its genetic susceptibility is likely to differ among various ethnic groups (6, 7). A large number of manuscripts concerning obesity-related genes have been reported (7). However, because there are also many papers reporting controversial results at these candidate loci, the genetic background of obesity still remains unclear.

As one of the Japanese Millennium Projects, a large-scale collaborative effort performed a search for gene-based single-nucleotide polymorphisms (SNPs) in a group of Japanese subjects and discovered approximately 190,000 genetic variations (JSNP database) (8), and subsequently our center developed a high-throughput SNP genotyping system that uses a combination of multiplex PCR and the Invader assay (9, 10) to effectively determine these variations' frequencies in the Japanese population. We performed an association study using a large number of SNPs selected from the JSNP database (62,663 SNPs in 11,932 genes, covering approximately 30% of the human genome) by genotyping Japanese obese and lean subjects and found that one SNP (SNP-1, rs3764220) showed the smallest *P* value and was significantly associated with obesity. This SNP existed in the 5′-flanking region of the secretogranin III (*SCG3*) gene. *SCG3* belongs to a family of acidic secretory proteins, known as granins, which are widely expressed in endocrine and neuronal cells (11). *SCG3* has been cloned from brain- and pituitary-specific mRNA and is expressed in the paraventricular nucleus (PVN) of the hypothalamus (12), which is known to be an important region for appetite regulation. *SCG3* is also expressed in pancreatic β-cells and participates in insulin secretion together with chromogranin (CHG) A (13). Interestingly, *SCG3* is located on chromosome 15q21, on which association with obesity has been previously indicated (14). Data from the Framingham Heart Study suggested a moderate linkage of the metabolic syndrome to this general region on chromosome 15q (15) on which the presence of a susceptibility gene for type 2 diabetes in the Japanese population has also been indicated (16).

In the present study, we demonstrate a significant association between functional SNPs in the *SCG3* gene and obesity. We found that *SCG3* was expressed together with appetite-regulating peptides such as orexin and melanin-concentrating hormone (MCH) in the lateral hypothalamic area (LHA) and neuropeptide Y (NPY) and POMC in the arcuate nucleus (ARC), suggesting that *SCG3* is a good candidate as an obesity-related gene.

Subjects and Methods

Subjects

The sample size of the first set of Japanese obese subjects (BMI ≥ 30 kg/m^2) was 94 (case 1; male to female ratio 39:55; age 47 ± 17 yr; BMI 36.3 ± 5.0 kg/m^2). The sample size of the first set of control individuals (control 1) was 658 and consisted of the Japanese general population as described in JSNP database [Institute of Medical Science (IMS)-Japan Science and Technology (JST) Agency Japanese SNP database] (8). The sample size of the second set of Japanese obese subjects (BMI ≥ 30 kg/m^2) was 796 (case 2; male to female ratio 379:417; age 49 ± 14 yr; BMI 34.3 ± 5.5 kg/m^2), whereas that of the second set of Japanese normal-weight controls (BMI ≤ 25 kg/m^2) was 711 (control 2; male to female ratio 267:444; age 52 ± 16 yr; BMI 21.6 ± 2.2 kg/m^2). Secondary obesity and obesity-related Mendelian disorders were excluded in this study. Patients with obesity caused by medications were also excluded. Control 2 subjects were Japanese normal-weight volunteers collected from subjects who had undergone a medical examination for common disease screening. We further collected 403 Japanese subjects with various BMIs [male to female ratio 144:259 females; age 48 ± 12 yr; BMI 29.7 ± 7.0 kg/m^2; visceral fat area (VFA) 126 ± 81 cm^2; sc fat area (SFA) 248 ± 117 cm^2] who agreed to undergo computed tomography examinations to measure the VFA and SFA. All subjects except control 1 were newly recruited for this study. Written informed consent was obtained from each subject, and the protocol was approved by the ethics committee of each institution and that of RIKEN.

DNA preparation and SNP genotyping

Genomic DNA was prepared from each blood sample according to standard protocols. Approximately 100,000 Invader probes (Third Wave Technologies, Madison, WI) could be made for SNPs of IMS-JST (8), and the SNPs were genotyped in case 1 by Invader assays as described previously (9, 17). Genotype and allele frequencies of these SNPs were compared with control 1. The SNPs selected by association study using case 1 and control 1 were submitted for further examination using independent case 2 and control 2 groups.

SNP discovery in around SNP-1

To identify additional variations in the genomic region around SNP-1, we generated a reference sequence of approximately 300 kb by assembling the relevant regions from the sequences with GenBank accession no. AC066613, AC020892, AC026770, and AC090971. We amplified appropriate fragments of genomic DNA by PCR and sequenced the products to identify SNPs within 300 kb genomic region using previously described methods (10, 18).

Cell culture

SH-SY5Y and BE(2)-C neuroblastoma cells and HIT-T15 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in advanced DMEM (Invitrogen, Carlsbad, CA) with 2 mM glutamine, 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Luciferase assay

We synthesized double-stranded oligonucleotides containing either a single copy or four concatenated copies of either the major or minor allele for a 19-bp region centered on SNP-1, SNP-2, SNP-5, SNP-9, SNP-11, or SNP-12 (Fig. 1B), with an *Nhe*I restriction site at the 5′ end and an *Xho*I restriction site at the 3′ end. We constructed luciferase reporter plasmids by cloning the oligonucleotides into the pGL3-promoter vector (Promega, Madison, WI) upstream of the Simian virus 40 promoter. pGL3-promoter vectors containing oligonucleotides were transfected into SH-SY5Y neuroblastoma cells together with the phRL-TK vector (Promega), an internal control for transfection efficiency, using lipofectamine 2000 reagent (Invitrogen). After 24 h, we

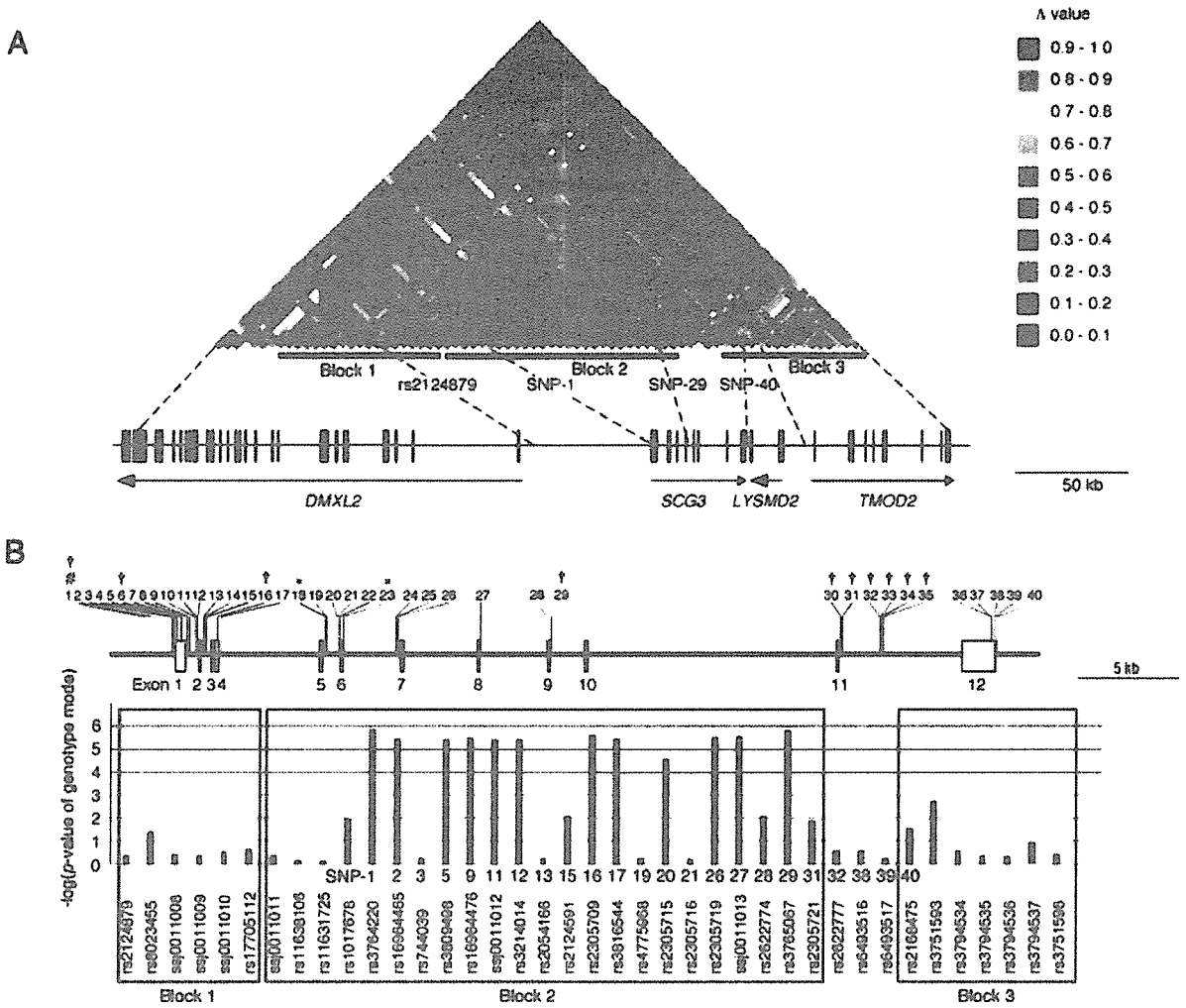

FIG. 1. LD mapping, polymorphisms, and *P* values identified around the *SCG3* gene. A, LD mapping around the *SCG3* gene. LD coefficients (Δ) between every pair of SNPs around SNP-1 (rs3764220, −1492A→G) were calculated. Minor allele frequencies of all SNPs used in this analysis are greater than 10%. Genomic structure is shown at the *bottom*. SNP rs2124879, SNP-1, SNP-29, and SNP-40 are indicated. B, Genetic variations and *P* values in the *SCG3* gene. #, SNP-1; *, insertion/deletion polymorphisms; †, SNPs analyzed in the first screening; no symbol, SNPs identified in the extensive search of the gene's genomic sequence. *P* values are represented as −logarithm of *P* values of genotype mode. Each SNP is labeled with its rs number, except for novel SNPs, which are indicated by JSNP ID (ssj0011008-0011013).

collected the cells and measured luciferase activity with the dual-luciferase reporter assay system (Promega).

Gel-shift assay

We prepared nuclear extract from SH-SY5Y cells using NE-PER extraction reagents (Pierce, Rockford, IL) and then incubated the extracts with 33-bp double-stranded oligonucleotides containing SNP-1, SNP-2, SNP-5, SNP-9, SNP-11, or SNP-12 (Fig. 1B) labeled with digoxigenin-11-ddUTP using the digoxigenin gel-shift kit (Roche Diagnostics, Indianapolis, IN). For competition studies, we incubated nuclear extract with unlabeled oligonucleotides (100-fold excess before adding digoxigenin-labeled oligonucleotide). Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer. The gel was transferred to nylon membrane, and the signal was detected with a chemiluminescent detection system (Roche Diagnostics) according to the manufacturer's instructions.

Double-labeling immunohistochemistry for SCG3, orexin, MCH, NPY, and POMC

Male mice (B57BL/6, 8 wk old) were purchased from CLEA Japan (Tokyo, Japan). After being anesthetized with sodium pentobarbital (100 mg/kg), mice were perfused with 10% neutral buffered formalin. The hypothalamic region was dissected from the brain, further fixed with tissue fixative (Genostaff, Tokyo, Japan), embedded in paraffin, and sectioned. Tissue sections (4 μm) were dewaxed and incubated at 4 C overnight with polyclonal goat anti-SCG3 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) together with either rabbit polyclonal antibody to orexin B (1:500; Chemicon, Temecula, CA), MCH (1:500; Phoenix Pharmaceuticals, Belmont, CA), NPY (1:200; Chemicon), or POMC (1:5000; Phoenix Pharmaceuticals). After washing, the sections were incubated at room temperature for 2 h with Alexa Fluor 568 donkey antigoat IgG (1:2000; Molecular Probes, Eugene, OR) and Alexa Fluor 488 donkey antirabbit IgG (1:2000; Molecular Probes) secondary antibodies. Double-immunofluorescence detection was carried out using a BX51 microscope (Olympus, Tokyo, Japan).

Expression of SCG3, orexin, MCH, NPY, and POMC in BE(2)-C neuroblastoma

The coding sequence of human SCG3 was amplified by RT-PCR from hypothalamus cDNA using primers with an added N-terminal *PstI* restriction site located before the start codon and a C-terminal *SalI* restriction site located after the stop codon. The PCR product was cloned between the *PstI* and *SalI* sites of the pBI vector (CLONTECH, Palo Alto,

CA). The coding region of human preproorexin, pro-*MCH*, *POMC*, and pro-*NPY* were also amplified but with primers that included an N-terminal *MluI* site located before the start codon and a C-terminal *Eco*RV site located after the stop codon. The PCR products were cloned between the *MluI* and *Eco*RV sites of the pBI-*SCG3* plasmid. The pBI-*SCG3*-preproorexin, pBI-*SCG3*-pro-*MCH*, pBI-*SCG3*-*POMC*, and pBI-*SCG3*-pro-*NPY* were transfected using lipofectamine 2000 reagent (Invitrogen) into a previously established cell line of BE(2)-C cells containing the pTet-Off vector (CLONTECH). For immunocytochemical detection, cells were fixed with 4% paraformaldehyde for 15 min then treated with 0.5% Triton X-100. Cells were incubated with polyclonal goat anti-SCG3 (1:200; Santa Cruz Biotechnology) together with rabbit polyclonal antibody to orexin B (1:500; Chemicon), MCH (1:500; Phoenix Pharmaceuticals), NPY (1:500; Progen Biotechnik, Heidelberg, Germany), or POMC (1:5000; Phoenix Pharmaceuticals) in PBS containing 1% BSA overnight at 4 C. We washed and incubated the cells at room temperature for 2 h with Alexa Fluor 488 donkey antigoat IgG (1:2000; Molecular Probes) and Alexa Fluor 568 donkey antirabbit IgG (1:2000; Molecular Probes) secondary antibodies. The cells were examined using an Olympus FV300 confocal laser-scanning microscope.

Statistical analysis

For each case-control study, the frequencies of the genotypes or the alleles were compared between cases and controls in four different modes. In the first mode (allele frequency mode), allele frequencies were compared between cases and controls using a 2 × 2 contingency table, whereas in the second mode (genotype mode), frequencies of the three genotypes were compared between cases and controls using a 2 × 3 contingency table. In the third mode (minor allele homozygotes mode), the frequencies of the homozygous genotype for the minor allele were compared using a 2 × 2 contingency table, whereas in the fourth mode (major allele homozygotes mode), the frequencies of the homozygotes for the major allele were compared using a 2 × 2 contingency table. Odds ratio and its 95% confidence interval (CI) were calculated by Woolf's method. Hardy-Weinberg equilibrium was assessed using the χ^2 test (19). We used the correlation coefficient Δ, calculated as reported previously (20), as the measure to evaluate the strength of linkage disequilibrium (LD). Haplotype phasing was estimated using the Expectation-Maximization algorithm (21). Haplotype blocks were estimated using Haploview 3.2 (22). Multiple linear regression analysis was performed using StatView 5.0 (SAS Institute Inc., Cary, NC) to test an independent effect of SNP-2 genotypes on SFA or VFA, considering the effects of other variables (age, BMI, and gender) that were assumed to be independent of the effect of the SNP. The significance of the association between an independent variable and the dependent variable was tested by *t* test. The relative luciferase activities and clinical data are expressed as mean ± SD. Differences in luciferase activities were analyzed with the unpaired *t* test.

Results

Case-control association study

A total of 62,663 IMS-JST SNPs covering 11,932 gene loci were successfully genotyped in 94 obese subjects (case-1). The genotype and allele frequencies were compared with 658 random Japanese subjects. According to the National Nutrition Survey, the proportion of the subjects with BMI of 30 kg/m^2 or greater was estimated to be 0.023 in males and 0.034 in females aged 20 yr and older (23), and the mean BMIs are approximately 23 kg/m^2 for ages 15–84 yr in Japan (24). Therefore, control 1 that was randomly selected from the Japanese subjects was not an inappropriate control for the initial analysis. A total of 2261 SNPs that possessed *P* values less than or equal to 0.02 by a test of independence using either genotype mode or allele frequency mode were further analyzed using another set of obese (case 2) and control subjects (control 2). Among the 2261 SNPs, we successfully completed genotyping of 2115 SNPs and identified a strong association with the obesity phenotype for SNP-1 (rs3764220, −1492A→G), which lies in the 5′ flanking region of the *SCG3* gene (Table 1). There were no gender- or age-related differences with respect to SNP-1 alleles. Because the *P* value of SNP-1 was the smallest ($P = 0.0000019$, genotype mode) among the 2115 SNPs, we considered this gene as a good candidate for further investigation.

LD blocks of the SCG3 locus

We identified 112 genetic variations (107 SNPs and five insertions/deletions) by sequencing in the approximately 300-kb genomic region around SNP-1, of which 38 SNPs and two insertions/deletions resided in the *SCG3* gene. Among the 107 SNPs, Invader probes could be synthesized for 81 SNPs, and 79 SNPs were successfully genotyped. Seven SNPs had minor allele frequency less than 5% and were excluded from LD analysis, whereas 10 SNPs had minor allele frequency less than 10% and were excluded from case-control association study. LD analysis revealed that SNP-1 in the *SCG3* gene was located in a 40-kb LD block (block 2, Fig. 1A), which did not contain any gene apart from *SCG3*. Because no association with obesity was observed for SNPs

TABLE 1. Association of SNP-1 (rs3764220, 5′ flanking −1492) in the *SCG3* gene with obesity in the first (case 1 *vs.* control 1) and the second (case 2 *vs.* control 2) set of experiments

Population	No. of subjects (%)			No. of chromosomes (%)		HWE test[a]	
	AA	AG	GG	A	G	χ^2	*P* value
Case 1 (n = 94)	81 (86.2)	13 (13.8)	0 (0.0)	175 (93.1)	13 (6.9)	0.5	0.47
Control 1 (n = 634)	486 (76.7)	134 (21.1)	14 (2.2)	1106 (87.2)	162 (12.8)	1.7	0.19
Case 2 (n = 796)	639 (80.3)	154 (19.3)	3 (0.4)	1432 (89.9)	160 (10.1)	3.9	0.05
Control 2 (n = 711)	522 (73.4)	164 (23.1)	25 (3.5)	1208 (85.0)	214 (15.0)	6.8	0.009

	Genotype mode[b]		Allele frequency mode[b]			Major allele homozygotes mode[b]			Minor allele homozygotes mode[b]		
	χ^2	*P*[c]	χ^2	*P*	OR (95% CI)	χ^2	*P*	OR (95% CI)	χ^2	*P*[c]	OR (95% CI)
Case 1 *vs.* control 1	5.2	0.09	5.3	0.02	1.97 (1.10–3.54)	4.3	0.04	1.90 (1.03–3.51)	2.1	0.24	ND
Case 2 *vs.* control 2	24.7	0.000002	17.3	0.00003	1.59 (1.27–1.97)	9.9	0.002	1.47 (1.16–1.88)	20.3	0.000004	9.63 (2.90–32.05)

The position of SNP in the 5′ flanking region is counted from the transcription initiation site. OR, Odds ratio; ND, not determined.
[a] Hardy-Weinberg equilibrium test.
[b] Association test was performed in four different modes as described in *Subjects and Methods*, and the results in the three modes are shown.
[c] Fisher's exact test.