does not exhibit ophthalmopathy. Furthermore, painless thyroiditis can occur in patients with Graves' disease[13,14]. Radioactive iodine uptake measurements are used to differentiate transient painless thyroiditis from Graves' disease.

The pertechnetate ion is transported into thyroid tissue by the thyroid iodide-concentrating mechanism, presumably because it has the same charge and is the same size as iodide. Unlike iodide, however, it is not metabolized and, therefore, quickly diffuses out of thyrocytes. Tc-99m, which has been used worldwide to study thyroid function, has several advantages, including its short half-life (6 hours), short residence time in the gland, and absence of β-emission[1-6,15]. Measurements of thyroid Tc-99m uptake coupled with scintigraphic images of the gland provides valuable diagnostic information in patients with hyperthyroidism[16].

An important factor that determines Tc-99m uptake, as with radioiodine, is iodine intake. Reinhardt et al. have reported that pertechnetate uptake is reduced to basal levels independent of the TSH concentration, when iodine excretion exceeds 500 μg/g creatinine[17]. According to a recent study, the median levels of urinary iodine in Japanese schoolchildren and adults are 362.6 μg/*l* and 612 μg/*l*, respectively[18,19]. In the present study, we examined Tc-99m uptake in patients who were not subject to iodine restriction. In such patients, pertechnetate uptake should be reduced. Furthermore, Tc-99m uptake in patients with Graves' disease may be within the normal range[9,10]. Nevertheless, Graves' disease could be distinguished from TH on the basis of Tc-99m uptake in our study. On the other hand, as shown by patient 3 (**Table 1**), it is difficult to differentiate spontaneous resolution of Graves' disease from painless thyroiditis. We believe immediate medical treatment is not necessary for such a patient. Therefore, our results suggest that iodine restriction and the establishment of a normal range are not necessary, at least for treatment decision. We have demonstrated that Tc-99m thyroid uptake can be examined without iodine intake restriction immediately after diagnosis of thyrotoxicosis and that differential diagnosis can be performed in 20 minutes. Tc-99m is readily available in most hospitals and has clear advantages over I-123.

For reasons that are unclear, all patients with SAT showed Tc-99m uptake of less than 0.5%, whereas some patients with TH showed Tc-99m uptake of more than 0.5%. The Tc-99m uptake did not correlate with levels of fT3 or fT4 in patients with TH, and in patients with SAT the levels of fT3 and fT4 were not significantly different from those in patients with TH or SAT. It has been reported that Tc-99m uptake in painless thyroiditis, which is included in TH and SAT, is usually markedly reduced[9,20], but in the case of SAT uptake is not always suppressed[21]. The reason for this apparent discrepancy is unknown. Further studies including larger numbers of patients may be needed.

Age is a factor influencing radioiodine uptake in patients with Graves' hyperthyroidism. In one study, 24-hour uptake fell within the normal range in 15% of patients younger than 65 years and in 27% of those older than 65 years[22]. In a study of 18 patients older than 75 years, 24-hour uptake was normal in 5 patients[23]. In the present study, we showed that Tc-99m uptake correlates with age in Graves' hyperthyroidism, i.e., uptake was lower in older subjects with Graves' disease. Although there is a possibility that thyroid weight[24] could be a factor in the effect of age, this is, to our knowledge, the first report demonstrating a significant correlation of the uptake with age.

In the present study, the factors associated with Tc-99m uptake in patients with Graves' disease were analyzed. We found significant correlations between Tc-99m uptake and serum levels of fT3, fT4, TBII, and TSAb at the beginning of the treatment period. These findings indicate that Tc-99m uptake reflects the severity of disease at this time to a degree similar to that seen with iodine uptake[25]. Why Tc-99m thyroid uptake TBII correlates more strongly with TBII than TSAb is unknown, but this phenomenon is compatible with other reports[9,24,26]. We have also demonstrated that uptake is correlated with a patient's response to methimazole. The time (number of days) required to taper the daily dose of methimazole to 5 mg was correlated significantly with Tc-99m uptake at the beginning of

treatment. It has been suggested that radioiodide or Tc-99m uptake could be used to predict a patient's responsiveness to antithyroid medication[9,27], although there have not been any reports to date which have demonstrated this, as we have done in this study. Pretreatment levels of fT3 and fT4 did not correlate with the time required to reduce the daily dose of methimazole to 5 mg. TBII levels were found to correlate with the number of days needed for patients to reach a methimazole dose of 5 mg, whereas TSAb levels did not. It has generally been acknowledged that it is unclear whether there is a correlation between pretreatment serum TSH receptor antibody values and treatment outcome[12,28].

Our data suggest that the determination of Tc-99m thyroid uptake is useful for the differential diagnosis of thyrotoxicosis and that neither establishing a normal range of Tc-99m uptake nor iodine intake restriction are necessary for this determination. The measurement of Tc-99m uptake has a lot of advantages over the use of iodide for the diagnosis of Graves' disease; it can be determined immediately after the diagnosis of thyrotoxicosis, and can indicate both the severity of disease and the effect of treatment.

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Gender Difference in Coronary Events in Relation to Risk Factors in Japanese Hypercholesterolemic Patients Treated With Low-Dose Simvastatin

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Background Gender differences between the risk factors for coronary heart disease and coronary events were examined in the Japan Lipid Intervention Trial, a 6-year observational study.

Methods and Results Men (12,575) and women (27,013) were analyzed for risk of coronary events (acute myocardial infarction and sudden cardiac death). Simvastatin reduced serum low-density lipoprotein cholesterol (LDL-C) by 27% in both genders, and increased serum high-density lipoprotein cholesterol (HDL-C) in men (5%) and women (4%). The incidence of coronary events was lower in women (0.64/1,000 patient-years) than in men (1.57/1,000 patient-years). The risk of coronary events increased by 18% in men and 21% in women with each 10 mg/dl elevation of LDL-C, and decreased by 39% in men and 33% in women with each 10 mg/dl elevation of HDL-C. The risk increased proportionally with aging in women, but not in men. Diabetes mellitus (DM) was more strongly related to the risk of coronary events for women (relative risk 3.07) than for men (relative risk 1.58).

Conclusions The incidence of coronary events is lower in women. Serum LDL-C is related to an increased risk of coronary events to the same extent in both genders. DM seems to be a more important risk factor in women, trading off the lower risk of coronary events among them. (*Circ J* 2006; **70:** 810–814)

Key Words: Coronary events; Hyperlipidemia; Risk factors; Serum cholesterol; Sex differences

Coronary heart disease (CHD), including myocardial infarction and cardiac sudden death, is one of the leading causes of death in Japan[1] The risk of developing CHD is known to be markedly different between men and women:[2,3] CHD incidence is 2 to 5 times higher among middle-aged men than women. In the Japan Lipid Intervention Trial (J-LIT);[4–7] we previously reported that serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) concentrations were positively and serum high-density lipoprotein cholesterol (HDL-C) concentration was inversely related to CHD or cerebrovascular disease risk in patients under treatment for hypercholesterolemia. The role of coronary risk factors in the development of CHD has been studied extensively in men[8–10] but relatively few studies have investigated women[2,11]

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This study aimed to assess gender differences in the association of risk factors with CHD in the J-LIT data. The J-LIT is a nationwide cohort study of 52,421 hypercholesterolemic patients treated with open-labeled low-dose simvastatin (5–10 mg/day)[4,5] The J-LIT included a large number of female patients, and we were able to investigate the gender difference in the role of risk factors in the occurrence of coronary events.

Methods

Study Design

The design of the J-LIT study has been previously described[12] Briefly, study patients with serum TC concentration ≥220 mg/dl, men aged 35–70 years and postmenopausal women aged 70 years or less, were treated with 5–10 mg/day of simvastatin. Body weight, serum lipid concentrations (TC, LDL-C, HDL-C, and triglyceride (TG)) were measured at baseline, and patients were interviewed as regards family history of CHD, number of cigarettes smoked, and the amount of alcohol ingestion. Serum lipid concentrations and CHD-related events (acute myocardial infarction and cardiac sudden death) were monitored every 6 months for 6 years in all patients, including those who discontinued simvastatin. Serum lipid concentrations were determined in each study institution, and the serum LDL-C concentration was calculated using the Friedewald formula for patients with TG concentration ≤400 mg/dl[13] Study physicians recommended dietary and exercise-therapy for hyperlipidemia to all patients. Additional lipid-lowering

agents were allowed only when an adequate response in serum TC concentration was not gained by simvastatin monotherapy. Each patient was informed of the purpose and method of the study, drug efficacy and the need for long-term treatment and they gave verbal, not written, informed consent.

Subjects

Patients who had been previously treated with a lipid-lowering agent were screened for eligibility after a washout period of at least 4 weeks. For patients previously treated with probucol, the washout period was at least 12 weeks. The exclusion criteria were the occurrence of acute myocardial infarction or stroke within the past month, concurrent uncontrolled diabetes mellitus (DM), serious hepatic or renal disease, secondary hypercholesterolemia, cancer or any other illness with potentially poor survival.

Of the 52,421 patients enrolled, 5,127 were excluded because of a history of CHD, 4,934 for lack of follow-up data, and 2,772 for missing data of the covariates. Therefore, data from 39,588 patients (12,575 men, 27,013 women) were used in the present study.

Endpoints

The primary endpoints were major coronary events, defined as nonfatal and fatal myocardial infarction and sudden cardiac death. Incidence of myocardial infarction or death was counted once for each patient during the treatment, and the follow-up data thereafter were excluded from the analysis. The events were reviewed and determined by the Endpoint Classification Committee.

Statistical Analysis

The mean lipid concentrations were calculated using data available at the follow-up points in time during the treatment period. The data of lipid concentrations after the onset of events were excluded. Data during the treatment period after discontinuation of simvastatin were also included for analysis. Mean values for serum lipid concentrations and age were tested with unpaired t-test, and the prevalence of baseline characteristics were tested with the chi-square test for comparison between men and women. Patients in each sex were categorized into 5–6 groups according to the mean lipid concentrations of treatment period for TC, TG, LDL-C and HDL-C with intervals of 20, 50, 20, 10 mg/dl, respectively, and for the LDL-C/HDL-C ratio with an interval of 0.5. The reference category for the relative risk was set on the group with the lowest lipid concentrations and the lowest value of LDL-C/HDL-C ratio. Relative risks and the 95% confidence intervals (CI) were calculated using the Cox proportional hazards model with adjustment for baseline characteristics such as sex, age, hypertension, DM, body mass index (BMI), ECG abnormality, family history of CHD, alcohol ingestion and cigarette smoking. Heterogeneity between men and women was evaluated by the likelihood ratio test. Two-sided p-value <0.05 was considered statistically significant. All the statistical calculations were performed using SAS software (version 8.02, SAS Institute, Inc, Cary, NC, USA).

Results

Serum Lipids and Other Risk Factors

There were no significant difference as regards the prevalence of obesity (BMI ≥25.0 kg/m^2), hypertension, ECG abnormality, and family history of CHD between men and women (Table 1). In men, the prevalence of DM was higher (p<0.001), and cigarette smoking and alcohol ingestion were much more frequent (p<0.001).

Table 1 Baseline Characteristics of the Subjects

	Men (n=12,575)	*Women (n=27,013)*
Age (years)	*54.0 (9.1)*	*59.5 (6.5)*
Obesity (%)[a]	*36.7*	*32.2*
Hypertension (%)[b]	*45.4*	*46.3*
Diabetes mellitus (%)[c]	*20.0*	*13.9*
ECG abnormality (%)[d]	*13.4*	*12.9*
Family history of CHD (%)[e]	*5.1*	*4.8*
Cigarette smoking (%)[e]	*43.8*	*4.1*
Alcohol use (%)[e]	*73.4*	*8.7*
Lipid profiles		
Baseline (mg/dl)		
TC	*268 (41)*	*271 (31)*
LDL-C	*178 (34)*	*184 (33)*
TG	*250 (241)*	*169 (111)*
HDL-C	*49 (15)*	*55 (15)*
During the treatment (mg/dl)		
TC	*218 (31)*	*221 (29)*
LDL-C	*130 (31)*	*135 (28)*
TG	*198 (133)*	*148 (77)*
HDL-C	*51 (13)*	*57 (14)*

Figs are mean±SD unless otherwise specified.
CHD, coronary heart disease; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol.
[a] Body mass index ≥25 kg/m^2. [b] Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. [c] Fasting plasma glucose ≥140 mg/dl or medication. [d] Study physician's diagnosis. [e] Self-reported information.

Lipid profiles at baseline and during the treatment period are shown for men and women in Table 1. Men had higher concentrations of serum TG and lower concentrations of serum HDL-C at baseline and during the treatment in comparison with women. Mean percent changes in the TC, LDL-C, TG, and HDL-C concentrations from baseline to during the treatment in men were −18.8% (p<0.001), −27.2% (p<0.001), −20.9% (p<0.001), and +4.7% (p<0.001), respectively, and the corresponding values in women were −18.2% (p<0.001), −26.6% (p<0.001), −12.8% (p<0.001) and +4.4% (p<0.001), respectively.

Incidence of Coronary Events

The incidence of coronary events was greater (105/12,575) in men than in women (93/27,013) during the treatment period. Incidence rates of coronary events per 1,000 patient-years were 1.57 in men and 0.64 in women. The age-adjusted relative risk of coronary events for men vs women was 2.81 (95% CI 2.10–3.76, p<0.001).

Serum Lipid Concentrations During the Treatment Period and Risk of Coronary Events

The risk of coronary events in relation to serum lipid concentrations is shown in Table 2. Increased risk for coronary events was evident at TC ≥240 mg/dl and LDL-C ≥160 mg/dl in both men and women. An increased risk of CHD associated with elevated concentration of TG (≥250 mg/dl) was noted in women but not in men. In men, the relationship between TG and CHD risk was not measurable. A lower risk of coronary events associated with elevation in HDL-C was seen in both sexes, but the protec-

Table 2 Relative Risk of Coronary Events According to Serum Lipid Concentrations During Treatment[a)]

	Men					Women				
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*
TC (mg/dl)										
<200	3,442	24	1.00	(Referent)		5,833	22	1.00	(Referent)	
200–219	3,643	23	0.99	(0.56–1.77)	0.984	8,194	14	0.52	(0.27–1.02)	0.057
220–239	3,029	25	1.46	(0.83–2.56)	0.192	7,070	18	0.88	(0.47–1.64)	0.687
240–259	1,431	15	2.01	(1.05–3.88)	0.036	3,668	22	2.19	(1.21–3.98)	0.010
260–	1,030	18	3.48	(1.86–6.52)	<0.001	2,248	17	2.82	(1.48–5.36)	0.002
LDL-C (mg/dl)										
<120	4,680	27	1.00	(Referent)		8,050	22	1.00	(Referent)	
120–139	3,542	23	1.24	(0.71–2.16)	0.456	8,418	17	0.83	(0.44–1.57)	0.566
140–159	2,406	21	1.84	(1.03–3.26)	0.038	6,185	19	1.42	(0.77–2.64)	0.263
160–179	1,057	12	2.60	(1.31–5.17)	0.006	2,673	17	3.29	(1.74–6.23)	<0.001
180–	648	17	6.58	(3.53–12.25)	<0.001	1,564	17	5.78	(3.03–11.00)	<0.001
TG (mg/dl)										
<100	1,521	11	1.00	(Referent)		6,337	18	1.00	(Referent)	
100–149	3,663	22	0.84	(0.41–1.74)	0.634	10,444	32	0.98	(0.55–1.76)	0.946
150–199	3,127	33	1.51	(0.76–3.02)	0.243	5,861	17	0.87	(0.44–1.71)	0.684
200–249	1,768	18	1.46	(0.68–3.15)	0.330	2,429	9	1.12	(0.50–2.53)	0.783
250–	2,494	21	1.24	(0.58–2.65)	0.572	1,921	17	2.62	(1.32–5.21)	0.006
HDL-C (mg/dl)										
<40	2,198	36	1.00	(Referent)		1,758	10	1.00	(Referent)	
40–44	2,133	23	0.64	(0.38–1.09)	0.099	2,794	17	1.12	(0.51–2.45)	0.776
45–49	2,207	17	0.44	(0.25–0.80)	0.006	4,101	24	1.09	(0.52–2.28)	0.819
50–54	1,956	13	0.39	(0.21–0.74)	0.004	4,440	13	0.57	(0.25–1.30)	0.179
55–59	1,402	8	0.33	(0.15–0.72)	0.005	4,053	13	0.66	(0.29–1.51)	0.324
60–	2,679	8	0.17	(0.08–0.36)	<0.001	9,867	16	0.33	(0.15–0.73)	0.006
LDL-C/HDL-C										
<2.0	2,851	11	1.00	(Referent)		7,426	11	1.00	(Referent)	
2.0–2.4	2,719	11	1.10	(0.48–2.55)	0.817	6,909	19	1.95	(0.92–4.10)	0.080
2.5–2.9	2,598	17	1.91	(0.89–4.10)	0.095	5,884	14	1.68	(0.76–3.72)	0.199
3.0–3.4	1,889	20	3.21	(1.53–6.74)	0.002	3,545	21	4.57	(2.19–9.54)	<0.001
3.5–4.0	1,082	13	3.87	(1.72–8.72)	0.001	1,728	12	5.04	(2.21–11.49)	<0.001
4.0–	1,194	28	8.06	(3.95–16.44)	<0.001	1,398	15	8.56	(3.88–18.88)	<0.001

RR, relative risk; CI, confidence interval. Other abbreviations see in Table 1.

a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

Table 3 Relative Risk of Coronary Events and Baseline Characteristics[a)]

	Men					Women					Heterogeneity
	N	*Event*	*RR*	*95%CI*	*p value*	*N*	*Event*	*RR*	*95%CI*	*p value*	*p value[b)]*
Age (years)											
<55	6,281	49	1.00	(Referent)		6,137	8	1.00	(Referent)		0.008
55–59	2,182	14	0.74	(0.41–1.34)	0.320	6,488	15	1.82	(0.77–4.29)	0.174	
60–64	2,164	17	0.87	(0.50–1.53)	0.627	7,112	29	3.02	(1.38–6.62)	0.006	
≥65	1,948	25	1.42	(0.86–2.34)	0.168	7,276	41	4.11	(1.92–8.82)	<0.001	
Obesity[c)]	4,621	40	0.99	(0.66–1.48)	0.956	8,700	32	0.91	(0.59–1.40)	0.663	0.676
Hypertension[d)]	5,705	68	2.15	(1.42–3.26)	<0.001	12,511	62	2.05	(1.32–3.18)	0.001	0.864
Diabetes mellitus[e)]	2,513	29	1.58	(1.03–2.43)	0.037	3,747	31	3.07	(1.99–4.74)	<0.001	0.019
ECG abnormality[f)]	1,681	26	1.86	(1.18–2.91)	0.007	3,473	23	1.67	(1.04–2.70)	0.035	0.972
Family history of CHD[g)]	637	10	2.00	(1.04–3.84)	0.038	1,289	13	3.34	(1.85–6.04)	<0.001	0.317
Cigarette smoking[g)]	5,506	52	1.46	(0.98–2.17)	0.063	1,105	9	2.94	(1.43–6.02)	0.003	0.148
Alcohol use[g)]	9,224	70	0.63	(0.41–0.96)	0.031	2,337	6	0.61	(0.26–1.45)	0.266	0.933

Abbreviations see in Tables 1,2.

a) Coronary events included acute myocardial infarction and sudden cardiac death. Adjustment for age, hypertension, diabetes mellitus, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use. b) Heterogeneity between men and women, based on the likelihood ratio test. c) Body mass index ≥25 kg/m². d) Systolic blood pressure ≥160 mmHg and/or diastolic blood pressure ≥95 mmHg or medication for hypertension. e) Fasting plasma glucose ≥140 mg/dl or medication. f) Study physician's diagnosis. g) Self-reported information.

tive association was more evident in men. The relative risk for coronary events was substantially increased in patients with LDL-C/HDL-C ≥3.0 in both men and women.

The increase in the risk of coronary events for each 10 mg/dl elevation of LDL-C concentration during the treatment period was 18% (95% CI 12–24%) in men and 21% (95% CI 15–27%) in women, and the decrease in CHD risk associated with each 10 mg/dl elevation of HDL-C concentration was 39% in men and 33% in women. The relationships of coronary events with baseline LDL-C and HDL-C concentrations were also examined, but were much weaker than those observed during the treatment period. With each 10 mg/dl elevation of LDL-C concentration at baseline, the increase in the relative risk was 7% for men

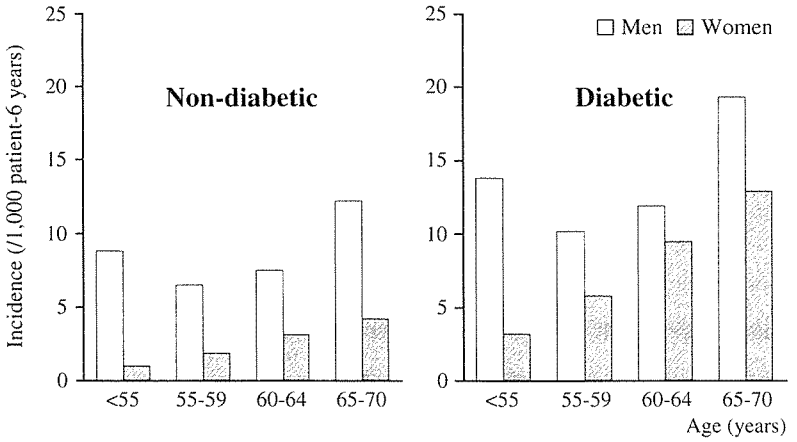

Fig 1. Estimated rates of coronary events according to age in men and women with and without diabetes mellitus (DM). Incidence rates were calculated from coronary heart disease (CHD) relative risks and the proportion of patients in each age category, for men and women separately, using Cox proportional hazards model, in which adjustment was made for age, hypertension, DM, body mass index, ECG abnormality, family history of CHD, cigarette smoking, and alcohol use.

and 9% for women and the decrease in risk with each 10 mg/dl elevation of HDL-C at baseline was 20% in both men and women.

Patient Baseline Characteristics and Risk of Coronary Events

The effect of age on the risk of coronary events was seen in women, but not in men (Table 3). Hypertension, DM, ECG abnormalities and a family history of CHD were also risk factors for coronary events in both men and women, but increased risks associated with DM and a family history of CHD were more marked for women than for men; the relative risk with DM was 1.58 in men and 3.07 in women, and the corresponding values for a family history of CHD were 2.00 in men and 3.34 in women. Obesity was unrelated to coronary events in either men or women. Although alcohol ingestion was protective in both men and women to the same extent, cigarette smoking was more strongly related to an increased risk of coronary events in women.

Discussion

This report addresses the gender differences in the relationship of serum lipid concentrations and other risk factors to CHD risk in Japanese patients under long-term treatment for hypercholesterolemia. Although serum TC and LDL-C concentrations were very similarly related to CHD risk in men and women, there was a difference between men and women in the relationship to serum TG and HDL-C concentrations. An inverse relationship of HDL-C to CHD risk was seen in men and women, but the HDL-C concentration showing a decreased risk of CHD differed by sex. The risk was significantly decreased at HDL-C ≥45 mg/dl in men and at HDL-C ≥60 mg/dl in women. The findings agree with observations published in the United States and Europe[2,3] and further indicate that the criterion of "low HDL-C" must be differential for men and women. An increased risk was observed only in women with an extremely high concentration of TG (≥250 mg/dl). Interpretation of this finding is difficult, and we do not have a clear idea about the implication of the present finding on serum TG.

In the present study, men did not show a clear increase in the risk of coronary events with increasing age, whereas there was a progressive increase in the risk with advancing age in women. The latter finding could be a reflection of the increase in serum TC and LDL-C concentrations with increasing age after menopause. The lack of an increasing trend in the association between age and coronary events in men is an unexpected finding, and may have been due to unknown characteristics of the male participants in the present study.

Whereas DM was related to increased CHD risk in both men and women, the increased risk was much greater in women, as indicated by a statistically significant interaction (p=0.019). These results did not change when further adjusted for TC or LDL-C. However, the risk difference between men and women for DM was not unique to the J-LIT patients. In a meta-analysis of 10 prospective studies, Lee et al showed that the effect of DM on the CHD risk was greater in women than in men![14] They showed that the relative risk of coronary death for DM patients vs non-DM patients was 2.58 (95% CI 2.05–3.26) in women and 1.85 (95% CI 1.47–2.33) in men (interaction p=0.045)![14] It was further noted in a later study that DM diminished the female advantage for lower CHD incidence![15] That DM is a stronger CHD risk factor in women may be related to the lower concentrations of HDL-C. Walden suggested that lower HDL-C concentrations in diabetic women as compared with men might be relevant to a stronger association between DM and CHD in women![16] In the present study, mean HDL-C concentrations in female diabetic patients were lower than those of non-diabetic patients (55.5 vs 57.5 mg/dl, p<0.001), but there was no difference in the HDL-C concentrations between the 2 groups in men (50.8 vs 51.3 mg/dl, p=0.09). The relative risk for DM was unchanged with adjustment for HDL-C. When the predicted rates of CHD incidence according to age were examined in men and women with and without DM (Fig 1), the increase in CHD incidence with aging was augmented in the presence of DM. Notably, DM diminished the women's advantage of having a lower CHD incidence in older patients.

Both cigarette smoking and family history of CHD were related to a greater increase in the risk of coronary events in women than in men. These differential increases in men and women may have been caused by random variation, as indicated by the lack of statistical significance for the interaction. As regards the effect of cigarette smoking, some studies suggest that smoking is a stronger risk factor in women than in men[2,17] but others have failed to find such a finding![18]

Finally, the present study results indicated that hypertension was an important risk factor in men and women equally, and that alcohol ingestion was protective in both sexes. These findings are in agreement with observations reported elsewhere![19–21]

In conclusion, the incidence of coronary events was 60% lower in women than in men among the J-LIT participants. Although the relationship of serum TC and LDL-C concentrations to coronary events was similar in men and women, the HDL-C concentration associated with a decreased risk of coronary events was slightly higher in women. DM was a stronger risk factor in women, and traded off the women's advantage of having a lower risk of coronary events, especially in aged patients.

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Original Article

Prevalence of Metabolic Syndrome in the General Japanese Population in 2000

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To determine the prevalence of metabolic syndrome in the Japanese general population, we analyzed data from a nationwide survey conducted in 2000. According to the Japanese new diagnostic criteria for metabolic syndrome in 2005, we analyzed 3,264 people aged from 20 to 79 (men, 1,917; women, 1,347) from the total participants. The incidence of metabolic syndrome was 7.8%. Men had a higher incidence (12.1%) than women (1.7%). Most of the women satisfying the criteria were 50 years old or over, while the incidence in men started to rise from their 30s. When we applied the criteria of Adult Treatment Panel III, the incidence was about 3-fold higher. In this population visceral obesity was associated with metabolic abnormalities, such as higher LDL-cholesterol, triglyceride, glucose, and blood pressure and lower HDL-cholesterol. Thus we determined the incidence of metabolic syndrome and each metabolic abnormality in the Japanese general population in 2000 and found an association of visceral obesity with metabolic abnormalities. Intervention to reduce the incidence of metabolic syndrome in Japan is necessary to reduce the risk of cardiovascular disease.

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Key words; Metabolic syndrome, Dyslipidemia, Visceral obesity, Japanese

Introduction

Metabolic syndrome is a constellation of multiple risk factors, such as dyslipidemia, elevated glucose, and elevated blood pressure. This syndrome has received increased attention due to its association with increased risk for cardiovascular disease and type 2 diabetes[1]. Although the pathogenesis of metabolic syn-

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drome has not been fully understood, the predominant underlying risk factor is considered to be visceral obesity due to an atherogenic diet and physical inactivity in the presence of some unknown genetic background[2-4]. In women the incidence of metabolic syndrome increases after menopause; therefore, hormonal imbalance and aging are also associated with the development of metabolic syndrome[5].

Along with the westernization of lifestyle, the incidence of metabolic disorders, such as dyslipidemia, hypertension, and diabetes is increasing in Japan. In spite of the availability of many drugs, such as statins, angiotensin-converting enzyme inhibitors, and aspirin, the incidence of cardiovascular disease is not decreasing in Japan, probably due to these metabolic abnormalities, especially dyslipidemia and diabetes along with obesity according to the national survey by the Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/toukei/saikin/hw/kenkou/jyunkan/jyunkan00/gaiyo.html). In 2000, we conducted a lipid survey in various districts in Japan[6]. What we found in this survey was that the level of triglyceride increased in middle-aged men along with increased body mass index (BMI) compared with the data in 1990[7]. This increase in BMI also suggests an increase in the incidence of visceral obesity and metabolic syndrome; therefore, knowing the incidence of metabolic syndrome is very important from the standpoint of preventive medicine.

In the last few years, several expert groups have attempted to set forth simple diagnostic criteria to be used in clinical practice to identify patients with metabolic syndrome. The committee of International Diabetes Federation (IDF) adopted waist circumference as the surrogate marker for visceral obesity as an essential component of this syndrome (http://www.idf.org/webdata/docs/IDF_Metasyndrome_definition.pdf). In Japan the committee established diagnostic criteria under the same principle as that used in the IDF criteria, except that the cutoff point for high glucose is 110 mg/dL instead of 100 mg/dL[8]. The cutoff of waist circumference for visceral obesity was adopted as $\geqq$85 cm in men and $\geqq$90 cm in women. Meanwhile, the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) criteria required no single factor for diagnosis, but instead required the presence of at least 3 out of 5 components for diagnosis[9]; thus, complete agreement on the definition and diagnostic criteria has not been achieved so far.

The purpose of this study is to examine the incidence of metabolic syndrome in the Japanese general population and the relationship with the risk factors included in the diagnostic criteria. We also compared the incidence of metabolic syndrome by using the NCEP-ATP III new diagnostic criteria.

Methods

Design and Data Collection

The Research Group on Serum Lipid Level Survey 2000 in Japan organized the members of 36 institutes from various areas around Japan. The project was designed to produce representative data about serum lipid levels in the civilian Japanese population. The subjects were people receiving annual health examinations in the general community, companies, and schools, and not patient-visiting hospitals. Among the 12,839 participants we measured the waist circumference of 3,264 people aged 20 to 79 (men 1,917; women, 1,357) and examined the incidence of metabolic syndrome.

Laboratory Methods

All serum and plasma samples were obtained in the fasting state. All lipid and other analyses were conducted on venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and triglyceride levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol were measured enzymatically using a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses in the four surveys were indirectly standardized according to the criteria of the CDC Lipid Standardization Program[10]. Thus, the cholesterol levels in these five surveys appear comparable. Plasma glucose was determined enzymatically and HbA1c was determined using a kit from Kyowa Medex Co.Ltd (Tokyo, Japan). Serum insulin was determined by immunoradiometric assay (Abbott Diagnostics Division, Abbot Park, IL). Waist circumference at the umbilical level was measured in the late exhalation phase in a standing position.

Definition of Metabolic Syndrome

According to the new definition released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome in April 2005, we defined metabolic syndrome as the presence of 2 or more abnormalities in addition to visceral obesity (waist circumference: 85 cm or more in men, 90 cm or more in women). These three abnormalities are as follows: 1, triglycerides $\geqq$150 mg/dL and/or HDL-cholesterol $<$40 mg/dL or under treatment for this type of dyslipidemia, 2, systolic blood pressure $\geqq$130 and/or diastolic blood pressure $\geqq$85, or under treatment for hypertension, 3, fasting glucose $\geqq$110 mg/dL or under treatment for diabetes. People treated for dyslipid-

Table 1. Clinical characteristics of the study population

	men (n=1,917)	women (n=1,347)
age	46.3 ± 0.30	45.7 ± 0.46
BMI	23.4 ± 0.07	22.4 ± 0.07*
waist circumference (cm)	84.1 ± 0.20	73.2 ± 0.29*
systolic blood pressure (mmHg)	125 ± 0.40	120 ± 0.49*
diastolic blood pressure (mmHg)	76.3 ± 0.27	72.3 ± 0.31*
T-cho (mg/dL)	201 ± 0.78	200 ± 0.97
TG (mg/dL)	145 ± 2.97	92.1 ± 1.64*
HDLc (mg/dL)	54.8 ± 0.33	64.6 ± 0.39*
LDLc (mg/dL)	118.0 ± 0.99	113.5 ± 1.22**
HbA1c (%)	4.86 ± 0.02	4.82 ± 0.14
fasting glucose (mg/dL)	97.8 ± 0.43	91.1 ± 0.36*
insulin (IU/mL)	6.28 ± 0.11	7.16 ± 0.21*

Data are expressed as the means ± SEM. T-cho; total cholesterol, TG; triglyceride, HDLc; HDL-cholesterol, LDLc; LDL-cholesterol. *$p <$ 0.001, **$p < 0.01$

Table 2. Incidence of metabolic syndrome and metabolic abnormalities by Japanese diagnostic criteria

	men (%)	women (%)	all (%)
metabolic syndrome	12.1	1.7	7.8
visceral obesity	48.2	9.7	32.3
hypertriglyceridemia	31.3	11.2	23.0
low HDL-cholesterolemia	12.4	2.2	8.2
dyslipidemia	35.2	12.1	25.6
hypertension	25.4	19.5	22.9
elevated fasting glucose	14.4	7.0	11.3

Dyslipidemia is defined as hypertriglyceridemia and/or low HDL-cholesterolemia

emia were excluded, because we could not obtain data as to whether they were treated for hypercholesterolemia or hypertriglyceridemia. We also analyzed the incidence of metabolic syndrome by ATP III criteria published in 2005[9]. We modified the criteria by using the Japanese cutoff of waist circumference. Other differences are fasting glucose ≧100 mg/dL and HDL-cholesterol <50 mg/dL in women. Metabolic syndrome in ATP III criteria was defined as the presence of at least 3 abnormalities among visceral obesity, hypertriglyceridemia, low HDL-cholesterolemia, hypertension, and glucose intolerance.

Data Analysis

The results are expressed as the mean value ± standard deviation, and categorical data by the incidence and relation between visceral obesity and various factors were expressed by the odds ratio and 95% confidence interval. Differences in the means were evaluated by analysis of variance (ANOVA) or analysis of covariance (ANCOVA). The relation between visceral obesity and various factors was examined using multiple, logistic regression analysis for multivariate analysis. Analysis was performed using the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan). A p value of 0.05 or less was considered to indicate significant difference.

Results

Table 1 shows the characteristics of the study population. The means of total cholesterol, triglyceride, HDL-cholesterol, and fasting glucose were 200 mg/dL, 123 mg/dL, 59 mg/dL, and 95 mg/dL. These data are almost the same as the means of the total participants (201, 115, 59, 95, respectively)[6]. The means of both genders were also equivalent to the means of the total participants, indicating that this population represents all participants in this Japanese lipid survey in 2000. Although we found no difference in the mean age, total cholesterol, and HbA1c between men and women, the means of BMI, waist circumference, blood pressure, triglyceride, LDL-cholesterol, and fasting glucose were higher in men than in women, while those of HDL-cholesterol and insulin were lower in men than in women.

Using the Japanese diagnostic criteria for metabolic syndrome we determined the incidence of metabolic syndrome (**Table 2**). The incidence of metabolic syndrome in all participants was 7.8%. The incidence in men and women was 12.1, 1.7%, respectively. The incidence was about 7-fold higher in men than in women, reflecting the difference in visceral obesity defined by waist circumference, 48.2% in men and 9.7% in women. The incidence of dyslipidemia, hypertension, and glucose intolerance was also higher in men than in women in this population, indicating a higher prevalence of metabolic abnormalities in men.

It is important for us to intervene from the period of visceral obesity to prevent cardiovascular disease due to these metabolic abnormalities. Therefore, we compared the incidence of visceral obesity, visceral obesity plus one metabolic abnormality, and metabolic syndrome. **Fig. 1** shows the incidence of visceral obesity, visceral obesity plus one metabolic abnormality, and metabolic syndrome. The incidence of visceral obesity plus one metabolic abnormality was about twice the incidence of metabolic syndrome both in men and women.

To compare the incidence of metabolic syndrome

by Japanese and ATP III criteria in this population, we determined the incidence of metabolic syndrome using these criteria in each generation from age 20s to 70s in men and women as shown in **Fig. 2**. The incidence of metabolic syndrome using ATP III criteria was about 3 times higher than that by the Japanese criteria. Using both criteria the incidence of metabolic syndrome started to rise in men in their 30s and reached a plateau after their 40s. Meanwhile, the incidence of metabolic syndrome in women started to rise after their 50s using both criteria, indicating the increased prevalence of metabolic syndrome after menopause.

We next examined whether visceral obesity contributed to metabolic abnormalities in this study population. **Fig. 3** shows the difference of lipid profiles and fasting glucose levels with or without visceral obe-

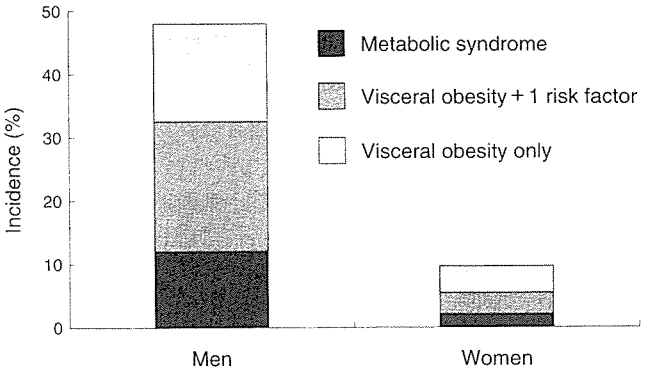

Fig. 1. Incidence of metabolic syndrome and visceral obesity in the lipid survey in 2000.

The percent incidence of metabolic syndrome, visceral obesity plus one risk factor, and visceral obesity in men and women is shown.

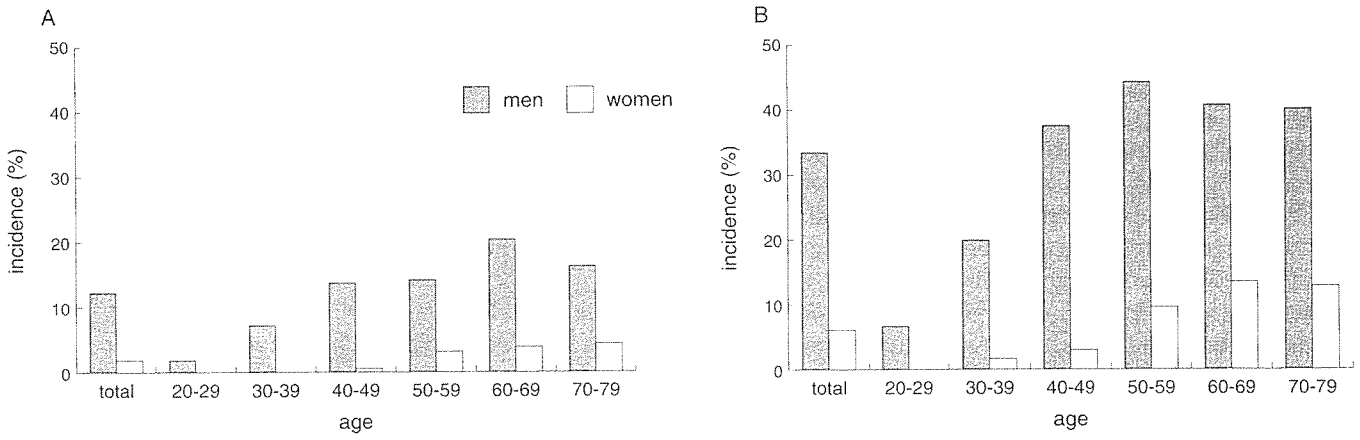

Fig. 2. Incidence of metabolic syndrome in each generation by Japanese and ATP III criteria.

Each column shows the incidence of metabolic syndrome in each generation in men (closed column) and women (open column) by Japanese (A) and ATP III (B) criteria. The incidence in the total population is shown on the left.

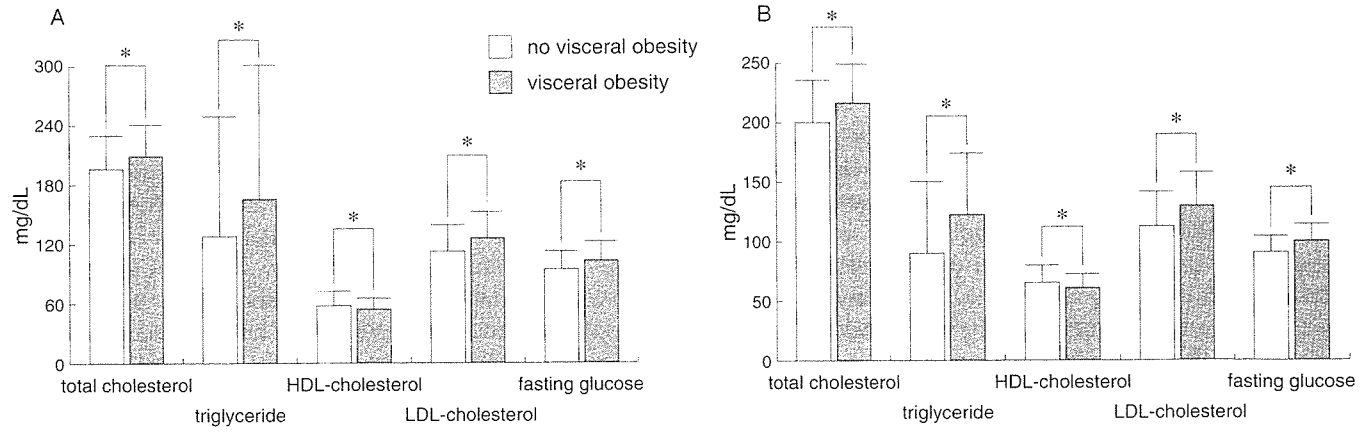

Fig. 3. Comparison of metabolic abnormalities with or without visceral obesity.

Each column shows the mean ± SD of total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and fasting glucose with or without visceral obesity in men (A) and women (B). $*p < 0.001$

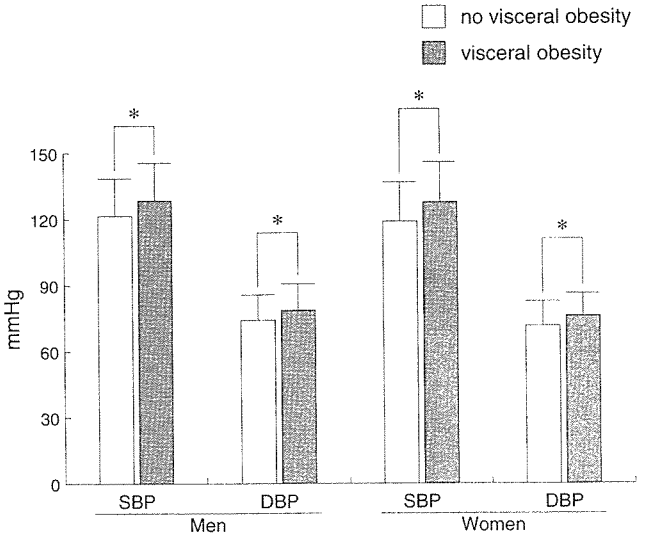

Fig. 4. Comparison of systolic and diastolic pressure with or without visceral obesity.

Each column shows the mean ± SD of systolic and diastolic blood pressure with or without visceral obesity in men and women. $^{*}p < 0.001$

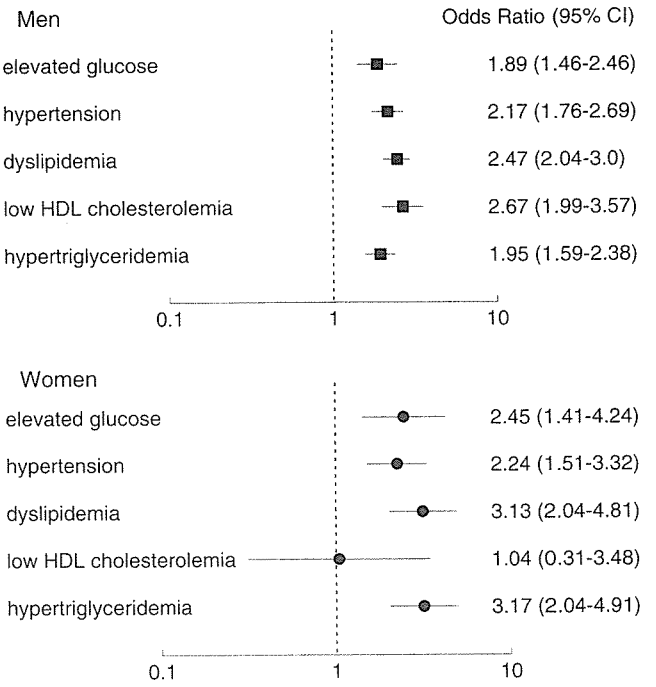

Fig. 5. Effect of visceral obesity on hypertriglyceridemia, low HDL cholesterolemia, dyslipidemia, hypertension, and glucose intolerance in men and women.

Odds ratios and 95% confidence interval are shown for each abnormality in the presence or absence of visceral obesity.

sity in this study population. The levels of total cholesterol, triglyceride, LDL-cholesterol, and fasting glucose were significantly higher, while the level of HDL-cholesterol was significantly lower in the group with visceral obesity than in the group without, indicating the contribution of visceral obesity to these metabolic abnormalities in both men and women. Systolic and diastolic blood pressure was also higher in the visceral obesity group in both genders (**Fig. 4**). We also determined the effect of visceral obesity on the development of each abnormality by calculating the odds ratios and 95% confidence interval (**Fig. 5**). Visceral obesity was significantly associated with the development of each metabolic abnormality in men and women except for low HDL-cholesterolemia in women. When we changed the cutoff of HDL-cholesterol to 50 mg/dL, visceral obesity was significantly associated with low HDL-cholesterolemia in women. The odds ratio was 2.10 and the 95% confidence interval was 1.35-3.27. Among dyslipidemia, hypertension, and glucose intolerance, visceral obesity was most associated with the development of dyslipidemia.

We also determined the age-adjusted difference of lipid profile in the presence or absence of visceral obesity in this population. Even after age adjustment we found a significant difference in total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol in men and in women, except for a difference in LDL-cholesterol in women (**Table 4**).

Discussion

In this study we determined the incidence of metabolic syndrome in the Japanese general population using a lipid survey performed in 2000 using new Japanese criteria to diagnose metabolic syndrome. We found that 3 times more people were diagnosed with metabolic syndrome using the new ATP III criteria than the Japanese criteria and that visceral obesity contributed to metabolic abnormalities, such as dyslipidemia, glucose intolerance, and hypertension.

In our study the incidence of metabolic syndrome in Japanese men and women was 12.1 and 1.7%, respectively. The incidence of metabolic syndrome in our survey is lower than that from the latest National Health and Nutrition survey in 2004. In that survey the incidence of metabolic syndrome in Japanese men and women was 23.0 and 8.9%, respectively. In this national survey they used HbA1c ($\geqq 5.5$) instead of FBS to diagnose glucose intolerance. This might explain the difference between the two surveys. This difference also indicates that the cutoff of FBS needs to be changed in the future. Although the mean age and the criteria used were different, Takeuchi *et al.*

Table 3. Incidence of each metabolic abnormality in the presence or absence of visceral obesity

	visceral obesity		no visceral obesity	
	men	women	men	women
hypertriglyceridemia	41.1%	25.4%	22.2%	9.7%
low HDL-cholesterolemia	17.6%	2.3%	7.4%	2.2%
dyslipidemia	45.7%	26.9%	25.4%	10.5%
hypertension	32.8%	33.1%	18.4%	18.1%
elevated fasting glucose	18.4%	14.6%	10.6%	6.2%

Dyslipidemia is defined as hypertriglyceridemia and/or low HDL-cholesterolemia

Table 4. Age-adjusted difference of lipid profile in the presence or absence of visceral obesity

		men		age-adjusted	women		age-adjusted	all		age-adjusted
		no visceral obesity	visceral obesity	*P*	no visceral obesity	visceral obesity	*P*	no visceral obesity	visceral obesity	*P*
T-cho	mean	195.6	205.9		198.8	214.2		197.3	206.9	
	number	994	923	<0.001	1217	130	0.082	2211	1053	<0.001
	SD	33.4	33.4		35.4	33.1		34.6	33.4	
TG	mean	128.7	162.0		88.9	121.7		106.8	157.0	
	number	994	923	<0.001	1217	130	<0.001	2211	1053	<0.001
	SD	119.3	138.8		60.2	51.5		93.7	131.8	
HDLc	mean	57.7	51.7		65.1	59.8		61.8	52.7	
	number	994	923	<0.001	1217	130	0.003	2211	1053	<0.001
	SD	14.2	13.9		14.5	12.5		14.8	14.0	
LDLc	mean	112.1	122.1		111.4	128.0		111.7	122.9	
	number	374	479	0.001	510	71	0.106	884	550	<0.001
	SD	26.0	30.1		29.0	28.8		27.8	30.0	

The mean, the number of samples, and SD are shown. *P* value was obtained by ANCOVA.

reported that the incidence of metabolic syndrome in men in the Tanno and Sobetsu study was 25.3%[11]. The mean age of their study population was 60.3 years, about 15 years older than that in our study population. Other studies reported a similar incidence of metabolic syndrome in Japanese. Considering that the incidence of metabolic syndrome in our population in their 60s was about 20%, the difference of the criteria used contributed to this difference. Similar to our study Urashima *et al.* reported an incidence of metabolic syndrome in Japanese men and women of 14.1% and 1.7%, respectively in central Tokyo[12]. Thus, the current incidence of metabolic syndrome in Japan would be around 15% in men and a few percent in women. In our study we found that about twice as many people with metabolic syndrome had visceral obesity and one risk factor in both men and women, indicating a potential for the incidence of metabolic syndrome to increase in the future. In our previous analysis we showed that the level of triglyceride in men dramatically increased from 1990 to 2000[6]. Therefore, we need to tackle this problem to prevent the increase in metabolic syndrome and cardiovascular disease in Japan.

In this population the incidence of metabolic syndrome in women was one seventh that in men. The incidence of visceral obesity, dyslipidemia, and glucose intolerance in women was one fifth, one third, and one half that in men, respectively. Furthermore, most of the women who satisfied this criteria were more than 50 years old, which means that few women are diagnosed with metabolic syndrome before the menopause. In Japan we adopted a cutoff of waist circumference of 90 cm for women, which is 5 cm more than that for men. This might explain why the incidence of metabolic syndrome in women was much less than in men. In contrast to the cutoff waist circumference in Japan, other criteria, such as in ATP Ⅲ,

generally have a larger cutoff in men than in women; however, our cutoff in women is based on the extensive study by Matsuzawa and his group using CT scan[13-15]. Therefore, in terms of detecting visceral obesity, 90 cm would be appropriate for Japanese women. However, we need to establish another method to select high-risk patients without visceral obesity. Our data also strongly indicate that visceral obesity using our cutoff is associated with metabolic abnormalities even after age adjustment, as shown in **Fig. 5** and **Table 4**. Therefore, we believe that visceral obesity is a useful surrogate marker for metabolic abnormalities and intervention to reduce abdominal circumference would lead to the prevention of cardiovascular disease. However, in terms of the cutoff of HDL-cholesterol, 50 mg/dL might be better than 40 mg/dL from the odds ratio in women (**Fig. 5** and Results) as in the cutoff of the ATP III criteria.

In summary we have shown that the incidence of metabolic syndrome in the Japanese general population is 7.8%, 12.1% in men and 1.7% in women. Intervention is required to prevent metabolic syndrome as well as metabolic abnormalities, such as dyslipidemia, hypertension, and glucose intolerance. The current criteria for metabolic syndrome should be assessed for the better diagnosis of women and elderly people.

Acknowledgements

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Growth Hormone-Releasing Hormone (GHRH) Neurons in the Arcuate Nucleus (Arc) of the Hypothalamus Are Decreased in Transgenic Rats Whose Expression of Ghrelin Receptor Is Attenuated: Evidence that Ghrelin Receptor Is Involved in the Up-Regulation of GHRH Expression in the Arc

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GH secretagogue (GHS)/ghrelin stimulates GH secretion by binding mainly to its receptor (GHS-R) on GHRH neurons in the arcuate nucleus (Arc) of the hypothalamus. GHRH, somatostatin, and neuropeptide Y (NPY) in the hypothalamus are involved in the regulatory mechanism of GH secretion. We previously created transgenic (Tg) rats whose GHS-R expression is reduced in the Arc, showing lower body weight and shorter nose-tail length. GH secretion is decreased in female Tg rats. To clarify how GHS-R affects GHRH expression in the Arc, we compared the numbers of GHS-R-positive, GHRH, and NPY neurons between Tg and wild-type rats. Immunohistochemical analysis showed that the numbers of GHS-R-positive neurons, GHRH neurons, and GHS-R-positive GHRH neurons were reduced in Tg rats, whereas the numbers of NPY neurons and GHS-R-positive NPY neurons did not differ between the two groups. The numbers of Fos-positive neurons and Fos-positive GHRH neurons in response to KP-102 were decreased in Tg rats. Competitive RT-PCR analysis of GHRH mRNA expression in the cultured hypothalamic neurons showed that KP-102 increased NPY mRNA expression level and that NPY decreased GHRH mRNA expression level. KP-102 increased GHRH mRNA expression level in the presence of anti-NPY IgG. GH increased somatostatin mRNA expression. Furthermore, GH and somatostatin decreased GHRH mRNA expression, whereas KP-102 showed no significant effect on somatostatin mRNA expression. These results suggest that GHS-R is involved in the up-regulation of GHRH and NPY expression and that NPY, somatostatin, and GH suppress GHRH expression. It is also suggested that the reduction of GHRH neurons of Tg rats is induced by a decrease in GHS-R expression. (*Endocrinology* 147: 4093–4103, 2006)

GH SECRETAGOGUES (GHSs), which were developed from the structure of met-enkephalin (1), strongly stimulate GH secretion by acting on GHRH neurons in the arcuate nucleus (Arc) of the hypothalamus, their main site of action, and on somatotrophs of the pituitary through the GHS receptor (GHS-R), a member of the G protein-coupled receptor superfamily (2). Ghrelin, an endogenous ligand for GHS-R, has been discovered in rat stomach extract (3). GHS-R mRNA is expressed in several hypothalamic nuclei, such as the Arc, paraventricular nucleus of the hypothalamus, ventromedial hypothalamic nucleus, supraoptic nucleus, suprachiasmatic nucleus, lateroanterior hypothalamic nucleus, and tuberomammillary nucleus, and other brain areas such as the hippocampus, substantia nigra, ventral tegmental area, and dorsal and median raphe nuclei (4). The systemic administration of GHSs induces c-*fos* mRNA expression in the neurons of Arc. Overall, of these c-*fos* mRNA-expressing neurons, 51% are neuropeptide Y (NPY) neurons, 23% are GHRH neurons, 11% are tyrosine hydroxylase (TH) neurons, 11% are proopiomelanocortin neurons, and 4% are somatostatin neurons (5, 6).

To clarify the physiological significance of GHS-R, we have created transgenic (Tg) rats expressing an antisense GHS-R mRNA that is designed to be specific for the region around the initiation codon of GHS-R, under the control of the promoter for TH (7). TH is a rate-limiting enzyme in catecholamine biosynthesis and is a marker for the dopaminergic neurons. TH is present in most neurons in the ventral portion of the Arc where GHRH neurons exist (8). TH mRNA-expressing neurons in the Arc of Tg rats have been shown to express antisense GHS-R mRNA (7). GHS-R mRNA is detected in NPY and GHRH neurons in the Arc (9–11). We have found that GHS-R is expressed in both GHRH and NPY neurons in the Arc of Tg rats and slc:SD wild-type (WT) rats and that GHS-R, as determined by Western blot analysis, in the Arc is reduced in Tg rats compared with WT rats (7). These Tg rats have lower body weight and

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Abbreviations: Arc, Arcuate nucleus; DNase, deoxyribonuclease; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; ICV, intracerebroventricular; NPY, neuropeptide Y; PeV, periventricular nucleus; Tg, transgenic; TH, tyrosine hydroxylase; WT, wild type.

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less adipose tissue, suggesting that GHS-R plays a role in the regulation of adiposity (7). They also have lower GH responses to iv administered KP-102, one of the GHSs, than WT rats (7). Furthermore, GH secretion and plasma insulin-like growth factor I levels are significantly reduced in female Tg rats, suggesting that GHS-R is involved in the regulation of GH secretion and that GHS-R plays a more important role in the regulatory mechanism of GH secretion in female than in male rats (7). However, the details of GHRH, GHS-R, and NPY expression in the Arc of Tg rats have not yet been examined. The relation of the GHS-R expression level with the expression of GHRH and NPY in the Arc has also not yet been studied. Recently, it has been reported that Tg mice overexpressing GHS-R1A in the GHRH neurons show an increase in hypothalamic GHRH expression (12), suggesting that GHS/GHS-R may regulate GHRH expression.

Therefore, in this study, we first compared the numbers of GHRH neurons, NPY neurons, GHS-R-positive neurons, GHS-R-positive GHRH neurons, and GHS-R-positive NPY neurons located in the Arc of Tg and WT rats to clarify whether the GHS-R expression levels affect GHRH and NPY expression. We then examined Fos expression of the neurons located in the Arc of Tg and WT rats in response to intracerebroventricular (ICV) injection of KP-102 to confirm that GHS-R is reduced in the Arc of Tg rats. We finally examined the effects of KP-102, NPY, somatostatin, and GH on the GHRH mRNA, NPY mRNA, or somatostatin mRNA expression level in primary cultured hypothalamic neurons of normal rats to clarify whether and how ghrelin/GHS-R affects the expression level of GHRH mRNA.

Materials and Methods

Animals

Twelve-week-old random-cycling female Tg rats (7) and WT rats were used in this study. The WT rats used in the present study were not littermates of Tg rats, although we used WT rats, which were littermates of Tg rats, in our previous studies (7). WT rats, which we used for these 5 yr, always showed similar results. The Tg rats, which are homozygote for transgene, also showed unchanged phenotype. There were significant differences in body weights [Tg rats (n = 14), 199.6 ± 2.6 g *vs.* WT rats (n = 14), 215.6 ± 2.3 g, $P < 0.001$] and in nose-tail length [Tg rats (n = 14), 36.9 ± 0.2 cm *vs.* WT rats (n = 14), 37.5 ± 0.2 cm, $P < 0.05$]. All animals were housed under controlled temperature and illumination (0800–2000 h) and allowed *ad libitum* access to food and water. To administer samples ICV, a polyethylene cannula was implanted into the right lateral ventricle under sodium pentobarbital anesthesia (50 mg/kg body weight ip injection), as described previously, 5 d before the experiment was to be done (13). All experimental procedures were conducted in accordance with the guidelines on the use and care of laboratory animals approved by the Local Ethics Committee of Nippon Medical School, Japan.

Effect of ICV injection of KP-102 on Fos expression

On the day of the experiment, rats with *ad libitum* access to food and water were given vehicle (2 μl saline) or KP-102 (100 pmol/2 μl) ICV. Ninety minutes after the injection, the rat brain was fixed for immunohistochemistry.

Immunohistochemistry of GHS-R, GHRH, and NPY

To compare the numbers of GHRH neurons, NPY neurons, and GHS-R-positive neurons of Tg and WT rats, the rats were injected ICV with colchicine (100 μg/5 μl saline) through the cannula. Tg and WT rats were anesthetized with pentobarbital (50 mg/kg body weight ip injection) and perfused via an intracardiac cannula with PBS followed by 4% paraformaldehyde 48 h after the injection of colchicine. The brain was removed, left overnight in 4% paraformaldehyde and then transferred to 20% sucrose/PBS. Coronal sections (20 μm) were cut with a cryostat and mounted onto gelatinized slides. Successive sections were used for immunohistochemistry of GHS-R, GHRH, and NPY.

Immunohistochemistry was performed with the avidin-biotin-peroxidase method using specific antiserum against rat GHRH (14), mouse GHS-R (15), and rat/human NPY (16). Briefly, sections were incubated with specific polyclonal antiserum against GHRH (1:1000), GHS-R (1:500) (7), or NPY (1:1000) overnight at 4 C. The tissues were then rinsed in PBS and incubated in biotinylated goat antirabbit IgG (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. This was followed by another 1-h incubation in avidin-biotin complex solution (Vectorstain ABC Elite kit, Vector Laboratories) at room temperature. The reaction product was visualized using a nickel-intensified diaminobenzidine reaction that gives a dark-brown precipitate. Briefly, sections were washed in 0.1 M sodium acetate buffer (pH 6.0) and incubated in the same buffer containing 2.5% nickel sulfate, 0.2% glucose, 0.04% ammonium chloride, 0.025% diaminobenzidine, and 30 U/ml glucose oxidase (Sigma-Aldrich, St. Louis, MO). The reaction was stopped by washing in 0.1 M acetate buffer under microscopic observation (17). The tissue was dehydrated through graded alcohols, cleared in xylenes, and then coverslipped with Vector Mount (Vector Laboratories). Preincubation of anti-GHRH serum with 1.0 μg GHRH, anti-GHS-R serum with 1.0 μg GHS-R, and anti-NPY serum with 1.0 μg NPY completely abolished the staining of GHRH, GHS-R, and NPY, respectively. Quantita-

TABLE 1. Primer sequences of the studied genes

Genes	Primers (5′–3′)	Length (bp)	GenBank accession no.
GHRH			
Sense	ATGCCACTCTGGGTGTTCTTTG	315	NM_031577
Antisense	TCAAGCCTCCGCTGAAAGCTTC		
Competitor sense	ATGCCACTCTGGGTGTTCTTTGGCCATGCAGACGCCATCTTCAC	211	
Competitor antisense	TCAAGCCTCCGCTGAAAGCTTCTCTGCTTGTCCTCTGCCCAC		
NPY			
Sense	CCGCTGCGCAGAGACCACAGCC	405	NP_036746
Antisense	TCAGACTGGTTTCACAGGATG		
Competitor sense	CCGCTGCGCAGAGACCACAGCCGCTCGTGTGTTTGGGCATTC	254	
Competitor antisense	TCAAGCCTCCGCTGAAAGCTTCTCTGCTTGTCCTCTGCCCAC		
Somatostatin			
Sense	ATGCTGTCCTGCCGTCTCCAGTG	351	NM_012659
Antisense	CTAACAGGATGTGAATGTCTTCCAG		
Competitor sense	ATGCTGTCCTGCCGTCTCCAGTGTCTGCATCGTCCTGGCTTTG	212	
Competitor antisense	CTAACAGGATGTGAATGTCTTCCAGGGCATCGTTCTCTGTCTGGTTG		
β-Actin			
Sense	TCATGAAGTGTGACGTTGACATCCGT	285	BC063166.1
Antisense	CCTAGAAGCATTTGCGGTGCACCGATG		

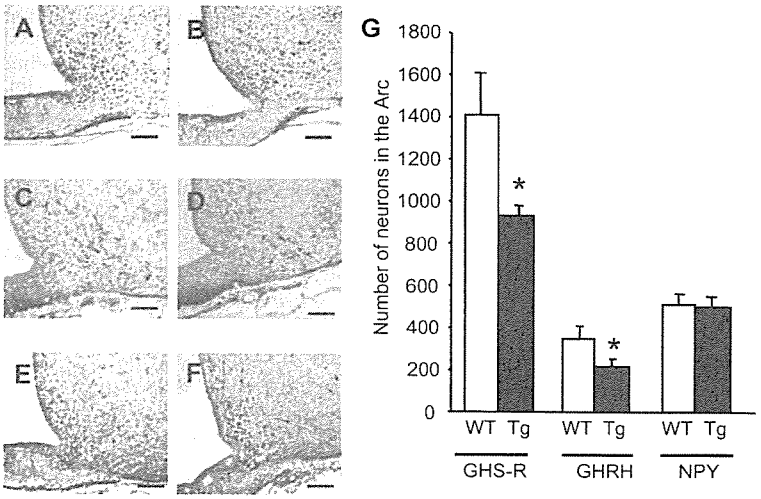

FIG. 1. Localization GHS-R-positive neurons, GHRH neurons, and NPY neurons in the Arc of female WT and Tg rats. GHS-R-positive neurons of female WT and Tg rats are shown in A and B, respectively, GHRH neurons of female WT and Tg rats in C and D, respectively, and NPY neurons of female WT and Tg rats in E and F, respectively. *Scale bars*, 100 μm. The numbers of GHS-R-positive neurons, GHRH neurons, and NPY neurons in the Arc are shown in G. *, $P < 0.05$ *vs.* WT rats. The number of rats of each group was five.

tive analysis of the number of GHS-R, GHRH, and NPY neurons was performed using an image analysis system (MCID Amersham Biosciences, Tokyo, Japan). Under light microscopy, at 200× magnification, the total number of positive neurons was counted in the Arc. Every three sections of 20 μm were used for the counting of GHS-R, GHRH, or NPY (eight sections for GHRH, GHS-R, and NPY, respectively, per rat).

Double-labeled immunohistochemistry for GHS-R with GHRH or NPY

Double-labeled immunofluorescence for GHS-R and GHRH or NPY coupled with confocal microscopic analysis was done using hypothalami of colchicine-treated rats. Coronal sections (10 μm) were mounted onto gelatinized slides. Sections were incubated in antiserum against GHS-R (1:500) overnight at 4 C. The tissues were then rinsed in PBS and incubated in fluorescein-conjugated goat antirabbit IgG (1:200, Vector Laboratories) for 3 h at room temperature. The sections were washed in PBS and subsequently incubated overnight at 4 C with the second antibody, GHRH or NPY. After washing in PBS, the tissues were incubated in Texas red-conjugated goat antirabbit IgG (1:200; Vector Laboratories) for 3 h at room temperature. The slides were coverslipped with VECTASHIELD Hard Set mounting medium (Vector Laboratories). Sections were examined using a Zeiss LSM 510 confocal microscope (Carl Zeiss Co. Ltd., Thornwood, NY). Immunofluorescence in tissue sections was visualized by using a Zeiss Axioplan photomicroscope with a multiband filter set for independent or simultaneous visualization of fluorescein (excitation range, 447–501 nm; emission range, 510–540 nm) and Texas red (excitation range, 560–596 nm; emission range, 610–655 nm) fluorophores. Double-labeled neurons for GHS-R and GHRH or NPY in the Arc were counted in five randomly selected sections under light microscopy at ×400 magnification. All images were processed by using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Immunohistochemistry for Fos

Twenty serial coronal sections (40 μm) were cut with a cryostat through the Arc. Immunohistochemistry was done using the free-floating method with the avidin-biotin-peroxidase method using Fos antibody (1:10000; rabbit polyclonal, Ab5; Oncogene, San Diego, CA). The antibody-peroxidase complex was visualized using diaminobenzidine (Vector DAB kit, Vector Laboratories).

Double-labeled immunohistochemistry for Fos and GHRH

Forty-micrometer sections were processed for immunohistochemical detection of Fos using the Vector DAB kit, and they were then incubated with GHRH antiserum (1:2000) overnight at room temperature. Immunostaining was visualized using the Vector SG substrate kit (Vector Laboratories), which gives a blue-gray precipitate. Quantitative analysis of the number of Fos-positive neurons was done using MCID image analysis system (MCID Amersham Biosciences). Under light microscopy at ×200 magnification, the total number of Fos-positive neurons was counted in the Arc. In total, nine sections per Arc were used for counting. Double-labeled neurons for Fos and GHRH in the Arc were counted in five randomly selected sections under light microscopy at ×400 magnification. Sections were viewed and photographed with an Olympus AX 80 microscope. All images were processed by using Adobe Photoshop software (Adobe Systems).

Primary culture of rat hypothalamic neurons

Newborn Wistar rats were killed by decapitation, and their hypothalami were removed under sterile conditions. Hypothalami were minced in a 1:1 mixture of DMEM and Ham's nutrient mix F-12 containing 10% fetal calf serum, penicillin, and streptomycin (DMEM/F12) (Sigma-Aldrich) for suspension. The minced tissues were then washed twice in PBS. The mixture was incubated in PBS containing 0.047 g/liter $MgCl_2$, 0.1 g/liter $CaCl_2$, and 0.01% Dispase (Godoshusei, Tokyo, Japan) with constant stirring for 30 min at room temperature. After being washed three times with PBS, 1-ml aliquots of cell suspension containing 5.0×10^4 cells in a DMEM/F12 were placed in the wells of 24-well plates that were coated with poly-D-lysine (Sigma-Aldrich). The cells were subsequently allowed to attach to the bottom surface in a humidified 95% air-5% CO_2 incubator for 6 d.

To test the effect of KP-102 on GHRH mRNA expression, cells were treated with KP-102 at concentrations ranging from 2–200 nM for 2 h, and cells were also treated with 20 nM KP-102 for 1–24 h to test the time course of the effect. To test the effect of KP-102 on NPY or somatostatin mRNA expression, cells were treated with 0.2, 2.0, or 20 nM KP-102 for 2 h or 20 nM KP-102 for 24 h.

To test the effect of NPY on GHRH mRNA expression, cells were treated with NPY at a concentration of 0.1 or 1.0 nM for 2 h. To delete the influence of NPY on KP-102-induced changes in GHRH mRNA expression in the culture system, anti-NPY IgG was used. To examine the binding capacity of anti-NPY IgG to NPY in the culture system, cells were treated for 2 h with 1 nM NPY plus anti-NPY IgG (3.6 μg/ml) or normal rabbit serum IgG (3.6 μg/ml) prepared from rabbit anti-NPY serum (16) or normal rabbit serum, respectively, using Protein A Sepharose 4FF (Pharmacia Biotech, Tokyo, Japan). Subsequently, the expression of GHRH mRNA was determined. To examine the effect of anti-NPY IgG on KP-102-induced changes in GHRH mRNA expression, cells were incubated with KP-102 plus anti-NPY IgG or normal rabbit serum IgG (3.6 μg/ml) for 2 h. To examine the effect of anti-NPY IgG on somatostatin-induced changes in GHRH mRNA expression, cells were incubated with 10 nM somatostatin plus anti-NPY IgG or normal rabbit serum IgG for 2 h.

To test the effect of somatostatin on GHRH mRNA expression, cells were treated with somatostatin at a concentration of 1, 10, or 100 nM for 2 h. To delete the influence of somatostatin on the KP-102-induced change in the GHRH mRNA expression level in the culture system, antisomatostatin IgG was used. Antisomatostatin serum was obtained from female New Zealand white rabbits by immunizing them with

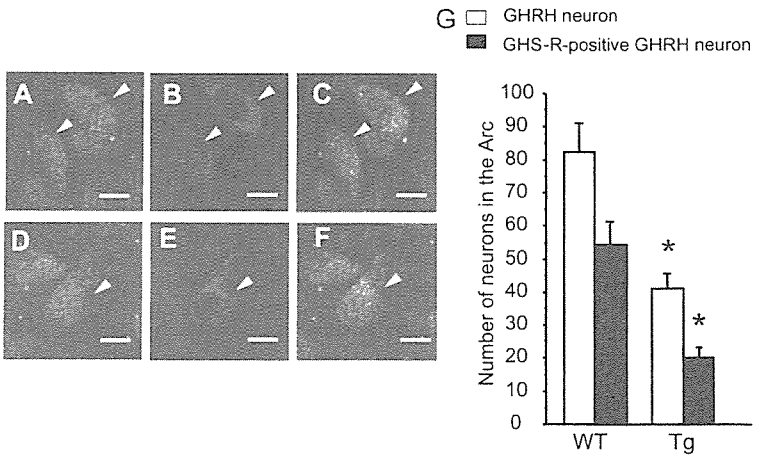

FIG. 2. Double-labeled immunohistochemistry of GHS-R and GHRH in the Arc of female WT and Tg rats. Confocal images of GHS-R (A), GHRH (B), and both (C) of WT rats and those of GHS-R (D), GHRH (E), and both (F) of Tg rats. *Arrows*, Positive neurons. G, Numbers of GHRH and GHS-R-positive GHRH neurons in the Arc. *Scale bars*, 10 μm. *, $P < 0.05$ *vs.* WT rats. The number of rats of each group was seven.

synthetic rat somatostatin-14 coupled with porcine thyroglobulin through water-soluble carbodiimide hydrochloride. The antiserum was used in the RIA at a final concentration of 1/380,000 and showed no cross-reactivity with GHRH, NPY, or corticotropin-releasing factor. The antisomatostatin IgG fraction was prepared using Protein A Sepharose 4FF (Pharmacia Biotech). To test the binding capacity of antisomatostatin IgG to somatostatin in the culture system, cells were treated with somatostatin (10 nM) plus antisomatostatin IgG (3.6 μg/ml) or normal rabbit serum IgG (3.6 μg/ml) for 2 h, and then the level of GHRH mRNA expression was determined. To examine the effect of antisomatostatin IgG on KP-102-induced changes in GHRH mRNA expression, cells were incubated with KP-102 plus antisomatostatin IgG or normal rabbit serum IgG (3.6 μg/ml) for 2 h. To examine the effect of somatostatin on NPY mRNA expression, cells were treated with somatostatin at a concentration of 10 nM for 2 h.

To test the effect of GH on GHRH, NPY, or somatostatin mRNA expression, cells were treated with human recombinant GH (ProSpec-Tany TechnoGene LTD, Rehovot, Israel) at concentrations ranging from 1–500 ng/ml for 2 h.

To test the effect of KP-102, NPY, or somatostatin on GHRH synthesis and release, cells were treated with KP-102 at concentrations ranging from 2–200 nM, NPY, or somatostatin at concentrations of 1 and 10 nM for 4–24 h.

RT-PCR

Total RNA was extracted from cells using Isogen according to the manufacturer's instructions (Takara, Shiga, Japan). To avoid false-positive results caused by DNA contamination, a deoxyribonuclease (DNAse) treatment for 60 min at 37 C using ribonuclease-free DNase (Takara) was done. First strand cDNA was synthesized using 1 μg denatured total RNA under conditions of 42 C for 30 min, 99 C for 5 min, and 5 C for 5 min using RT-PCR kit (Takara). PCR was carried out under conditions of denaturation at 94 C for 10 sec, annealing at 50 C for 5 sec, and extension at 72 C for 60 sec for 30 cycles, using specific primers for GHRH, NPY, and somatostatin (Table 1). After amplification, the PCR products were subjected to 2% agarose gel electrophoresis, stained with 0.5 μg/ml ethidium bromide, and were then visualized under UV illumination. All PCR-amplified DNAs were sequenced for purposes of confirmation.

Competitor construction

Homologous competitive internal standards that shared the same primer binding sites but contained a shortened internal sequence with respect to the endogenous target RNA for GHRH, NPY, or somatostatin were prepared as follows. The products resulting from PCR were subcloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The cloned pGEM-T vector was linearized by *Nco*I restriction digestion and transcribed into the cRNA template by SP6 RNA Polymerase (Promega). The DNA template was removed after transcription, and the cRNA product was quantified and used as an internal standard in RT-PCR for GHRH, NPY, and somatostatin gene expression.

Competitive RT-PCR

After DNase treatment, the amount of mRNA present in the samples was normalized using β-actin primers as an internal reference standard (Table 1). To test for possible pseudogene or genomic DNA contamination, either the RT enzyme or RNA was omitted from the reaction tube. To confirm that RNA competitor is not contaminated with DNA, we performed RT-PCR using only RNA competitor (10^9 copies) at the maximum amount. The reaction mixture and RNA competitor were added to each tube. RT reaction was carried out under conditions of 42

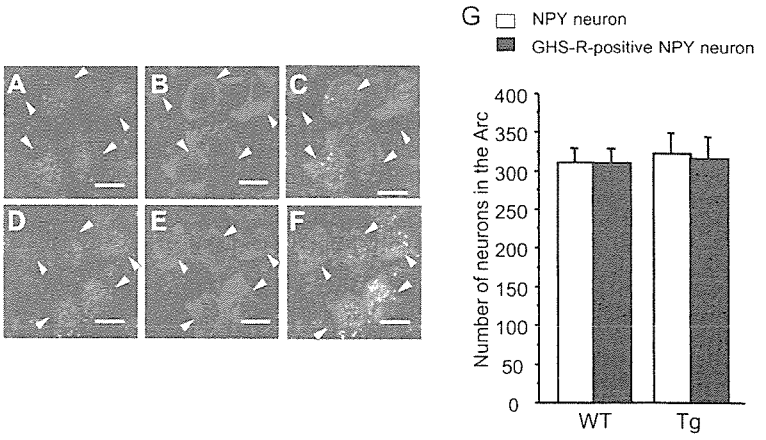

FIG. 3. Double-labeled immunohistochemistry of GHS-R and NPY in the Arc of female WT and Tg rats. Confocal images of GHS-R (A), NPY (B), and GHS-R-positive NPY neurons (C) of WT rats and GHS-R (D), NPY (E), and GHS-R-positive NPY neurons (F) of Tg rats. *Arrows*, Positive neurons. The numbers of NPY and GHS-R-positive NPY neurons in the Arc are shown in G. *Scale bars*, 10 μm. The number of rats of each group was seven.

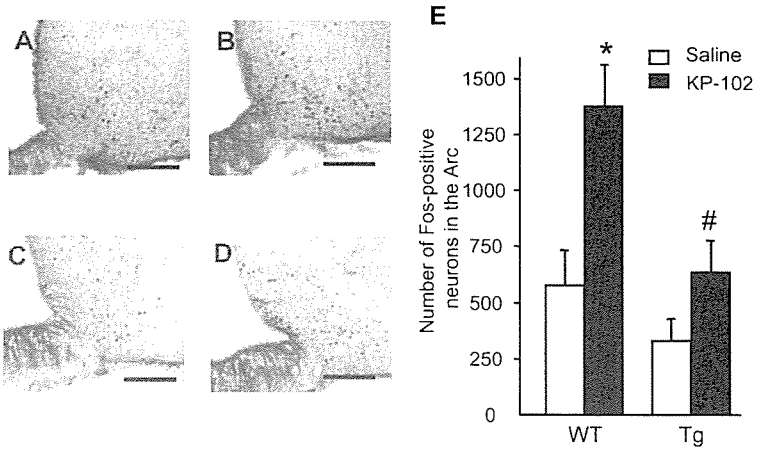

FIG. 4. Changes of Fos-expressing neurons in the Arc of female WT and Tg rats after KP-102 administration. Fos-positive neurons in the Arc 90 min after ICV administration of 2 μl saline (A and C) and 100 pmol KP-102 (B and D) were immunohistochemically determined in WT (A and B) and Tg (C and D) rats. *Scale bars*, 200 μm. E, Statistical analysis of the Fos-positive neurons in the Arc of WT and Tg rats. *, $P < 0.01$ *vs.* WT rats treated with saline; #, $P < 0.01$ *vs.* WT rats treated with KP-102. The number of rats of each group was seven.

C for 30 min, 99 C for 5 min, and 5 C for 5 min. Then, PCR mixture was dispensed into each tube, which contained RT reactant. PCR was carried out under the following conditions: denaturation at 94 C for 10 sec, annealing at 60 C for 5 sec, and primer extension at 72 C for 60 sec for 30 cycles. PCR products were separated on 2% agarose gel and visualized with ethidium bromide. The intensities of the bands of the PCR products of GHRH, NPY, and somatostatin were quantified using National Institutes of Health image software. The ratio of internal standard to endogenous area was plotted as a function of the competitor concentration added to each PCR. The concentrations of GHRH, NPY, and somatostatin mRNA were determined at the point where the ratio of the internal standard and the endogenous area of each gene were equal to 1 (the equivalence point). Experiments of same protocol were repeated twice or three times, and the results were combined for statistical analysis.

RIA for GHRH

RIA for GHRH was performed as described previously (14). In short, synthetic rat GHRH was iodinated using the chloramine-T method and purified on a column of Sephadex G-50. PBS [0.1 M (pH 7.5)] containing 0.01% Nonidet P-40 (Nacalai Inc., Kyoto, Japan), 5 mM EDTA-Na, and 0.02% NaN_3 was used for RIA. Standard synthetic rat GHRH or sample was incubated with antirat GHRH antiserum in 3-ml plastic tubes for 24 h at 4 C. ^{125}I-labeled GHRH was then added to each tube and incubated for another 24 h. Goat antirabbit IgG was used to separate tracer bound to antiserum from free tracer. The anti-GHRH antiserum was used for RIA at a final concentration of 1:400,000 to yield a maximum binding of approximately 30%.

Image analysis

Image analysis was performed with an Olympus AX-80 microscope and a digital camera (DP50, Olympus, Tokyo, Japan). Images were assembled using Lumina Vision (Mitani Corp., Tokyo, Japan).

Statistical analysis

Data are expressed as mean ± SEM. The statistical analysis was completed using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA); for *in vivo* study, one-way ANOVA followed by a *post hoc* Fisher's test was performed, whereas for the *in vitro* study, ANOVA followed by Fisher's PLSD was done. $P < 0.05$ considered statistically significant.

Results

Distribution and number of GHS-R-positive, GHRH, and NPY neurons in the Arc

GHS-R-positive neurons were widely distributed in the Arc of the WT and Tg rats (Fig. 1, A and B). The mean number of GHS-R-positive neurons was significantly less in Tg rats than in WT rats (Tg rats, 927 ± 57 *vs.* WT rats, 1409 ± 199, $P < 0.05$) (Fig. 1G). GHRH neurons were distributed in the ventral and lateral part of the Arc of the WT and Tg rats (Fig. 1, C and D). The mean number of GHRH neurons was significantly less in Tg rats than that in WT rats (Tg rats, 206 ± 33 *vs.* WT rats, 326 ± 59, $P < 0.05$) (Fig. 1G). NPY neurons

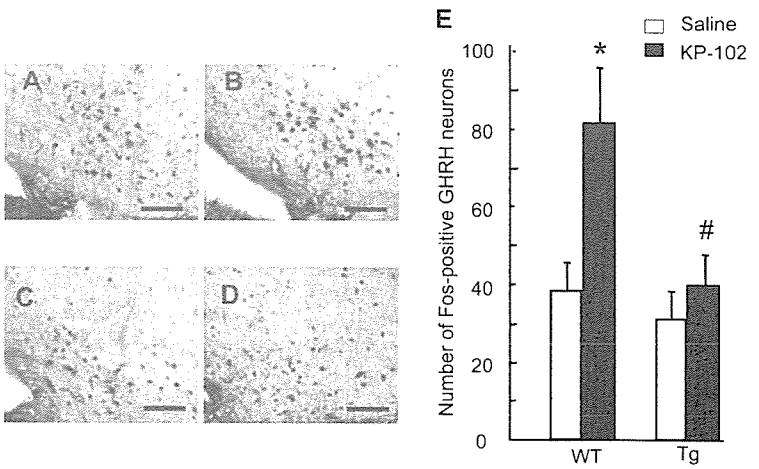

FIG. 5. Distribution and number of Fos-positive GHRH neurons in the Arc in response to saline or KP-102 in female WT and Tg rats. A, Fos-positive GHRH neurons in the Arc 90 min after ICV administration of 2 μl saline (A and C) and 100 pmol KP-102 (B and D) were immunohistochemically determined in WT (A and B) and Tg (C and D) rats. *Scale bars*, 100 μm. E, Statistical analysis of the Fos-positive GHRH neurons in the Arc of WT and Tg rats. *, $P < 0.05$ *vs.* WT rats treated with saline. #, $P < 0.05$ *vs.* WT rats treated with KP-102. The number of rats of each group was eight.