sensus on whether or not smoking increases the risk of stroke in Japanese [4–8]. Furthermore, some studies have evaluated the interaction between smoking and hypercholesterolemia, which is also an important risk factor for CVD, but their conclusions have not been consistent [4, 9–13]. The purposes of the present study were to assess the effect of smoking on the development of stroke and CHD, and to clarify the interactions between smoking and hypercholesterolemia as well as other risk factors in a population-based cohort study in Japan.

Methods

Study Subjects

In 1988, a screening examination for the present study was performed in the town of Hisayama, a suburban community in the Fukuoka metropolitan area on Kyushu Island, Japan. A detailed description of this examination was published previously [14, 15]. Briefly, a total of 2,587 residents aged 40–79 years (80.2% of the total population in this age range) participated in the examination. After the exclusion of 88 subjects with a history of stroke or CHD, 77 who did not complete a 75-gram oral glucose tolerance test, and 1 who died before the initiation of follow-up, the remaining 2,421 (1,037 men and 1,384 women) were enrolled in the present study. This study was conducted with the approval of the ethics committee of the Faculty of Medicine, Kyushu University, and written informed consent was obtained from the subjects.

Risk Factors

At the baseline examination, each subject completed a self-administered questionnaire covering medical history, treatment for hypertension and diabetes, smoking status, alcohol intake and leisure time activity. The smoking status was classified into 4 categories: never smokers, former smokers, current light smokers (<20 cigarettes per day) and current heavy smokers (≥20 cigarettes per day). Alcohol intake was defined as customary drinking of an alcoholic beverage at least once a month. Subjects engaging in sports or other forms of exertion ≥3 times a week during their leisure time made up a regular exercise group.

The sitting blood pressure was measured 3 times using a standard mercury sphygmomanometer after rest for at least 5 min. The mean of the 3 measurements was used for the analysis. Hypertension was defined as blood pressure ≥140/90 mm Hg or current use of antihypertensive agents. Obesity was defined as body mass index ≥25. Electrocardiogram abnormalities were defined as left ventricular hypertrophy (Minnesota Code, 3–1), ST depression (4–1, 2 or 3) or atrial fibrillation (8–3).

We performed the 75-gram glucose tolerance test after at least a 12-hour overnight fast. The plasma glucose levels were determined by the glucose-oxidase method. Diabetes mellitus was defined as any of the following: fasting plasma glucose ≥7.0 mmol/l, 2-hour postload glucose ≥11.1 mmol/l, or current use of oral hypoglycemic agents or insulin. The total cholesterol levels were determined enzymatically. Hypercholesterolemia was defined as total cholesterol ≥5.69 mmol/l.

Follow-Up Survey

The subjects were followed up prospectively for 14 years from December 1988 to November 2002 by repeated health examinations. Their health status was checked yearly by mail or telephone for any subjects who did not undergo a regular examination or who had moved out of town. We also established a daily monitoring system among the study team and local physicians or members of the town's health and welfare office. Using this system, we gathered information on new events of stroke and CHD, including suspected cases. When a new CVD event occurred or was suspected, physicians in the study team examined the subject and evaluated his or her detailed clinical information, including medical history and physical, neurological, laboratory and radiological examinations, to determine whether or not this event met the definition of an outcome. In addition, when a subject died, an autopsy was usually performed at the Department of Pathology of Kyushu University. During the follow-up period, 1 subject was lost to follow-up and 418 died, of whom 312 (74.6%) underwent autopsy.

Study Outcomes

Study outcomes were the development of CVD consisting of stroke and CHD. Stroke was defined in principle as a sudden onset of nonconvulsive and focal neurological deficit persisting for ≥24 h and was classified into 3 subtypes: ischemic stroke, intracerebral hemorrhage and subarachnoid hemorrhage. All stroke events were morphologically examined by computed tomography, magnetic resonance imaging or autopsy findings. CHD included acute and silent myocardial infarction, sudden cardiac death within 1 h after the onset of acute illness, and coronary artery disease treated by coronary artery bypass surgery or angioplasty. Acute myocardial infarction was diagnosed when a subject met at least 2 of 4 criteria: (1) typical symptoms including prolonged severe anterior chest pain; (2) evolving diagnostic electrocardiographic changes; (3) cardiac enzyme levels more than twice the upper limit of the normal range; (4) morphological changes (local asynergy of cardiac wall motion on echocardiography, persistent perfusion defect on cardiac scintigraphy, or myocardial necrosis or scars ≥1 cm long accompanied by coronary atherosclerosis at autopsy). Silent myocardial infarction was defined as a morphological change of the myocardium without any historical indication of clinical symptoms or abnormal cardiac enzyme changes. During the follow-up period, 281 subjects developed CVD for the first time. These included 194 cases of all forms of stroke (132 ischemic stroke, 43 intracerebral hemorrhage and 19 subarachnoid hemorrhage) and 112 cases of CHD.

Statistical Analysis

SAS software version 9.2 was used to perform all statistical analyses. The frequency of each risk factor at baseline across the smoking status was adjusted for age and sex by a direct method and compared by logistic regression analysis. The age- and sex-adjusted mean of each risk factor at baseline was estimated and compared by the analysis of covariance. The age- and sex-adjusted and multivariate-adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) were estimated using the Cox proportional hazards model. The interaction between smoking and each of the other risk factors was tested by adding an interaction term to the relevant Cox model. $p < 0.05$ was considered statistically significant.

Table 1. Age- and sex-adjusted mean values or frequencies of cardiovascular risk factors by smoking status at baseline

	Never smoker (n = 1,477)	Former smoker (n = 332)	Current smoker	
			<20 cigarettes/day (n = 348)	≥20 cigarettes/day (n = 264)
Age, years (sex-adjusted)	57 ± 12	61 ± 12*	59 ± 11	55 ± 12*
Men, % (age-adjusted)	14.3	92.6*	77.0*	94.8*
Systolic blood pressure, mm Hg	133 ± 23	133 ± 22	130 ± 21	129 ± 22
Diastolic blood pressure, mm Hg	79 ± 13	79 ± 12	75 ± 12*	75 ± 12*
Hypertension, %	38.6	43.0	35.2	21.1*
Fasting plasma glucose, mmol/l	5.8 ± 1.5	6.0 ± 1.5	5.8 ± 1.4	5.7 ± 1.4
Two-hour postload glucose, mmol/l	7.5 ± 4.3	7.9 ± 4.1	7.5 ± 3.8	7.4 ± 4.0
Diabetes, %	10.3	12.1	12.5	11.0
Total cholesterol, mmol/l	5.37 ± 1.27	5.44 ± 1.21	5.20 ± 1.14	5.40 ± 1.19
Hypercholesterolemia, %	35.3	38.0	37.1	22.5
Body mass index	23.1 ± 3.7	23.3 ± 3.5	22.3 ± 3.3*	22.5 ± 3.4*
Obesity, %	25.1	27.1	19.8*	23.9
Electrocardiogram abnormalities, %	17.4	16.1	16.3	13.4
Current alcohol intake, %	26.6	56.0*	46.3*	51.9*
Regular exercise, %	10.0	10.9	9.9	3.5

Values presented are means ± SD or percentages. * $p < 0.05$ compared with never smokers.

Results

The baseline characteristics of the study subjects are summarized in table 1. Compared with never smokers, the mean age was higher in former smokers but lower in current heavy smokers. The proportions of men and alcohol drinkers were higher in former and current smokers. Current heavy smokers had a lower prevalence of hypertension. Current light and heavy smokers had a lower body mass index.

In men, the risk for the development of CVD was significantly higher in current smokers than in never smokers (age-adjusted HR = 1.65; 95% CI = 1.04–2.63), and the risk of CVD was almost the same in women as in men (age-adjusted HR = 1.68; 95% CI = 0.94–2.98). Because there was no evidence of interaction between sex and current smoking (p for interaction = 0.97), we analyzed both sexes together in the following evaluations.

Table 2 shows the effects of smoking on the development of CVD, total stroke and CHD. The age- and sex-adjusted HRs for CVD and total stroke were significantly higher in current light and heavy smokers, and that for CHD was significantly higher in current heavy smokers than in never smokers. Former smoking was not a significant risk factor for each outcome. After adjusting for risk factors (age, sex, systolic blood pressure, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, alcohol intake and regular exercise), both current light and heavy smokers had a significantly higher risk of each outcome than never smokers. Table 3 shows the effects of smoking on the risks of stroke subtypes. Current light and heavy smokers were combined here because of the limited number of events. Current smoking was an independently significant risk factor for ischemic stroke and subarachnoid hemorrhage, but not for intracerebral hemorrhage, after adjustment for confounding factors.

As shown in table 4, we assessed the combined and separate effects of smoking and each of the other established risk factors on the development of CVD. Compared with nonsmokers (never or former smokers) without hypercholesterolemia, current smokers with hypercholesterolemia had significantly higher multivariate-adjusted HRs for CVD. However, no significant elevations in HRs were observed in nonsmokers with hypercholesterolemia or in current smokers without hypercholesterolemia. A significant interaction between smoking and hypercholesterolemia was revealed in the risk of CVD, while we failed to detect any significant interaction between current smoking and hypertension, diabetes, obesity, alcohol intake or regular exercise. Table 5 shows the interaction analyses between current smoking and hypercholesterolemia on the development of stroke and CHD. The combination of current smoking and hyper-

Table 2. Risks for the development of cardiovascular disease, total stroke and coronary heart disease according to smoking status

	Events/ population	Age- and sex-adjusted			Multivariate-adjusted		
		HR	95% CI	p	HR	95% CI	p
Cardiovascular disease							
Never smoker	137/1,477	1.00			1.00		
Former smoker	50/332	1.26	0.83–1.91	0.29	1.25	0.80–1.93	0.32
Current smoker (<20 cigarettes/day)	54/348	1.60	1.09–2.34	0.02	1.80	1.21–2.66	0.004
Current smoker (≥20 cigarettes/day)	40/264	1.88	1.20–2.95	0.006	2.04	1.29–3.24	0.003
Total stroke							
Never smoker	104/1,477	1.00			1.00		
Former smoker	34/332	1.52	0.91–2.52	0.11	1.53	0.90–2.61	0.12
Current smoker (<20 cigarettes/day)	34/348	1.70	1.07–2.71	0.02	1.90	1.18–3.06	0.009
Current smoker (≥20 cigarettes/day)	22/264	1.87	1.05–3.32	0.03	2.01	1.11–3.65	0.02
Coronary heart disease							
Never smoker	43/1,477	1.00			1.00		
Former smoker	23/332	1.19	0.63–2.26	0.60	1.10	0.56–2.15	0.78
Current smoker (<20 cigarettes/day)	25/348	1.61	0.88–2.93	0.12	1.88	1.02–3.47	0.04
Current smoker (≥20 cigarettes/day)	21/264	2.07	1.07–4.01	0.03	2.31	1.17–4.57	0.02

Multivariate-adjusted: adjusted for age, sex, systolic blood pressure, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, alcohol intake and regular exercise.

Table 3. Risks for the development of stroke subtypes according to smoking status

	Events/ population	Age- and sex-adjusted			Multivariate-adjusted		
		HR	95% CI	p	HR	95% CI	p
Ischemic stroke							
Never smoker	69/1,477	1.00			1.00		
Former smoker	26/332	1.72	0.95–3.12	0.08	1.70	0.90–3.20	0.10
Current smoker	37/612	1.78	1.05–3.01	0.03	2.03	1.18–3.49	0.01
Intracerebral hemorrhage							
Never smoker	24/1,477	1.00			1.00		
Former smoker	7/332	1.00	0.34–2.91	>0.99	1.11	0.37–3.33	0.85
Current smoker	12/612	1.20	0.48–3.00	0.70	1.21	0.47–3.15	0.70
Subarachnoid hemorrhage							
Never smoker	11/1,477	1.00			1.00		
Former smoker	1/332	0.86	0.09–8.32	0.89	0.92	0.09–9.08	0.95
Current smoker	7/612	3.39	1.00–11.54	0.051	3.85	1.05–14.13	0.04

Multivariate-adjusted: adjusted for age, sex, systolic blood pressure, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, alcohol intake and regular exercise.

cholesterolemia significantly increased the risks of total stroke and CHD, and their interactions were statistically significant. In regard to stroke subtypes, similar findings were observed in the risks of ischemic stroke and subarachnoid hemorrhage, although interaction was significant only for subarachnoid hemorrhage.

Discussion

In the present study of a population-based cohort in Japan, current smoking was an independently significant risk factor for the development of stroke and CHD. In regard to stroke subtypes, current smoking was clear-

Table 4. Combined and separate effects of smoking and each risk factor on the development of cardiovascular disease

	Events/ population	Multivariate-adjusted		
		HR	95% CI	p
Hypercholesterolemia				
Current smoking (–)/hypercholesterolemia (–)	108/1,099	1.00		
Current smoking (–)/hypercholesterolemia (+)	79/710	1.08	0.80–1.47	0.60
Current smoking (+)/hypercholesterolemia (–)	57/435	1.36	0.96–1.93	0.08
Current smoking (+)/hypercholesterolemia (+)	37/177	2.68	1.81–3.95	<0.001
p for interaction				0.001
Hypertension				
Current smoking (–)/hypertension (–)	67/1,103	1.00		
Current smoking (–)/hypertension (+)	120/706	1.87	1.36–2.57	<0.001
Current smoking (+)/hypertension (–)	44/388	1.83	1.22–2.76	0.003
Current smoking (+)/hypertension (+)	50/224	2.97	1.98–4.45	<0.001
p for interaction				0.22
Diabetes				
Current smoking (–)/diabetes (–)	142/1,602	1.00		
Current smoking (–)/diabetes (+)	45/207	1.74	1.23–2.46	0.002
Current smoking (+)/diabetes (–)	73/524	1.70	1.24–2.34	0.001
Current smoking (+)/diabetes (+)	21/88	2.83	1.73–4.63	<0.001
p for interaction				0.82
Obesity				
Current smoking (–)/obesity (–)	139/1,348	1.00		
Current smoking (–)/obesity (+)	48/461	0.93	0.66–1.30	0.66
Current smoking (+)/obesity (–)	68/480	1.49	1.07–2.07	0.02
Current smoking (+)/obesity (+)	26/132	2.10	1.34–3.28	0.001
p for interaction				0.07
Alcohol intake				
Current smoking (–)/alcohol intake(–)	138/1,402	1.00		
Current smoking (–)/alcohol intake (+)	49/407	0.81	0.55–1.19	0.29
Current smoking (+)/alcohol intake (–)	39/250	1.19	0.79–1.80	0.40
Current smoking (+)/alcohol intake (+)	55/362	1.15	0.75–1.75	0.52
p for interaction				0.58
Regular exercise				
Current smoking (–)/regular exercise (–)	166/1,622	1.00		
Current smoking (–)/regular exercise (+)	20/185	0.81	0.51–1.29	0.38
Current smoking (+)/regular exercise (–)	89/556	1.83	1.36–2.48	<0.001
Current smoking (+)/regular exercise (+)	5/56	0.57	0.23–1.42	0.23
p for interaction				0.08

Current smoking (–) includes both never and former smoking. Multivariate-adjusted: adjusted for age, sex, systolic blood pressure, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, alcohol intake and regular exercise. The variable relevant to the subgroup was excluded from each model.

ly associated with the development of ischemic stroke and subarachnoid hemorrhage, but not with intracerebral hemorrhage. These findings are concordant with previously reported meta-analyses based mainly on Caucasian populations [1, 2]. In addition, we demonstrated that hypercholesterolemia strengthened the harmful effects of smoking on these outcomes, but such effects were not observed for other risk factors: hypertension, diabetes, obesity, alcohol intake and regular exercise.

Several injurious effects of cigarette smoking on arteries have been demonstrated. Smoking causes direct injury to endothelial cells [16], oxidation of low-density lipoprotein [17], and acceleration of thrombus formation through increased plasma fibrinogen [18], increased platelet aggregability [19] and decreased fibrinolytic ac-

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p for interaction				0.22
Diabetes				
Current smoking (–)/diabetes (–)	142/1,602	1.00		
Current smoking (–)/diabetes (+)	45/207	1.74	1.23–2.46	0.002
Current smoking (+)/diabetes (–)	73/524	1.70	1.24–2.34	0.001
Current smoking (+)/diabetes (+)	21/88	2.83	1.73–4.63	<0.001
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ly associated with the development of ischemic stroke and subarachnoid hemorrhage, but not with intracerebral hemorrhage. These findings are concordant with previously reported meta-analyses based mainly on Caucasian populations [1, 2]. In addition, we demonstrated that hypercholesterolemia strengthened the harmful effects of smoking on these outcomes, but such effects were not observed for other risk factors: hypertension, diabetes, obesity, alcohol intake and regular exercise.

Several injurious effects of cigarette smoking on arteries have been demonstrated. Smoking causes direct injury to endothelial cells [16], oxidation of low-density lipoprotein [17], and acceleration of thrombus formation through increased plasma fibrinogen [18], increased platelet aggregability [19] and decreased fibrinolytic ac-

population prevalence of hypercholesterolemia was low. The other possible reason is a change in the distribution of ischemic stroke subtypes. In Hisayama, while the proportion of lacunar infarctions among all ischemic stroke events has decreased during the past 4 decades, the proportions of atherothrombotic and cardioembolic stroke have increased [25]. These changes might affect the influence of smoking on the development of ischemic stroke.

In previous studies, the relationship between smoking and the risk of intracerebral hemorrhage has been reported to be inconsistent. A few cohort studies [26, 27] showed that current smoking increased the risk of intracerebral hemorrhage, while other studies [5–8, 12, 28], including a meta-analysis [1] and ours, found no discernible association between the two. The reasons for these inconsistent conclusions are unknown. However, because smoking increases hypercoagulability rather than bleeding tendency [18–20], the effect of smoking on the risk of intracerebral hemorrhage seems to be weak, if any.

Because smoking oxidizes low-density lipoprotein [17], it is reasonable to think that the combination of smoking and hypercholesterolemia may accelerate the progression of atherosclerosis and the development of ischemic stroke and CHD. Some studies have evaluated the interaction between smoking and hypercholesterolemia in relation to CVD outcomes. However, the conclusions have not been consistent [4, 9–13]. In the present study, the synergistic effect of smoking and hypercholesterolemia on the development of CHD was significant, and a similar tendency was observed for ischemic stroke. Another Japanese cohort study [9] also demonstrated positive interactions between smoking and cholesterol for ischemic stroke and CHD mortality. On the other hand, the first cohort of the Hisayama study, established in 1961 [4], as well as the Asia Pacific Cohort Studies Collaboration [10], confirmed a positive interaction for CHD but not for ischemic stroke. Two Korean cohort studies [11, 12] and a meta-analysis of mainly Caucasian studies [13] did not find any interactions for CVD outcomes. These inconsistent conclusions may be explained in part by ethnicity and differences in average cholesterol levels. The effects of smoking and cholesterol on CVD outcomes may differ between Asians and Caucasians. Among Asian studies, the average cholesterol levels were lower in the first cohort of Hisayama [4], Pacific Cohort Studies Collaboration [10] and 2 Korean studies [11, 12] compared with the present study. We have no clear explanation of the synergistic effect of smoking and hypercholesterolemia on the risk of subarachnoid hemorrhage. In any case, we cannot draw any conclusion from the present results because of the small number of subarachnoid hemorrhages in our study. Our finding should be confirmed in larger cohort studies.

The advantages of the present analyses include accurate measurement of risk factors at baseline, the longitudinal population-based study design, the long duration of follow-up, perfect follow-up of study subjects and accurate diagnoses of CVD. However, a possible limitation should be discussed. Because we did not consider changes in smoking habits and other risk factors or treatments that occurred during the follow-up, our results may underestimate the effects of smoking and other risk factors on the risk of CVD.

In conclusion, we demonstrated that current smoking increases the risk of ischemic stroke, subarachnoid hemorrhage and CHD, especially in individuals with hypercholesterolemia. Although the smoking rates in Japanese men and women have been decreasing in the past 4 decades [25], Japanese men still have a higher smoking rate than people in Western countries [3]. Our findings highlight the importance of smoking cessation to reduce the burden of CVD in Japan, where the prevalence of hypercholesterolemia is escalating rapidly [25].

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Plasma levels of *n*-decanoyl ghrelin, another acyl- and active-form of ghrelin, in human subjects and the effect of glucose- or meal-ingestion on its dynamics

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ABSTRACT

Besides *n*-octanoyl ghrelin (O-ghrelin), there is another acyl-form of ghrelin; *n*-decanoyl ghrelin (D-ghrelin), which has a decanoic acid modification. In this study, we examined the kinetics of D-ghrelin immunoreactivity in human plasma in comparison to O-ghrelin or total ghrelin by using a D-ghrelin-specific radioimmunoassay. The dynamics of plasma D-ghrelin was assessed following glucose- or meal-ingestion in healthy, non-obese subjects (5 males and 5 females). Correlations were also analyzed between the levels of plasma D-ghrelin and anthropometric or metabolic indicators in healthy human subjects ($n=111$, BMI 17.4–34.3). The plasma levels of D-ghrelin, like O- or T-ghrelin, significantly declined ($p<0.05$ for male and $p<0.01$ for female) 60 min after the ingestion of glucose in non-obese subjects. However, in the same subjects, no significant decline was noted in the levels of D-ghrelin, unlike O- or T-ghrelin, upon the meal ingestion. A significant increase was observed in the proportion of plasma D-ghrelin levels to that of T-ghrelin ($p<0.05$) in the healthy human subjects as BMI increased, unlike the proportion of O-ghrelin to T-ghrelin, which did not change. Since D-ghrelin possesses almost the same potential as that of O-ghrelin with regard to the feeding-stimulation, these differences between the dynamics of D- and O-ghrelin in human plasma might influence appetite-control, especially in those with increased BMI.

1. Introduction

Ghrelin is a brain and gut hormone primarily produced in the stomach [1,2]. Ghrelin exerts various physiological functions through the growth hormone secretagogue (GHS) receptor (GHS-R) [3,4]. The third amino acid in the peptide, which is the serine residue (Ser^3), is modified by an acyl group; this modification is essential for the activity of ghrelin [5–7] through the functional ghrelin receptor, GHS-R1a [8,9]. The primary acyl-form of the ghrelin peptide in various mammalian species [1,10–14] and other vertebrates [15–18] has been reported to be *n*-octanoyl ghrelin (O-ghrelin), the Ser^3 of which is acyl-modified with *n*-octanoyl group (C8:0; an eight-carbon chain containing no double bonds).

However, other acyl-forms of ghrelin also existed in almost all of the species examined [11–20]. These include *n*-decanoyl ghrelin (D-ghrelin): a ghrelin acylated with an *n*-decanoyl group (C10:0, a 10-carbon chain lacking double bounds) or *n*-decenoyl ghrelin: a ghrelin modified with *n*-decenoyl group (C10:1, a 10-carbon chain with one double bound). In a tilapia (*oreochromis mossambicus*), a teleost fish,

Abbreviations: AcOH, acetic acid; BMI, body mass index; C8:0, *n*-octanoyl group (an eight-carbon chain without a double bond); C10:0, *n*-decanoyl group (a ten-carbon chain without a double bond); C10:1, *n*-decenoyl group (a ten-carbon chain with one double bond); C18-RP-HPLC, reverse-phase HPLC equipped with a C18-column; CH_3CN, acetonitrite; D-ghrelin, *n*-decanoyl ghrelin; *des*-acyl, without acyl-modification; ghrelin delivertives, ghrelin peptides except *des*-acyl or *n*-octanoyl ghrelin; GHS, GH secretagogue; GHS-R, GHS receptor (ghrelin receptor); HOMA-IR, homeostatic model assessment of insulin resistance; intact ghrelin, ghrelin with the complete N- and C-terminal peptide sequence; O-ghrelin, *n*-octanoyl ghrelin; O-ghrelin RIA, radioimmunoassay for the N-terminal of sequence *n*-octanoyl ghrelin (residues 1–11) that measures mainly the content of O-ghrelin; T-ghrelin, total ghrelin (*des*-acyl- and *acyl*-ghrelin peptides with intact C-terminal sequence); T-ghrelin RIA, RIA for the C-terminal sequence of ghrelin (residues 13–28) that measures the content of ghrelin molecules with intact C-terminal sequence irrespective of their acylation status; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid.

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the main acyl-form of ghrelin has been reported to be the D-ghrelin [20].

Concerning the biological activities and the physiological functions of D-ghrelin, previous *in vitro* studies demonstrated that D-ghrelin possessed almost the same potential as did O-ghrelin to activate GHS-R1a [6,11]. These findings indicated that D-ghrelin was one of the active forms of the ghrelin peptide, and that it could interact with the receptor in a manner similar to that of O-ghrelin. Previous *in vivo* studies also demonstrated that the D-ghrelin stimulated the feeding [19] and the secretion of GH [11] to nearly the same degree of magnitude.

In humans and other species, plasma concentrations of acyl-ghrelin (reflecting mainly the level of O-ghrelin) together with that of total ghrelin (all ghrelin peptides with intact C-termini irrespective of their acyl-modifications) decline significantly following the ingestion of glucose or other nutrients [21,22]. These findings indicate that the short-term regulation of the levels of plasma acyl-ghrelin was almost the same as that of total ghrelin. Contrary to the above findings, the long-term regulation of the respective levels of plasma acyl- or non-acyl-ghrelin was somewhat different. In normal human subjects, the plasma concentration of total (acyl-plus non-acyl) ghrelin exhibited an inverse correlation to the values of body mass index (BMI) [5,23]. On the other hand, Barazzoni et al. [24] and Pacifico et al. [25] reported that the absolute concentrations of acyl-ghrelins, as well as the proportion of acyl-ghrelin to non-acyl (*des*-acyl) ghrelin, increased in subjects suffering from insulin resistance and/or morbid obesity in comparison to non-obese, non-insulin-resistant subjects. However, previous studies by the above two groups did not clarify which forms of acyl-ghrelin (O-, D- or other acyl-forms of ghrelin) contributed to the net increase of acyl-ghrelin in the plasma that were dependent upon the increase in the index for insulin resistance (i.e., HOMA-IR) or in the index for obesity (i.e., BMI).

We have recently established a D-ghrelin-specific radioimmunoassay (D-ghrelin RIA), and reported the dynamics of D-ghrelin immunoreactivity in mice upon fasting [19]. By using this D-ghrelin RIA, we have revealed that D-ghrelin kinetics in the stomachs of mice upon fasting differ in comparison to those of O-ghrelin [19]. We have also demonstrated a comparatively longer half-life of D-ghrelin immunoreactivity in the plasma of mice compared to that of O-ghrelin. However, until now, there have been no reports concerning the dynamics of D-ghrelin in human plasma. In this study, to further elucidate the regulation on the acyl-modification of ghrelin peptides, we examined the kinetics of D-ghrelin in human plasma (healthy volunteers) in comparison to the dynamics of O-ghrelin measured by the ghrelin N-terminal RIA as well as that for total ghrelin (T-ghrelin) measured by the ghrelin C-terminal RIA. To examine the short-term regulation of the levels of D-ghrelin in human plasma, we examined the kinetics of D-ghrelin in plasma following the 75 g-oral glucose test (75 g-OGTT), and also examined the dynamics of the peptide after the test-meal ingestion. To compare the long-term regulation of the levels of the different forms of ghrelins D-, O- and T-ghrelin in human plasma, correlations between the values for anthropometric or metabolic parameters and the levels of D-ghrelin in plasma were examined, and the respective correlations between the above parameters and the plasma levels for O-ghrelin or T-ghrelin were compared.

2. Materials and methods

2.1. Subjects

To estimate the correlations among anthropometric variables, serum lipid parameters and the respective plasma concentrations of D-, O- or T-ghrelin, we enrolled one hundred and eleven healthy volunteers (64 males and 47 females, aged 18–49 y.o.) with varying body mass index (BMI: 17.4–34.3) as control, normal subjects. We defined healthy subjects as those without any symptoms of hypertension, diabetes mellitus, familial dyslipidemia, endocrine disorders, general disorders of the liver or kidneys and inflammatory disorders. We also enrolled twelve T2-DM patients (2 males and 10 females; mean age 65.2 ± 17.0 y.o.; HbA1c 8.74 ± 2.31) to compare their fasting plasma concentrations of D-, O- and T-ghrelin with those in healthy control subjects. To examine the nutrient-related regulation of the levels of plasma D-ghrelin, we performed the 75 g-OGTT or the meal-ingestion test (the precise protocol for the respective tests are described below) in healthy subjects (n = 10; age: 20–22 y.o.; BMI: 20.5 ± 1.9) without insulin resistance (HOMA-IR, 0.93 ± 0.41) (n = 10). All study subjects were recruited under the consent form approved by the Institutional Review Board of Kurume University School of Medicine (Approved No.08083), and data regarding their height and weight, together with their fasting blood samples, were obtained. Informed consent was obtained from all of the subjects.

2.2. Schedule for the 75 g-OGTT or the meal-ingestion test

Fasting blood samples were obtained from each subject after a 12-h overnight fast. For the 75 g-OGTT, 10 healthy subjects (5 males and 5 females) were given 75 g/300 ml glucose solution after overnight fasting, and blood samples were collected 5–10 min before (0 min), and 30, 60 and 120 min after the glucose ingestion. For the meal-ingestion test, 10 healthy subjects (5 males and 5 females) were given 296 kcal/115 g of test meal cookie containing 50% carbohydrate, 43% fat and 7% protein (Neo-Cookie, SRL Co. Ltd., Osaka, Japan) after overnight fasting, and blood samples were collected in the same way as 75 g-OGTT.

2.3. Preparation of plasma samples for D-, O- or T-ghrelin RIA

Plasma samples from the subjects were prepared as previously described [23,26,27] with minor modifications. In brief, blood samples were immediately transferred to chilled polypropyrene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 kallikrein inactivator units/ml) and centrifuged at 4 °C. The plasma was separated and concentrated HCl was added to make a final concentration of 0.1 N and the acidified plasma was diluted with an equal volume of 0.9% NaCl solution. The preparation of all plasma samples described above was carried out on ice and all procedures were done within 5 min after sampling. The diluted sample was passed through a Sep-pak Plus™ C18 cartridge (Waters Corp., Milford, MA) as described previously and was extracted with a 42% CH_3CN-0.1% TFA solution. The extract was then lyophilized after evaporating the CH_3CN. The lyophilized sample was dissolved in 1000 μl of RIA buffer, and the immunoreactivity for D-, O- or T-ghrelin in each 100 μl solution (equivalent to 100 μl of the original plasma sample) was measured using the D-, O- or T-ghrelin RIA.

2.4. RIAs for D-ghrelin, O-ghrelin and T-ghrelin

The RIA for D-ghrelin was prepared as previously described [19]. The anti-D-ghrelin [1–11] antiserum was used at final dilutions of 1/100,000. All assays were performed in duplicate. The antibody in this anti-D-ghrelin antiserum exhibited complete cross-reactivity with human, mouse, and rat D-ghrelin. The anti-D-ghrelin antibody didn't recognize *des*-acyl- or O-ghrelin, although the antibody did cross-react considerably (15–20%) with *n*-decenoyl ghrelin (C10:1-ghrelin) [19].

The RIA for total ghrelin (T-ghrelin RIA) that recognized all ghrelin peptides with intact C-terminal sequences irrespective of their acylation status, as well as the RIA for *n*-octanoyl ghrelin (O-ghrelin RIA) that mainly recognized N-terminal sequence of *n*-octanoyl ghrelin (O-ghrelin), were performed as described previously [10,27]. All assays were performed in duplicate. Both antibodies in

these anti-sera exhibited complete cross-reactivity with human, mouse and rat ghrelin. The anti-rat O-ghrelin [1–11] antibody recognized O-ghrelin, but did not recognize *des*-acyl ghrelin. However, this antibody cross-reacted considerably (10–20%) with other acyl-forms of ghrelin, including D-ghrelin [10]. The anti-rat ghrelin [13–28] antibody equally recognized both the *des*-acyl and all acyl-forms of ghrelin with intact C-termini, such as *des*-acyl-, O- and D-ghrelin [10,27].

2.5. Measurement of the levels of blood glucose, plasma insulin, plasma C-peptide, hemoglobin A1c and serum lipids

Blood samples were obtained from the anterocubital vein into fluoride tubes for analysis of blood glucose or hemoglobin A1c, into EDTA-2Na tubes for insulin or C-peptide, and into plain silliconized tubes for other measurements. The levels of fasting plasma glucose were measured using an electro-enzymatic glucose sensor (Glutest-Ace, Sanwa-Kagaku Co. Ltd., Osaka, Japan) [28]. The proportion of hemoglobin A1c (% Hb_{A1c}) was measured by HPLC (Automatic Glycated Hemoglobin Analyzer HLC-723GHbIIIs; Tosoh, Tokyo, Japan) at an external laboratory. The levels of serum lipids (total cholesterol, triglycerides, free fatty acids, and HDL-cholesterol) were measured by standard enzymatic methods [29] using an automated analyzer (BM8060, Nihon-Densi Co. Ltd., Tokyo, Japan). The plasma insulin levels or C-peptide levels were determined using commercially available RIA kits (Insulin RIA beads II, Yamasa Co. Ltd., Japan; C-peptide kit III, TFB-Japan Co. Ltd., Japan). The degree of insulin resistance was estimated by homeostasis model assessment (HOMA-IR), and was calculated using a Matthews's formula: [fasting insulin (μU/ml) x fasting glucose (mg/dl)]/405 [30].

2.6. Measurement of the relative levels of D-ghrelin and O-ghrelin in human plasma using C18-RP-HPLC combined with T-ghrelin RIA

The relative concentrations of plasma D-ghrelin, O-ghrelin, and *des*-acyl ghrelin, as well as those of other ghrelin molecules with intact C-termini, were measured by using a reverse-phase HPLC (Gilson, Villiers le Bel, France) equipped with a C18-column (Symmetry 300™, Waters, Milford, MA) (C18-RP-HPLC), which was combined with the T-ghrelin RIA as described previously [19,27]. In brief, semi-purified plasma samples eluted from the Sep-Pak plus™ C18 cartridge (7.0 ml plasma equivalent) were injected onto the C18-RP-HPLC using a linear gradient from 10 to 60% CH_3CN-0.1% TFA at a flow rate of 1.0 ml/min for 30 min, and every 500 μl, fractions (fraction 1–60) were collected and lyophilized [19]. The lyophilized samples in each fraction were dissolved in 700 μl of RIA buffer to perform T-ghrelin RIA (1.0 ml plasma equivalent per 100 μl of RIA buffer per RIA tube). The levels of D-ghrelin or O-ghrelin in each fraction of the C18-RP-HPLC were also measured by D-ghrelin RIA or O-ghrelin RIA, respectively.

2.7. Statistical analysis

The data for ghrelins (D-, O-, T-ghrelin) and triglycerides, were log-transformed before statistical analysis because of their skewed distribution. Respective data for D-, O-, or T-ghrelin upon OGTT or meal-ingestion were compared using the repeated-measures ANOVA, followed by a *post hoc* test (Scheffe's test). The Welch's *t*-test was used to compare the differences in anthropometric values between the indicated groups. An age-adjusted partial Pearson's correlation was used to evaluate the correlations between the concentrations of T-, O- or D-ghrelin and each parameter for anthropometric or metabolic conditions. A 2-tailed *P* value <0.05 was considered to be statistically significant. All statistical analyses were performed using the SAS (ver.9.2) for Windows software package (SAS institute Inc. Cary, NC).

3. Results

3.1. Characterization of the D-ghrelin RIA

The basic characteristics of this D-ghrelin RIA were the same as those we reported previously [19]. The anti-D-ghrelin antiserum reacted only minimally with the *des*-acyl ghrelin or *n*-octanoyl ghrelin (O-ghrelin), but cross-reacted considerably with *n*-decenoyl ghrelin (C10:1-ghrelin) (Fig. 1). The concentrations of D-ghrelin or C10:1-ghrelin, which inhibited the half-maximal binding of the radiolabeled tracer to this antiserum (IC_{50}) was 19.1 and 87.2 fmol/tube. The sensitivity of this RIA was 0.5 fmol D-ghrelin/100 μl sample, based on a 95% confidence limit of the mean for ten replicate tests for zero standards. The intra- and inter-assay coefficients of variation were 6.4% and 5.2%, respectively. The dilution curve obtained from human plasma samples paralleled the competition curves obtained using the D-ghrelin standard (Fig. 1), thus suggesting the existence of D-ghrelin immunoreactivity in human plasma. D-ghrelin from humans, rats and mice were detected with the same sensitivity by this D-ghrelin RIA (data not shown).

3.2. Levels of D-, O-, and T-ghrelin in fasting plasma from healthy subjects and T2-DM patients

The plasma level of D-ghrelin was found to be 20–30% of T-ghrelin (Table 1). The plasma levels of O-ghrelin and T-ghrelin were higher in healthy female subjects than in healthy males ($p<0.01$ for O-ghrelin; $p<0.05$ for T-ghrelin) after adjusting for age and BMI (Table 1). Although no significant differences were detected in the plasma levels of D-ghrelin between male and female subjects, a trend toward a slightly higher concentration of plasma D-ghrelin in female subjects than male subjects was observed (Table 1). The plasma concentration of D-ghrelin, together with that of T-ghrelin, in the fasting plasma of T2-DM patients were significantly higher ($p<0.01$ for both D- and T-ghrelin) than those in control normal healthy subjects, even after adjusting for sex, age and BMI (Table 2).

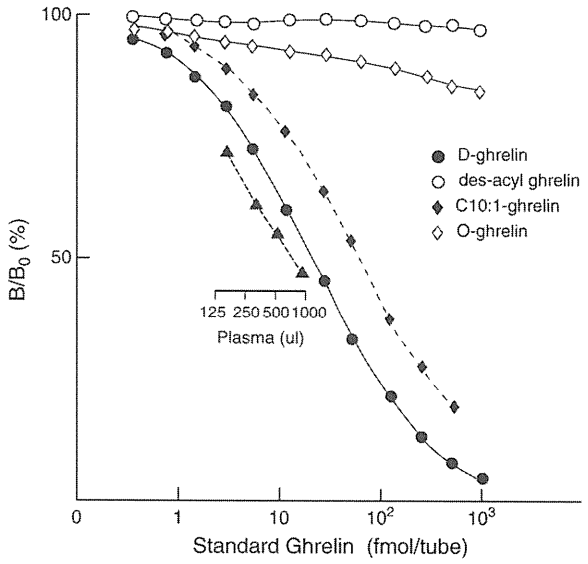

Fig. 1. Competition curves of human *n*-decanoyl ghrelin (D-ghrelin) and other forms of ghrelin (*des*-acyl-, *n*-octanoyl or *n*-decenoyl ghrelin) for the binding of [^{125}I] Tyr^{12}-D-ghrelin [1–11] to the anti-D-ghrelin antiserum. The RIA was performed as described in the Materials and methods section. Three independent experiments were done in duplicate, and the results were expressed as a percentage of specific binding (B) in the absence of peptides (B_0) (mean; n = 3). A parallel dilution curve to the D-ghrelin standard curve was drawn from the Sep-Pak-C18 extract of fasted human plasma (black triangle). D-ghrelin, *n*-decanoyl ghrelin (black circle); *des*-acyl ghrelin (white circle); C10:1-ghrelin, *n*-decenoyl ghrelin (black diamond); O-ghrelin, *n*-octanoyl ghrelin (white diamond).

Table 1
Basic characteristics and plasma concentrations of ghrelins (D-, O-, T-ghrelin) in healthy human subjects (n = 111).

	Male (n = 64)	Female (n = 47)
	Mean ± SD	Mean ± SD
Age (years)	34.0 ± 21.5	34.8 ± 16.1
Weight (kg)	67.9 ± 11.0	51.0 ± 7.5**
Height (cm)	170.2 ± 6.1	157.0 ± 7.2**
BMI (kg/m^2)	23.3 ± 3.2	20.7 ± 2.8**
	Median (range)	Median (range)
D-Ghrelin (fmol/mL)	44.5 (13.9–136.4)	51.8 (23.6–110.0)
O-Ghrelin (fmol/mL)	17.0 (4.4–105.2)	31.3 (6.5–75.8)**
T-Ghrelin (fmol/mL)	141.9 (12.0–613.0)	207.4 (18.0–548.2)*

D-ghrelin, O-ghrelin or T-ghrelin represents the immunoreactivity for *n*-decanoyl-, *n*-octanoyl- or total ghrelin, respectively. Male, healthy male subjects; Female, healthy female subjects. The healthy subjects were defined as described in the Materials and methods. The Welch's *t*-test was used to compare the respective anthropometric values in each group (male or female). The Wilcoxon's rank-sum test was used by adjusting for age and BMI to estimate the differences between the values (medians) for ghrelins in each group. * $p<0.05$, ** $p<0.01$ *vs.* the indicated values in Males.

3.3. Dynamics of D-, O-, and T-ghrelin levels in plasma during the 75 g-OGTT

The plasma levels of D-ghrelin in both male and female subjects was significantly decreased at 60 min ($p<0.05$ for males, $p<0.01$ for females) after the glucose-load compared with basal levels at 0 min (Fig. 2A). In female subjects, the level of plasma D-ghrelin at 120 min was still lower ($p<0.05$ *vs.* 0 min) than the basal level (Fig. 2A). The plasma levels of O-ghrelin in both male and female subjects was significantly decreased at 30 min ($p<0.05$ for males, $p<0.01$ for females) and 60 min ($p<0.05$ for males, $p<0.01$ for females) after the glucose-load in comparison to basal levels. (Fig. 2B). The level of plasma T-ghrelin in male subjects was significantly decreased at 60 min ($p<0.05$) and 120 min ($p<0.05$) after the glucose-load in comparison to the basal level at 0 min (Fig. 2C). The plasma T-ghrelin level in female subjects was significantly lower 120 min after the glucose-load ($p<0.05$) compared with the basal level (Fig. 2C). In both sexes, the proportion of plasma O-ghrelin to T-ghrelin (O/T-ratio) was significantly lower at 30 min ($p<0.01$) and 60 min ($p<0.01$) during the 75 g-OGTT compared to the basal levels at 0 min (Fig. 3A). In contrast to this, no significant changes were noted in the D/T-ratio or D/O-ratio throughout the 75 g-OGTT (Fig. 3B,C).

Table 2
Basic characteristics and plasma concentrations of ghrelins (D-, O-, T-ghrelin) in T2-DM and control subjects.

	Control (n = 111)	T2-DM (n = 12)
	Mean ± SD	Mean ± SD
Age (years)	35.8 ± 18.6	65.2 ± 17.0**
Weight (kg)	60.4 ± 12.6	63.1 ± 16.1
Height (cm)	164.4 ± 9.3	155.4 ± 7.7**
BMI (kg/m^2)	22.2 ± 3.3	25.9 ± 5.1**
	Median (range)	Median (range)
D-Ghrelin[a] (fmol/mL)	45.8 (13.9–136.4)	86.74 (47.2–189.3)**
O-Ghrelin[a] (fmol/mL)	22.6 (4.4–105.6)	15.90 (2.6–24.9)
T-Ghrelin[a] (fmol/mL)	170.0 (12.0–613.0)	221.8 (76.9–420.4)**

Control, normal healthy subjects (64 males and 47 females, the same subjects shown in Tables 1 and 2); T2-DM, patients with type 2-diabetes mellitus (2 male and 10 female; HbA1c 8.74 ± 2.31). [a], The data for ghrelins were compared after adjustment for sex, age and BMI, and log-transformed values were used for the statistical analysis. The rank-based analysis of covariance (ANCOVA) was used to estimate the differences between the values for ghrelins in normal subjects and T2-DM patients. The Welch's *t*-test was used to compare the respective anthropometric values in each group. * $p<0.05$, ** $p<0.01$ *vs.* control.

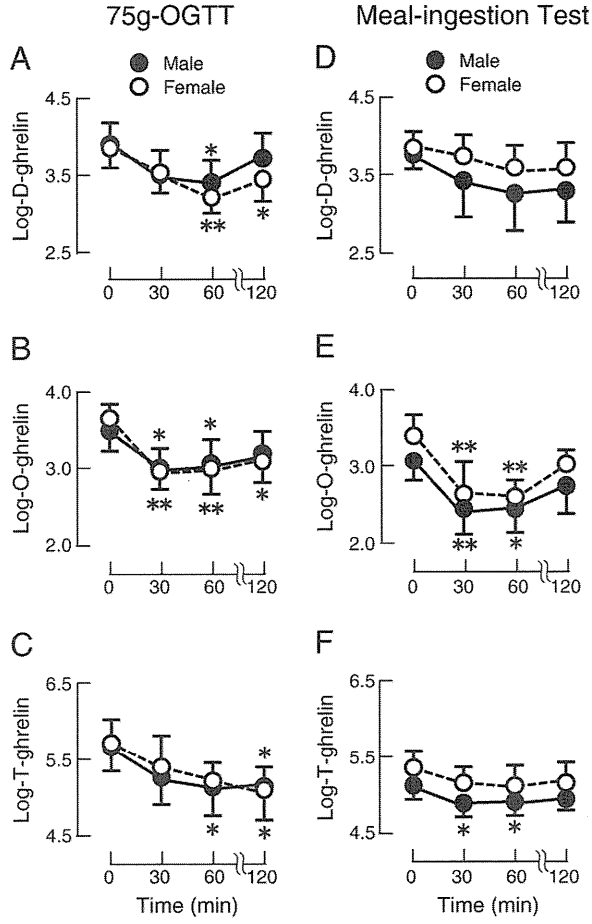

Fig. 2. Dynamics of the levels of D-ghrelin (A, D), O-ghrelin (B, E) and T-ghrelin (C, F) in plasma of normal human subjects upon the 75-g OGTT (A, B, and C) or the meal-ingestion test (296 kcal) (D, E, and F). Values are represented as the means ± S.D. (n = 10) (5 male, black circles; 5 female, white circles). * $p<0.05$, ** $p<0.01$ *vs.* values at 0 min.

3.4. Dynamics of D-, O-, and T-ghrelin levels in plasma induced by the meal-ingestion test

Plasma D-ghrelin levels in both male and female subjects were the same at all time points throughout the meal-ingestion test (Fig. 2D). The plasma O-ghrelin level in male subjects was significantly lower at 30 min ($p<0.01$ *vs.* basal level) and 60 min ($p<0.05$ *vs.* basal level), then returned to the basal level at 120 min. (Fig. 2E). The plasma O-ghrelin level in female subjects was also significantly lower at 30 min ($p<0.01$ *vs.* basal level) and 60 min ($p<0.01$ *vs.* basal level) after the meal ingestion, then returned to the basal level by 120 min after ingestion (Fig. 2E). The plasma T-ghrelin level in males was decreased significantly at 30 and 60 min ($p<0.05$ *vs.* basal level) after the test-meal ingestion, and then returned to the basal level at 120 min (Fig. 2F). In female subjects, the T-ghrelin levels did not change at all after the test-meal ingestion. (Fig. 2F). After the test-meal ingestion, the proportions of plasma O-ghrelin to T-ghrelin (O/T-ratio) in both sexes were significantly lower at 30 min ($p<0.01$ for males and $p<0.05$ for females) and 60 min ($p<0.05$ for both males and females) in comparison to the basal levels at 0 min (Fig. 3D). The proportions of plasma D-ghrelin to T-ghrelin (D/T-ratio) in both sexes were the same at all time points throughout the meal-ingestion test (Fig. 3E). The proportions of plasma D-ghrelin to O-ghrelin (D/O-ratio) increased significantly in both male ($p<0.05$) and female ($p<0.01$) subjects at 30 min in comparison to the respective basal D/O-ratio at 0 min (Fig. 3F).

3.5. Partial correlations between the levels of D-, O-, and T-ghrelin in plasma and the values for BMI or HOMA-IR

A significant inverse correlation was noted between the values for BMI and the respective levels of D-ghrelin ($r=-0.251$, $p<0.01$), O-ghrelin ($r=-0.398$, $p<0.01$) and T-ghrelin ($r=-0.238$, $p<0.01$) in all subjects (male + female) after adjustment for age (Table 3). Plasma levels of D-ghrelin correlated inversely ($r=-0.335$) and significantly ($p<0.01$) with HOMA-IR in male subjects. A significant inverse correlation was also noted between the values for HOMA-IR and the respective levels of O-ghrelin ($r=-0.361$, $p<0.01$) or T-ghrelin ($r=-0.235$, $p<0.05$) in plasma of male subjects. Whereas no significant correlations were noted in the female subjects between the BMI values and the respective levels of D-ghrelin ($r=0.022$, $p=0.891$), O-ghrelin ($r=0.183$, $p=0.252$), and T-ghrelin ($r=0.046$, $p=0.776$).

3.6. Partial correlations between the levels of serum lipids and the respective levels of D-, O-, and T-ghrelin in plasmann

Positive correlations were noted between the serum levels of HDL-cholesterol and the concentrations of plasma D-ghrelin ($r=0.224$, $p<0.05$), O-ghrelin ($r=0.434$, $p<0.01$) and T-ghrelin ($r=0.264$, $p<0.05$) in all subjects (male + female) (Table 4). In female subjects, a comparatively stronger correlation was noted between the level of HDL-cholesterol and that of D-ghrelin ($r=0.342$, $p<0.05$) than in male subjects ($r=0.108$). A significant positive correlation was also noted between the levels of HDL-cholesterol and O-ghrelin in both male ($r=0.316$, $p<0.05$) and female ($r=0.332$, $p<0.05$) subjects. The plasma levels of D-ghrelin exhibited a positive correlation ($r=0.315$, $p<0.05$) with the levels of total cholesterol only in female subjects. However, no significant correlations were noted in the male subjects ($r=-0.198$, $p=0.163$). An inverse correlation ($r=-0.242$, $p<0.05$) was noted between the levels of serum triglycerides and the levels of O-ghrelin in all subjects. In addition, a positive correlation ($r=0.223$, $p<0.001$) was noted between the levels of serum free fatty acids and the levels of T-ghrelin in all subjects.

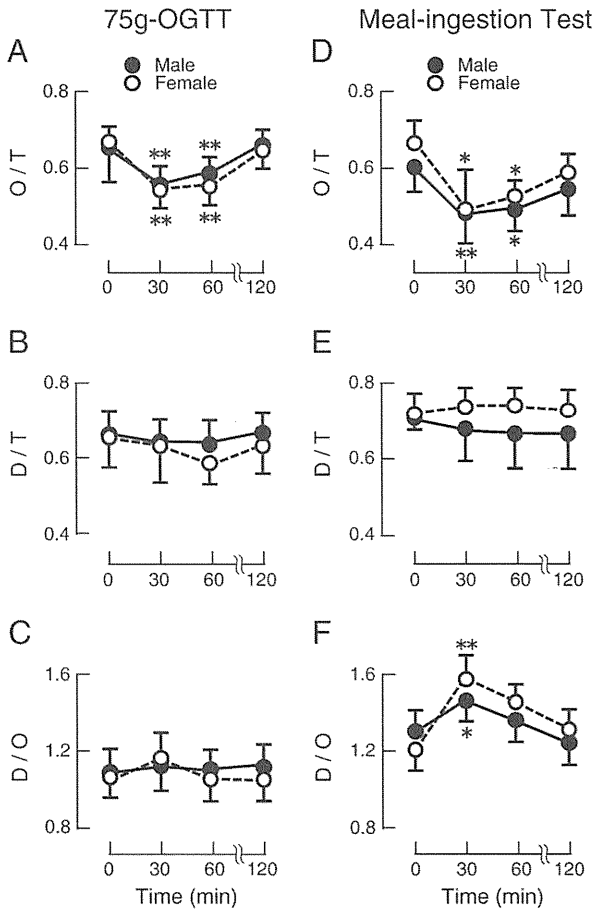

Fig. 3. The kinetics for the ratio of the plasma O-ghrelin level to that of T-ghrelin (O/T-ratio), the D-ghrelin level to that of T-ghrelin (D/T-ratio) and the D-ghrelin level to that of O-ghrelin (D/O-ratio) after the 75 g-OGTT (A–C) or after the test-meal ingestion (D–F) in normal subjects (5 male and 5 female; same subjects represented in Fig. 1). Values are represented as the means ± S.D. (male, black circles; female, white circles). * $p<0.05$, ** $p<0.01$ *vs.* values at 0 min (before ingestion).

Table 3
Partial correlations between plasma concentrations of ghrelins and the body mass index or the index for insulin resistance in healthy subjects.

		BMI (kg/m^2)	HOMA-IR [a]
D-ghrelin [a]	Male (n = 64)	- 0.177	- 0.335**
	Female (n = 47)	- 0.230	0.022
	Total (n = 111)	- 0.251**	- 0.205*
O-ghrelin [a]	Male (n = 64)	- 0.248*	- 0.361**
	Female (n = 47)	- 0.281	0.183
	Total (n = 111)	- 0.398**	- 0.181
T-ghrelin [a]	Male (n = 64)	- 0.153	- 0.235*
	Female (n = 47)	- 0.257	0.046
	Total (n = 111)	- 0.238**	- 0.155

Each value represents the coefficient for the partial correlation between the indicated data. Correlations between the values of BMI or HOMA-IR against the respective values of D, O- or T-ghrelin were calculated after adjusting for age. D-ghrelin, O-ghrelin or T-ghrelin represents the immunoreactivity for *n*-decanoyl-, *n*-octanoyl- or total ghrelin, respectively.[a], Log-transformed values were used for the statistical analysis (Pearson's partial correlation). * $p<0.05$, ** $p<0.01$, *vs.* partial correlation coefficient.

3.7. Correlations between the values for BMI and the proportions of D, O- and T-ghrelin (the D/T-ratio, O/T-ratio or D/O-ratio)

The proportion of plasma D-ghrelin to T-ghrelin (D/T-ratio), which was calculated as follows: [log (D-ghrelin)]/[log (T-ghrelin)], exhibited a positive correlation with the values for BMI in all subjects (male + female) ($r=0.215$, $p<0.05$) as well as in female subjects ($r=0.381$, $p<0.01$) (Fig. 4A), whereas not in the male subjects ($r=0.046$, $p=0.726$). A positive correlation was also noted between the values for BMI and the proportion of plasma D-ghrelin to O-ghrelin (D/O-ratio), calculated as: [log (D-ghrelin)/log (O-ghrelin)], in all (male + female) subjects ($r=0.324$, $p<0.01$) and in female subjects ($r=0.386$, $p<0.01$) (Fig. 4C). In contrast, no significant correlations were noted between the values for BMI and the

Table 4
Partial correlations between plasma concentrations of ghrelins (D-, O-, T-ghrelin) and serum lipid parameters after adjustment for age.

		TG [a]	HDL-Chol	T-Chol	FFA
D-ghrelin [a]	Male (n = 64)	- 0.230	0.108	- 0.178	0.009
	Female (n = 47)	0.102	0.342*	0.315*	0.151
	Total (n = 111)	- 0.151	0.224*	0.002	0.089
O-ghrelin [a]	Male (n = 64)	- 0.207	0.316*	0.059	0.100
	Female (n = 47)	- 0.033	0.332*	0.233	0.030
	Total (n = 111)	- 0.242*	0.434**	0.158	0.141
T-ghrelin [a]	Male (n = 64)	- 0.091	0.081	0.023	0.179
	Female (n = 47)	0.088	0.253	0.214	0.194
	Total (n = 111)	- 0.113	0.264**	0.127	0.223**

Each value represents the coefficient for the correlation between the indicated parameters. D-ghrelin, O-ghrelin or T-ghrelin represents the immunoreactivity for *n*-decanoyl-, *n*-octanoyl- or *n*-total ghrelin, respectively. [a], Log-transformed values were used to for the statistical analyses (Pearson's partial correlation). TG, triglyceride; HDL-chol., cholesterol in high-density lipoprotein; T-Chol, total cholesterol; FFA, free fatty acids. * $p<0.05$, ** $p<0.01$, *vs.* partial correlation coefficient.

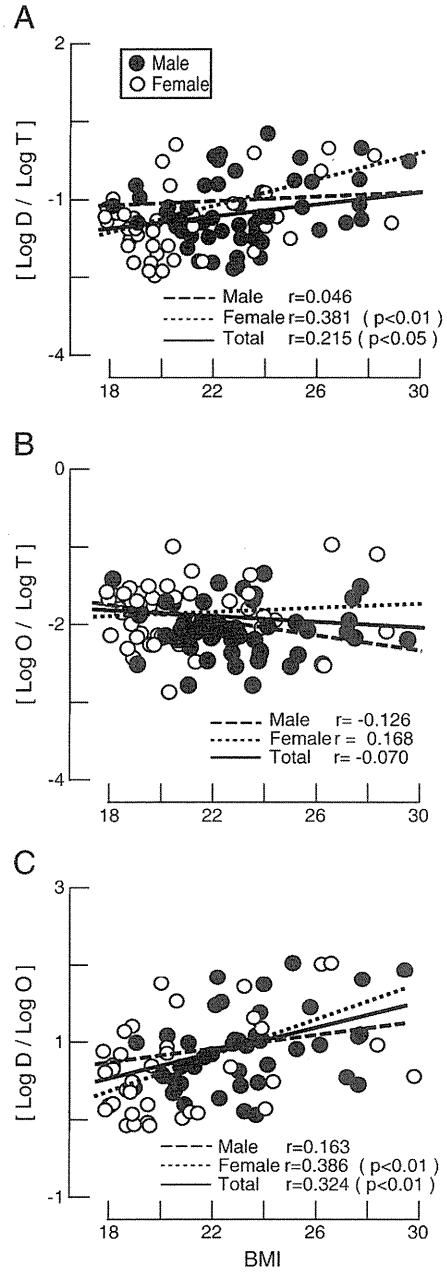

Fig. 4. Correlations between the values for BMI and the plasma D-ghrelin to T-ghrelin ratio ([log (D-ghrelin)]/[log (T-ghrelin)]) (A), between BMI and the plasma O-ghrelin to T-ghrelin ratio ([log (O-ghrelin)]/[log (T-ghrelin)]) (B), and between BMI and the plasma D-ghrelin to O-ghrelin ratio ([log (D-ghrelin)]/[log (O-ghrelin)]) (C) (n = 111, 64 male and 47 female). Closed circles represent data from male subjects and open circles represent data from female subjects. Solid lines represent simple regression lines drawn from all (male + female) subjects, while the broken lines or dotted lines represent simple regression lines from male or female subjects, respectively.

proportion of plasma O-ghrelin to T-ghrelin (O/T-ratio), which was calculated as: [log (O-ghrelin)/log (T-ghrelin)] (Fig. 4B).

3.8. Relative levels of plasma D-ghrelin and O-ghrelin in healthy human subjects measured by C18-RP-HPLC plus T-ghrelin RIA

To confirm the results of the D-ghrelin RIA concerning the proportion of plasma D-ghrelin to that of O-ghrelin or T-ghrelin (all ghrelin molecules with intact C-termini), we utilized another assay system with C18-RP-HPLC combined with T-ghrelin RIA (Fig. 5, A_1– A_3) [19,27]. We analyzed the proportion in the levels of D-ghrelin or O-ghrelin to that of T-ghrelin in the plasma of three healthy human subjects with different sex, age or BMI (Cases 1–3 in Fig. 5). The HPLC profiles of Case 1 (male, 12 y.o., BMI 17.6), Case 2 (female, 28 y.o., BMI 21.0) and Case 3 (male, 45 y.o., BMI 27.5) corresponded to Fig. $5A_1$, A_2 or A_3, respectively. Based on the respective elution profile of the synthetic *des*-acyl ghrelin (I), O-ghrelin (II) or D-ghrelin (III), peaks b_1, b_2 and b_3 corresponded to those of *des*-acyl ghrelin. Peaks c_1, c_2 and c_3 corresponded to those of O-ghrelin. The peaks d_1, d_2 and d_3 corresponded to those of D-ghrelin. Peaks a_1, a_2 and a_3 (Fig. $5A_1$–A_3) were estimated to be those corresponding to the C-terminal fragments of ghrelin without acylation. The estimated elution point for C10:1-ghrelin was between peak c_{1-3} and peaks d_{1-3} in Fig. 5 A_1– A_3 [27]. The proportions of the plasma O-ghrelin level to the level of total ghrelin in Case 1, 2 or 3, each of which was calculated as [the level of peak c]/[the level of (peak a + peak b + peak c + peak d)], was 0.131, 0.167 and 0.109, respectively. The proportion of the plasma D-ghrelin level to that of total ghrelin in Case 1, 2 or 3, each of which was calculated as [the level of peak d]/[the levels of (peak a + peak b + peak c + peak d)], were 0.136, 0.138 and 0.180, respectively. The proportions of plasma D-ghrelin level to that of O-ghrelin in Case 1, 2 or 3, each of which was calculated as [the level of peak d]/[the level of peak c], were 1.043, 0.828 and 1.66, respectively. To confirm the results from C18-RP-HPLC plus ghrelin T-RIA, the contents of O-ghrelin or D-ghrelin within the same C18-RP-HPLC fractions (the same samples measured by T-ghrelin RIA) were also measured by using O-ghrelin RIA or D-ghrelin RIA. The levels of O-ghrelin in Case 1, 2 or 3 (Fig. $5B_1$–B_3) within the corresponding fractions (peak e_1, peak e_2 or peak e_3), which were measured by O-ghrelin RIA, were 16.46, 16.28 and 15.32 fmol/fraction (plasma 1.0 ml equivalent), respectively. The levels of D-ghrelin in Case 1, 2 or 3 (Fig. $5C_1$–C_3) within the corresponding fractions (peak g_1, peak g_2 or peak g_3), which were measured by D-ghrelin RIA, were 13.88, 10.82 and 18.30 fmol/fraction (plasma 1.0 ml equivalent), respectively.

4. Discussion

The short-term dynamics of plasma D-ghrelin level following the glucose-ingestion were similar to those in O-ghrelin or T-ghrelin (Fig. 2). The levels of D-ghrelin in plasma, like those of O- or T-ghrelin, declined after consumption of glucose, and recovered thereafter. However, the extent of the decrease in the levels of plasma D-ghrelin upon ingestion of the test-meal (296 kcal; almost the same caloric intake as was used during the 75 g-OGTT) was far smaller than that of O-ghrelin, and even smaller than the extent of decrease in the D-ghrelin level during the glucose-ingestion test under the same caloric conditions (75 g-glucose = 300 kcal). One can suppose that this discrepancy in the dynamics of D-ghrelin between the glucose- and the meal-ingestion test is due to the different potentials of the respective dietary components (carbohydrates, proteins or fats) on the secretion process and/or the degradation process and the resulting half-life of the ghrelins [21,22,31–33]. Comparatively longer half-life of D-ghrelin in plasma than that of O-ghrelin might also account for the discrepancy between the dynamics of plasma D- and O-ghrelin upon the test meal-ingestion [19].

Plasma concentrations of D-ghrelin in our normal subjects, like those of O- or T-ghrelin, were positively correlated with the levels of HDL cholesterol (Table 4). Furthermore, stronger correlations were noted between the levels of ghrelins (D-, O-, and T-ghrelin) and the levels of HDL cholesterol than the correlations of plasma ghrelin levels with other lipid parameters. This might be due to the differences in the type of binding to the different serum lipids, since ghrelin interacts via its N-and C-terminal with HDL-cholesterol, whereas the presence of the N-terminal acyl-group is necessary for ghrelin's interactions with triglycerides and other lipid components [34]. It is possible that the circulating lipoproteins and D-ghrelin interact with each other, similar to O-ghrelin and *des*-acyl ghrelin, modulating the

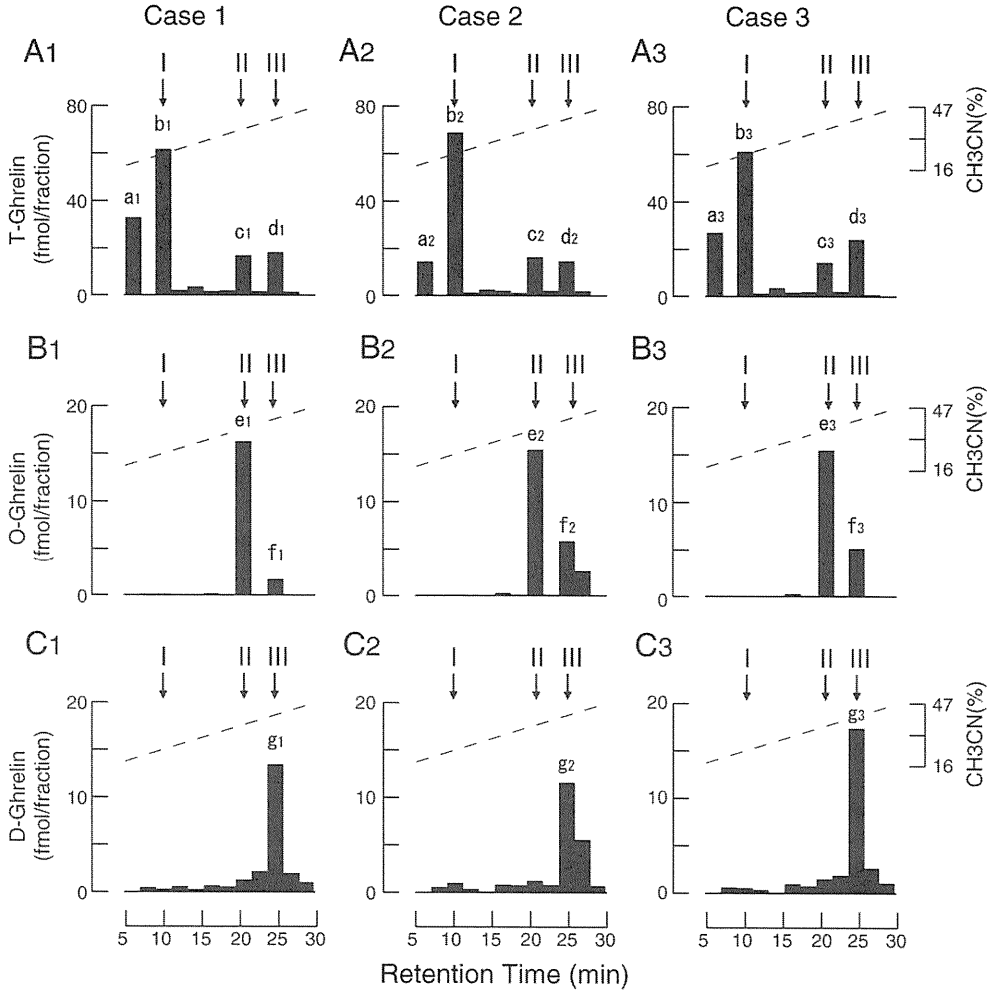

Fig. 5. RP-HPLC profiles for the immunoreactivity of total ghrelin (T-Ghrelin; A_1, A_2 and A_3), *n*-octanoyl ghrelin (O-Ghrelin; B_1, B_2 and B_3) and *n*-decanoyl ghrelin (D-Ghrelin; C_1, C_2 and C_3) in plasma from normal human subjects (Cases 1–3). The RP-HPLC profiles shown in Fig. 5A_1, B_1 and C_1 were those from Case 1 (12 y.o., male, BMI = 17.6), those in Fig. 5A_2, B_2 and C_2 were from Case 2 (28 y.o., female, BMI = 21.0) and those in Fig. 5A_3, B_3 and C_3 were from Case 3 (45 y.o., male, BMI = 27.5). Arrows I, II and III indicate the elution point of synthetic *des*-acyl ghrelin, synthetic *n*-octanoyl ghrelin (O-ghrelin) and synthetic *n*-decanoyl ghrelin (D-ghrelin), respectively. Based on the retention times (elution points) of these synthetic peptides (I, II and III), peaks b_1, b_2 and b_3 corresponded to those of *des*-acyl ghrelin (Fig. 5 A_1–A_3), while peaks c_1, c_2 and c_3 (Fig. 5A_1–A_3) as well as peaks e_1, e_2 and e_3 (Fig. 5 B_1–B_3) corresponded to O-ghrelin. Peaks d_1, d_2 and d_3 (Fig. 5 A_1–A_3), peaks f_1, f_2 and f_3 (Fig. 5 B_1–B_3), as well as peaks g_1, g_2 and g_3 (Fig. 5 C_1–C_3) all corresponded to D-ghrelin. Peaks a_1, a_2 and a_3 (Fig. 5A_1–A_3) were estimated to be those correspond to C-terminal fragments of ghrelin without acylation. The estimated elution point for C10:1-ghrelin was between peak c_{1-3} and peaks d_{1-3} in Fig. 5 A_1–A_3 [27].

levels of these components in plasma, thereby influencing their biological effects [34–36].

As for fasting plasma levels of D-ghrelin, inverse correlations were observed between the value for HOMA-IR and the levels of D-ghrelin in male subjects (Table 3). Inverse correlations were also noted in male subjects between the value for HOMA-IR and the levels of O- or T-ghrelin, which was in line with the previous reports on the long-term regulation of plasma ghrelin levels [37–39]. However, in female subjects, no significant correlations were observed between the value for HOMA-IR and the respective levels of D-, O- or T-ghrelin. This might be due to the menstrual cycle in our female subjects, since the menstrual cyclicity exerts a profound influence on glucose homeostasis [40,41].

Inverse correlations were also detected between the value for BMI and the respective levels of D-ghrelin, O- and T-ghrelin in normal (male + female) subjects (Table 3). These findings might imply that the long-term regulation of the plasma levels of D-ghrelin was almost the same as that of O-ghrelin or T-ghrelin under similar chronic nutritional conditions. However, we were able to find a concomitant increase in the proportion of the D-ghrelin level to that of T-ghrelin with increased values for BMI, whereas no significant changes were noted in the proportion of the plasma O-ghrelin level to that of T-ghrelin in the same setting (Fig. 4). These findings indicate that the long-term regulation of the plasma D-ghrelin level was, to some degree, different from that of O-ghrelin. The precise mechanism underlying the discrepancy between the long-term regulations of the levels of D- and O-ghrelin is unknown. However, one may speculate that intrinsic factors, the activity of which are influenced by chronic nutritional conditions, may differentially modulate the production and/or the degradation of the different forms of ghrelin (e.g. D- and O-ghrelin) to cause this discrepancy.

Our present study has several limitations. First, we used a ghrelin N-terminal RIA (O-ghrelin RIA) to measure the levels of O-ghrelin in plasma. However, the antibody utilized for the O-ghrelin RIA exhibited 10–20% cross-reactivity to D-ghrelin [10]. Consequently, the levels of O-ghrelin measured by this RIA reflected the net concentrations of O-ghrelin plus 10–20% of D-ghrelin (Fig. 5B_{1-3}). Similarly, the levels of D-ghrelin in plasma measured by our D-ghrelin RIA reflected the net concentration of D-ghrelin plus 15–20% of *n*-decenoyl ghrelin (C10:1-ghrelin) together with a small proportion of acyl-ghrelin delivertives (i.e., the N-terminal fragment of D-ghrelin) (Fig. 5C_{1-3}). Consequently, the precise levels of intact D-ghrelin in plasma may be 70–80% that measured by D-ghrelin RIA (Table 1). Nonetheless, we think that our data accurately reflect the

dynamics of D-ghrelin in human plasma, since our D-ghrelin RIA was only modestly affected by the levels of O-ghrelin [19], and since the plasma levels of acyl-ghrelins except O- or D-ghrelin seemed to be smaller than that of D- or O-ghrelin, at least in humans (Fig. 5 A_{1-3}). Previous reports by our group have confirmed that the ratio of D-ghrelin to O-ghrelin in human stomachs, the main source of circulating ghrelins, was 1:3, the ratio of which was almost equal to that of the bullfrog [15] but was significantly higher than the ratio observed in mice (1:20 in fed condition and 1:3 in a 48 h-fasted condition) [19] or rats (approximately 1/5 those in mice; our unpublished data). Comparatively higher ratios of D-ghrelin to O-ghrelin in the stomachs of humans together with our preliminary finding that D-ghrelin has relatively longer half-life in plasma [19] might, in part, support our present findings that plasma D-ghrelin levels in human subjects exceeds that of O-ghrelin under such conditions as "after a meal" or "with an increased BMI". In this study, we enrolled only "Oriental" subjects (Japanese) and did not include "Caucasian" or "African" subjects for the measurement of plasma ghrelin levels. Since the production- and the acylation-process of ghrelin peptides are highly influenced by the ingested nutrients [22,27] and since these components are very different between the Western- and Eastern diets, it may be necessary to consider possible ethnic differences in the regulation of plasma ghrelin levels. A comparatively small number of subjects (5 males and 5 females) was used for the 75 g-OGTT and the meal-ingestion test (Figs. 2, 3), possibly decreasing the accuracy of our findings of the short-term regulation of D-ghrelin levels in plasma. The amount of calories consumed during our single test-meal ingestion (296 kcal) was approximately half of that consumed during each meal (breakfast, lunch or supper), so that the kinetics of plasma D-ghrelin upon our meal-ingestion test might differ from those seen upon daily food consumption. In this respect, however, we have also confirmed a significantly smaller declining-trend of the plasma D-ghrelin level to that of O-ghrelin upon the food-intake study (600 Kcal/each meal, 3-times a day, $n=5$) (data not shown here). We therefore believe that this observation supports our hypothesis that the kinetics of plasma D-ghrelin levels after meal-ingestion differs from that of O-ghrelin. Further studies on the short-term dynamics of D-ghrelin with a larger number of subjects should be done to reveal the fine-tuned kinetics of the D-ghrelin levels in human plasma.

The clinical implications for the measurement of D-ghrelin in human plasma still needs to be established, however, it is possible to think that the concentration of plasma D-ghrelin, as well as the ratio of the peptide to the levels of O-ghrelin, reflects the metabolic conditions of the subjects examined, since the lipid and carbohydrate metabolism regulate the amount (or production rate) of the substrates for the ghrelin-acyl-modification (i.e., *n*-octanoyl or *n*-decanoyl derivative) [27,42–46]. In this respect, our present findings on the increase of plasma D-ghrelin levels in T2DM patients, unlike those of O-ghrelin, is interesting. One may speculate that catabolic process of lipids, the rate of which is increased in T2-DM as well as in fasting [19], interferes the acylation/de-acylation process of ghrelin peptides [33,47,48]. Local metabolic conditions in the stomachs of T2-DM patients might also modify the production rate of D- or O-ghrelin within ghrelin-producing cells through the acylation-process of the ghrelins. It is also possible to think that an increase in the proportion of D-ghrelin to that of T- or O-ghrelin in plasma, along with the increase in BMI, may modulate the net hormonal activity of ghrelins through the D-ghrelin's relatively longer half-life in plasma [19], because the peptide has bioactivity comparable to that of O-ghrelin [6,11,19]. Consequently, one may speculate that the higher proportion of D-ghrelin in the fasting plasma of human subjects with increased BMI, together with D-ghrelin's relatively larger proportion (within acyl-forms of ghrelin) even after a meal accounts for the abnormality in appetite which is often seen in these subjects [49].

Recently, differential roles for D-ghrelin and O-ghrelin were proposed in appetite and metabolic regulation by using a warm water teleost (fish), *Oreochromis mossambicus* [50,51]. The main acyl-form of ghrelin in these fish has been reported to be D-ghrelin. It might be possible that the D-ghrelin exerts different biological functions from that of O-ghrelin in humans and other mammalian species.

In conclusion, we have herein provided the first measurement of the dynamics of D-ghrelin in human plasma by using our D-ghrelin-specific RIA. A considerable amount of D-ghrelin was detected in human plasma, and the regulation on the levels of D-ghrelin was slightly but significantly different from that of O-ghrelin after a meal as well as under the metabolic conditions reflected by the value for BMI. Further investigations on the dynamics and the biological effects of D-ghrelin should be done to increase our understanding of the biological regulation of ghrelin (together with its acyl- or non-acyl isoforms) and their relationship to the value for BMI, appetite and energy metabolism.

Disclosure statement

The authors of this manuscript have nothing to disclose.

Acknowledgments

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ORIGINAL ARTICLE

Body mass index and stroke incidence in a Japanese community: the Hisayama study

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Although obesity is one of the major risk factors for coronary heart disease, its role in the development of stroke remains controversial. A total of 2421 residents, aged 40–79 years of a Japanese community were followed up prospectively for 12 years. The subjects were divided into four groups according to body mass index (BMI) levels (<21.0, 21.0–22.9, 23.0–24.9 and ⩾25.0 kg m^{-2}). During the follow-up, 107 ischemic and 51 hemorrhagic strokes occurred. The age-adjusted incidence of ischemic stroke for men significantly increased with increasing BMI levels (*P* for trend=0.005). This association remained substantially unchanged even after adjustment for other risk factors: namely, systolic blood pressure, electrocardiogram abnormalities, diabetes, total cholesterol, high-density lipoprotein-cholesterol, triglycerides, smoking habits, alcohol intake and regular exercise (*P* for trend<0.001). Compared with that of the BMI levels of <21.0 kg m^{-2}, the multivariate-adjusted risk of ischemic stroke was significant even in the BMI levels of 23.0–24.9 kg m^{-2} (multivariate-adjusted hazard ratio (HR)=3.12; 95% confidence interval (CI), 1.24–7.87; *P*=0.02) as well as in the BMI levels of ⩾25 kg m^{-2} (multivariate-adjusted HR=5.59; 95% CI, 2.09–14.91; *P*<0.001). In stratified analyses, the risk of ischemic stroke for men synergistically increased in subjects having both obesity and diabetes or a smoking habit. We found no significant associations between BMI levels and ischemic stroke in women and between BMI levels and hemorrhagic stroke in either sex. In conclusion, our findings suggest that overweight and obesity are independent risk factors for ischemic stroke in Japanese men.
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Keywords: body mass index; incidence; obesity; prospective study; stroke

INTRODUCTION

Stroke is a leading cause of death[1] and permanent disability in middle-aged and elderly people in Japan[2–4] as well as in other developed countries.[5] In Japan, the prevalence of obesity has increased rapidly along with the westernization of lifestyle,[6] although it remains considerably lower than that in Western populations.[7] Increased body mass index (BMI) is tightly related to an increased risk of coronary heart disease,[8] but its association with stroke is less well recognized because of conflicting results reported in the literature. Some cohort studies have found a positive association between BMI and the risk of stroke,[8–14] whereas others have shown no apparent association[15–18] or have even reported an inverse or a U-shaped association.[19–22] In Japan, no prospective study has provided incidence data on this issue nor observed a positive association between BMI and the risk of stroke until now.[21,22] Based on its pathogenesis, stroke is divided into several clinical subtypes, and the effects of BMI on stroke are considered to be different among these subtypes.[8,19] In addition, obesity is an important risk factor for hypertension, diabetes mellitus and dyslipidemia, which are known as major risk factors for stroke,[23,24] and therefore, whether obesity itself independently increases the risk of stroke remains controversial.

In the present article, we investigated the association between BMI and the occurrence of stroke by its subtype based on records of a prospective study of a general Japanese population, taking other known risk factors into account.

METHODS

Study population

In 1988, a screening survey for the present study was performed in the town of Hisayama, a suburb of the Fukuoka metropolitan area in southern Japan. Of a total of 3227 residents aged 40–79 years on the town registry, 2587 consented to participate in the examination (participation rate, 80.2%) and underwent a comprehensive assessment. After excluding 82 subjects who had already had breakfast, 10 who were on insulin therapy and 15 due to complaints of nausea or general fatigue during the ingestion of glucose, a total of 2480 subjects completed a 75-g oral glucose tolerance test. From a total of 2490 subjects including 10 on insulin therapy, 68 who had a history of stroke or coronary heart disease based on questionnaires and medical records, and one who died

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before follow-up was started were excluded. The remaining 2421 (1037 men and 1384 women) were enrolled in this study.

Baseline data collection

At baseline, body height and weight were measured in light clothing without shoes, and BMI (kg m ^{2}) was calculated as an indicator of obesity. Information on antihypertensive treatment, smoking habits, alcohol intake and regular exercise were obtained with the use of a standard questionnaire. Subjects who reported smoking at least one cigarette per day were defined as current smokers, and subjects who reported consuming alcohol at least once a month were regarded as current drinkers. Subjects engaging in sports at least three times a week during their leisure time made up a regular exercise group. Sitting systolic and diastolic blood pressures were measured three times after a rest of at least 5 min by a standard mercury sphygmomanometer with a standard cuff. The average of three measurements was used for data analysis. Hypertension was defined as a systolic blood pressure $\geqslant$140 mm Hg, a diastolic blood pressure $\geqslant$90 mm Hg or current use of antihypertensive agents. ECG abnormalities were defined as left ventricular hypertrophy (Minnesota code 3–1), ST depression (4–1, 2 and 3) and/or atrial fibrillation (8–3). Blood samples were drawn after an overnight fast of at least 12 h. Fasting and 2-h post-load plasma glucose levels were determined by the glucose-oxidase method. Diabetes mellitus was defined as fasting plasma glucose $\geqslant$7.0 mmol l $^{-1}$, 2-hour post-load plasma glucose $\geqslant$11.1 mmol l $^{-1}$, or current use of insulin or oral medication for diabetes. Total cholesterol, high-density lipoprotein-cholesterol and triglyceride levels were all determined enzymatically.

Follow-up survey

The subjects were followed up prospectively for 12 years from December 1988 to November 2000 by repeated health examinations and by a daily monitoring system established by the study team and local physicians or members of the Health and Welfare Office of the town. Health status was checked once yearly by mail or telephone for any subjects who did not undergo a regular examination or who moved out of town. Study-team physicians performed physical and neurological examinations on all subjects who developed stroke and collected the relevant clinical information, including that on the disease course. During the follow-up period, only one subject was lost to follow-up, and 339 subjects died; among those who died, autopsy was performed on 253 (74.6%).

Stroke, defined as sudden onset of a non-convulsive and focal neurological deficit persisting for >24 h, was classified as ischemic stroke, cerebral hemorrhage, subarachnoid hemorrhage or undetermined type.[25] The clinical diagnosis of stroke and its subtypes was determined on the basis of a detailed history, neurological examination and ancillary laboratory examinations. In this paper, we focused on ischemic and hemorrhagic stroke (cerebral hemorrhage and subarachnoid hemorrhage). During the follow-up period, we identified 107 cases of first-ever ischemic stroke (47 men and 60 women) and 51 cases of first-ever hemorrhagic stroke (21 men and 30 women), consisting of 34 cases of cerebral hemorrhage and 17 cases of subarachnoid hemorrhage. All of the stroke cases were examined by computed tomography and/or magnetic resonance imaging.

Statistical analysis

All statistical analyses were performed with the SAS program package Ver 9.2 (SAS Institute Inc, Cary, NC, USA). All tests were two-sided, and values of $P<0.05$ were considered statistically significant in all analyses. The subjects were divided into four groups according to BMI levels (<21.0, 21.0–22.9, 23.0–24.9 and $\geqslant$25.0 kg m ^{2}). Because of the skewed distribution of serum triglycerides, this value was log-transformed for statistical analysis. The age-adjusted mean values of risk factors were calculated by the analysis of covariance method, and their trends across BMI levels were tested by multiple regression analysis. Frequencies of risk factors were adjusted for age by the direct method and were examined for trends by the Cochran–Mantel–Haenszel test. The incidence of stroke was calculated by the person-year method and was adjusted for the age distribution of the study population by the direct method. Differences in the incidence of stroke among BMI levels were tested by the Cox proportional hazards model. The age- and multivariate-adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs) were also calculated using the Cox proportional hazards model. The multivariate adjustment was made for age, systolic blood pressure, ECG abnormalities, diabetes, total cholesterol, high-density lipoprotein-cholesterol, triglycerides, smoking habits, drinking status and regular exercise. To assess whether synergistic effect was observed between obesity and each of other risk factors, we added a multiplicative interaction term to the relevant Cox model.

Ethical considerations

The study protocol was approved by the Human Ethics Review Committee of Kyushu University Graduate School of Medical Sciences, and a written informed consent was obtained from the study participants.

RESULTS

Characteristics of the subjects

The age-adjusted mean values or frequencies of risk factors by BMI levels at baseline are shown by sex (Table 1). Mean age significantly decreased with rising BMI levels for men, but such an association was not observed for women. In both sexes, the mean values of systolic and diastolic blood pressures, total cholesterol and triglycerides, and the frequencies of hypertension, antihypertensive drug use and diabetes increased significantly, whereas the mean high-density lipoprotein-cholesterol levels decreased significantly with increasing BMI levels. The frequency of smoking habits for men and that of ECG abnormalities for women decreased significantly with increasing BMI levels. No dose-response relationships were observed between BMI levels and the frequencies of alcohol intake or regular exercise for both sexes.

Impact of BMI on stroke

As shown in Figure 1, the age-adjusted incidence of ischemic stroke for men increased with increasing BMI levels: the difference was significant between the BMI level of $<21.0\,kg\,m^{-2}$ and that of $\geqslant25.0\,kg\,m^{-2}$ (age-adjusted HR=3.32; 95% CI, 1.43–7.72; P=0.005; Table 2). This association remained substantially unchanged even after adjustment for other risk factors (Table 2). The multivariate-adjusted risk of ischemic stroke was significant even in the subjects with BMI levels of 23.0–24.9 $kg\,m^{-2}$ (multivariate-adjusted HR=3.12; 95% CI, 1.24–7.87; P=0.02) as well as in those with BMI levels of $\geqslant25\,kg\,m^{-2}$ (multivariate-adjusted HR=5.59; 95% CI, 2.09–14.91; $P<0.001$). We found no significant associations between BMI levels and the incidence of ischemic stroke in women and between BMI levels and the incidence of hemorrhagic stroke in either sex (Figure1 and Table 2).

Combined effects of obesity and other risk factors

Because hypertension, diabetes and smoking habits are major risk factors for ischemic stroke and are concurrently associated with obesity, we examined the combined effects of obesity and these risk factors on the development of ischemic stroke for men after adjustment for the above-mentioned confounding factors, except for the factor which was used for the grouping. As shown in Table 3, multivariate-adjusted HRs of ischemic stroke were significantly higher in the group of obese subjects irrespective of the presence or absence of hypertension. On the other hand, the risk of ischemic stroke synergistically increased in obese subjects with diabetes compared with non-obese subjects without diabetes (multivariate-adjusted HR=7.91; 95% CI, 3.08–20.28; $P<0.001$), whereas such an increased risk was not observed in non-obese subjects with diabetes or in obese subjects without diabetes. A similar synergistic pattern was observed for the coexistence of obesity and smoking habits (multivariate-adjusted HR=3.62; 95% CI, 1.39–9.43; P=0.008). A significant interaction between obesity and diabetes was revealed in the risk of ischemic

Table 1 Age-adjusted baseline characteristics according to body mass index level by sex, the Hisayama Study, 1988

	Body mass index, kg m^{-2}				
	<21	*21–22.9*	*23–24.9*	*⩾25*	*P for trend*
Men					
No at risk	283	255	247	252	—
Age (years)	60.5 (0.6)	56.8 (0.6)	56.2 (0.7)	54.4 (0.6)	<0.001
SBP (mm Hg)	127.1 (1.1)	132.2 (1.2)	135.5 (1.2)	141.2 (1.2)	<0.001
DBP (mm Hg)	75.5 (0.6)	79.3 (0.7)	82.0 (0.7)	86.3 (0.7)	<0.001
Hypertension (%)	32.6	37.4	46.9	58.7	<0.001
Antihypertensive drug (%)	9.0	10.8	15.1	23.6	<0.001
ECG abnormalities (%)[a]	20.6	20.9	19.3	18.7	0.28
Diabetes (%)	10.1	16.9	13.6	20.9	0.005
Total cholesterol (mmol l^{-1})	4.95 (0.06)	5.05 (0.07)	5.13 (0.07)	5.31 (0.07)	<0.001
HDL cholesterol (mmol l^{-1})	1.37 (0.02)	1.30 (0.02)	1.22 (0.02)	1.14 (0.02)	<0.001
Triglycerides (mmol l^{-1})	1.01 (0.94–1.07)	1.28 (1.20–1.37)	1.46 (1.36–1.56)	1.77 (1.65–1.90)	<0.001
Smoking (%)	68.7	47.0	44.5	36.6	<0.001
Drinking (%)	59.7	65.9	64.7	58.6	0.63
Regular exercise (%)[b]	12.8	11.1	11.0	10.9	0.34
Women					
No at risk	380	347	318	339	
Age (years)	59.1 (0.5)	57.0 (0.6)	57.0 (0.6)	57.6 (0.6)	0.052
SBP (mm Hg)	125.2 (1.0)	130.2 (1.0)	131.1 (1.1)	136.9 (1.0)	<0.001
DBP (mm Hg)	71.8 (0.5)	74.4 (0.6)	77.0 (0.6)	80.0 (0.6)	<0.001
Hypertension (%)	24.2	30.9	34.5	50.3	<0.001
Antihypertensive drug (%)	7.5	14.1	14.2	21.5	<0.001
ECG abnormalities (%)[a]	15.2	14.3	9.4	11.6	0.03
Diabetes (%)	7.5	6.8	8.8	16.6	<0.001
Total cholesterol (mmol l^{-1})	5.31 (0.05)	5.54 (0.06)	5.74 (0.06)	5.66 (0.06)	<0.001
HDL cholesterol (mmol l^{-1})	1.44 (0.01)	1.35 (0.02)	1.30 (0.02)	1.26 (0.02)	<0.001
Triglycerides (mmol l^{-1})	0.88 (0.84–0.92)	1.04 (0.99–1.09)	1.15 (1.10–1.21)	1.24 (1.18–1.30)	<0.001
Smoking (%)	8.1	3.5	6.6	8.1	0.72
Drinking (%)	9.5	10.3	5.1	10.7	0.79
Regular exercise (%)[b]	9.4	10.5	8.9	6.3	0.11

Abbreviations: DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure.
Data are shown as the means (standard error) or a percentage. Geometric mean values and 95% confidence intervals of serum triglycerides are shown attributable to the skewed distribution. Mean age was not age-adjusted.
[a]Minnesota codes: 3–1, 4–1, 2, 3 or 8–3
[b]Engaging in sports or other forms of exertion regularly ⩾three times a week during leisure time.

stroke (P=0.01), whereas the interactions between obesity and hypertension and between obesity and smoking habits were not significant.

DISCUSSION

In this prospective study of a community-dwelling Japanese population, we demonstrated that higher BMI was a significant risk factor for the development of ischemic stroke in men. This association remained unchanged even after adjustment for other risk factors. In addition, the combinations of obesity plus diabetes or obesity plus a smoking habit synergistically increased the risk of ischemic stroke. However, there was no significant association between BMI levels and the risk of hemorrhagic stroke in either sex.

Some cohort studies have shown an increased risk of total stroke or ischemic stroke with elevating BMI,[8–14] which is in accord with the findings of the risk of ischemic stroke in our male subjects. On the other hand, other studies have found no association,[15–18] an inverse or a U-shaped association.[19–22] One possible explanation for this difference in findings may be that stroke was not evaluated by its subtype in all these studies, as the effect of obesity is different among stroke subtypes. Another explanation may be that most of these studies used mortality data as an endpoint. Our previous study showed that lower BMI was a significant risk factor for death after total stroke and ischemic stroke.[26] Epidemiological studies of body weight and mortality are affected by methodological problems, such as failure to control the harmful biological effects of smoking and subclinical diseases resulting in weight loss. Thus, the association of BMI with stroke mortality should be interpreted with caution.

In the literature, the associations between BMI levels and the risk of hemorrhagic stroke have been inconsistent, with some studies showing a positive association,[8,11,14] and others showing no, a negative or a U-shaped, association.[9,12,13,16,19,21,22] In the present study, we did not find a clear association between BMI levels and hemorrhagic stroke in men or women. The lack of a clear consensus on this association may be partly due to the low number of cases of hemorrhagic stroke in most of the studies, including our present work, or differences in ethnicities, study populations or study methods. Future studies will be needed to resolve this issue.

A number of studies have reported that the association between BMI and total or ischemic stroke was attenuated or eliminated after adjustment for potential mediators, such as hypertension, diabetes

and dyslipidemia.[9,10,12–14,19,22] In our study, however, the association between BMI and ischemic stroke was not attenuated even after adjusting for these risk factors. This finding indicates an independent effect of overweight and obesity on the development of ischemic stroke. A similar independent association has been observed in other studies of stroke.[10,12,14] These findings, together with our present results, suggest a link between overweight/obesity and ischemic stroke independent of established risk factors. Some investigators have proposed that the increase in prothrombotic factors[27–29] and inflammatory markers,[30–33] and the enhancement of insulin resistance and metabolic syndrome[34] observed among overweight and obese individuals may have a role in their increased risk of ischemic stroke.

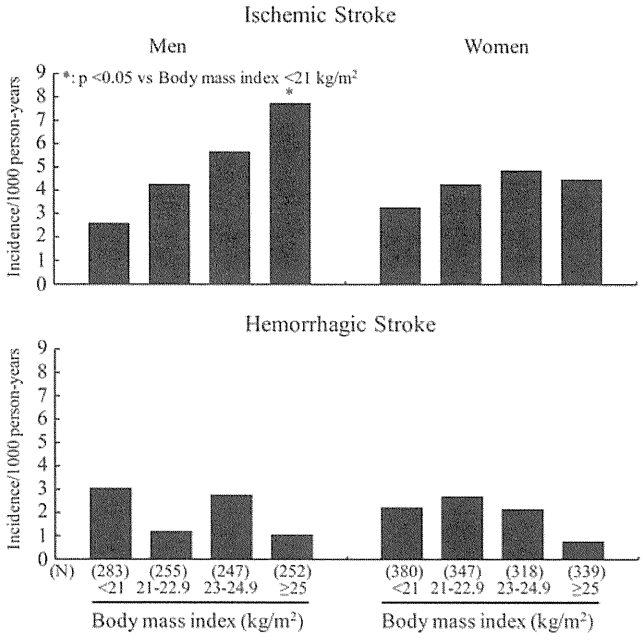

Figure 1 Age-adjusted incidence of stroke by body mass index levels during 12-year follow-up, the Hisayama Study, 1988–2000.

Our stratified analysis showed an extremely increased risk of ischemic stroke in men who have both obesity and diabetes or smoking habits. Although the mechanisms underlying this phenomenon are not clearly understood, a possible explanation can be proposed. Because diabetes and smoking are strong risk factors for the progression of systemic arteriosclerosis, it is reasonable to consider that subjects with these risk factors already have vascular injuries to some extent. Obesity-related disorders, such as inflammation, insulin resistance and metabolic syndrome, may accelerate the progression of preexisting vascular injuries, resulting in an increased risk of ischemic stroke. However, in the present study we did not find that obesity enhanced the effect of hypertension on stroke risk. Although the precise reason for this is not known, the popularization of antihypertensive treatment in our study population might have weakened the synergistic effects of these factors.

In our female subjects, we did not observe a significant association between BMI and the risk of ischemic stroke. Several cohort studies have also examined the effects of BMI on the risk of ischemic stroke in women,[9,13–15,21,22] but the findings were inconsistent, with some studies showing a positive association,[9,13,14] and others showing no association[15,21] like our study. Further studies will be needed to clarify the true association between BMI and stroke in women.

Table 2 Adjusted hazard ratio for stroke incidence according to body mass index level by sex, the Hisayama Study, 1988–2000

Body mass index, kgm^{-2}	*Person year*	*No. of events*	*Age-adjusted HR*	*95% CI*	*Multivariate-adjusted HR*[a]	*95% CI*
Men						
Ischemic stroke						
<21.0	2907	9	1.00	Referent	1.00	Referent
21.0–22.9	2736	10	1.70	0.69–4.20	2.34	0.91–6.00
23.0–24.9	2692	12	2.09	0.88–5.00	3.12	1.24–7.87
25.0≥	2790	16	3.32	1.43–7.73	5.59	2.09–14.91
P for trend			0.005		<0.001	
Hemorrhagic stroke						
<21.0	2907	9	1.00	Referent	1.00	Referent
21.0–22.9	2736	3	0.44	0.12–1.63	0.38	0.10–1.50
23.0–24.9	2692	6	0.89	0.31–2.55	0.90	0.28–2.87
25.0≥	2790	3	0.47	0.12–1.80	0.36	0.08–1.57
P for trend			0.41		0.31	
Women						
Ischemic stroke						
<21.0	4214	15	1.00	Referent	1.00	Referent
21.0–22.9	3935	15	1.41	0.69–2.90	1.37	0.65–2.88
23.0–24.9	3652	15	1.51	0.73–3.10	1.56	0.71–3.43
25.0≥	3794	15	1.41	0.69–2.91	1.27	0.58–2.80
P for trend			0.32		0.55	
Hemorrhagic stroke						
<21.0	4214	10	1.00	Referent	1.00	Referent
21.0–22.9	3935	10	1.26	0.52–3.04	1.32	0.52–3.35
23.0–24.9	3652	7	0.94	0.36–2.49	1.13	0.39–3.25
25.0≥	3794	3	0.38	0.10–1.39	0.35	0.09–1.35
P for trend			0.16		0.16	

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.
[a]Multivariate adjustment was made for age, systolic blood pressure, ECG abnormalities, diabetes, total and high-density lipoprotein-cholesterols, triglycerides, smoking, drinking and regular exercise.