

## 2.2. Measurements

The plasma glucose level was measured by glucose oxidase method using the Hitachi Automatic Clinical Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured by two-site radioimmunoassay (Dainabot, Tokyo, Japan) reported previously [18]. Serum total cholesterol, HDL cholesterol, and triglycerides levels were measured as reported previously [18].

We used the index as parameters of insulin secretion: insulinogenic index, which represents the capacity of insulin secretion [14,15]. To measure insulin sensitivity, we used the index: ISI composite, which correlates with systemic insulin sensitivity [16]. The formulas of each index are as follows.

Insulinogenic index =  $[30\text{-min serum insulin} - \text{FIRI (pmol/l)}] / [30\text{-min plasma glucose} - \text{FPG (mmol/l)}]$  [14,15].

ISI composite =  $10000 / [\text{FPG (mg/dl)} \times \text{FIRI } (\mu\text{U/ml)} \times \text{mean OGTT plasma glucose (mg/dl)} \times \text{mean OGTT serum insulin } (\mu\text{U/ml})]^{0.5}$  [16].

## 2.3. Statistical analysis

All analyses were performed using STATVIEW 5 system (StatView, Berkeley, CA, USA). The age, BMI, plasma glucose, and insulin level during 75g OGTT, HbA<sub>1c</sub>, triglycerides, total cholesterol, HDL-cholesterol, insulinogenic index, and ISI composite

were compared among the NGT, IFG, and DM/IFH groups by general analysis of variance (ANOVA). Insulin levels during OGTT also were compared among the NGT, IFG, DM/IFH, and isolated IGT groups by ANOVA. For comparison between any two groups among these three or four groups, unpaired Student's *t*-test was performed as post-hoc analysis. Probability (*P*) values of less than 0.05 were considered statistically significant. Data are expressed as mean  $\pm$  standard error (S.E.).

## 3. Results

The clinical and metabolic characteristics of subjects with NGT, IFG, DM/IFH, and isolated IGT are shown in Table 1. The average age and BMI are  $50.6 \pm 0.5$  and  $23.3 \pm 0.1$  with S.E., respectively. The mean ages in the IFG and isolated IGT groups are significantly higher than those in the NGT group. The mean BMI in the DM/IFH group is significantly greater than that in the NGT group. The values in the IFG and isolated IGT groups are not greater than those in the NGT group. The isolated IGT group has significantly higher triglycerides levels than the NGT group, the isolated IGT and DM/IFH groups have significantly lower HDL cholesterol levels than the NGT group, but there is no significant difference in total cholesterol levels among the NGT, IFG, DM/IFH, and isolated IGT groups.

Table 1  
Demographic/metabolic characteristics of NGT, IFG, and DM/IFH groups

	NGT	IFG	DM/IFH	Isolated IGT
<i>N</i>	319	76	42	130
Age (years)	48.1 $\pm$ 0.7	54.4 $\pm$ 1.0 <sup>†</sup>	50.4 $\pm$ 1.4	54.5 $\pm$ 0.8 <sup>§</sup>
BMI (kg/m <sup>2</sup> )	23.0 $\pm$ 0.2	23.5 $\pm$ 0.3	24.5 $\pm$ 0.6 <sup>†</sup>	23.6 $\pm$ 0.2
FPG (mmol/l)	5.3 $\pm$ 0.0	6.4 $\pm$ 0.0 <sup>§</sup>	7.6 $\pm$ 0.1 <sup>§</sup>	5.6 $\pm$ 0.0 <sup>†</sup>
2-h PG (mmol/l)	5.9 $\pm$ 0.1	6.2 $\pm$ 0.1	8.6 $\pm$ 0.3 <sup>§</sup>	9.0 $\pm$ 0.1 <sup>§</sup>
Fasting insulin (pmol/l)	30 $\pm$ 1	32 $\pm$ 2	39 $\pm$ 3 <sup>*</sup>	34 $\pm$ 2 <sup>†</sup>
2-h insulin (pmol/l)	176 $\pm$ 8	169 $\pm$ 14	162 $\pm$ 18	234 $\pm$ 11 <sup>†</sup>
HbA <sub>1c</sub> (%)	5.1 $\pm$ 0.0	5.5 $\pm$ 0.1 <sup>§</sup>	6.1 $\pm$ 0.1 <sup>§</sup>	5.5 $\pm$ 0.1 <sup>§</sup>
Triglycerides (mmol/l)	1.32 $\pm$ 0.06	1.28 $\pm$ 0.13	1.80 $\pm$ 0.19	2.28 $\pm$ 0.46 <sup>†</sup>
Total cholesterol (mmol/l)	5.24 $\pm$ 0.06	5.27 $\pm$ 0.17	5.29 $\pm$ 0.16	5.41 $\pm$ 0.08
HDL-cholesterol (mmol/l)	1.52 $\pm$ 0.03	1.58 $\pm$ 0.06	1.33 $\pm$ 0.06 <sup>*</sup>	1.35 $\pm$ 0.04 <sup>†</sup>

Data are mean  $\pm$  S.E.

\* *P* < 0.05.

† *P* < 0.01.

‡ *P* < 0.001.

§ *P* < 0.001 vs. NGT.

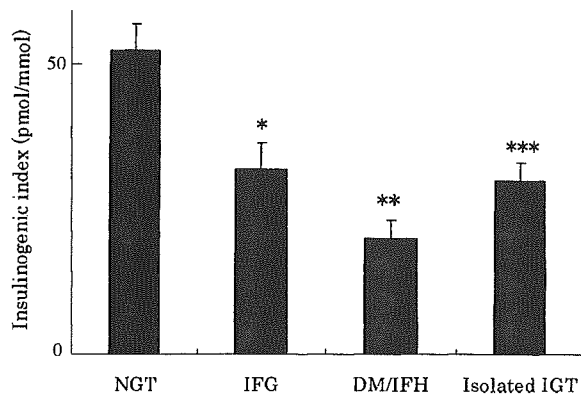


Fig. 1. Insulinogenic index in subjects with NGT, IFG, DM/IFH, and isolated IGT. Data are mean  $\pm$  S.E. \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. NGT.

Comparisons of insulinogenic index are shown in Fig. 1. The insulinogenic index in the IFG and DM/IFH groups are lower than that in the NGT group. Insulinogenic index in the isolated IGT group is also significantly lower than that in the NGT group, and degrees of absolute-valued decline from NGT are similar for IFG and isolated IGT groups (29.8 pmol/mmol versus 31.9 pmol/mmol). Comparisons of ISI composite among the groups are shown in Fig. 2. ISI composite in the IFG and DM/IFH groups are lower than that in the NGT group. Though ISI composite in isolated IGT group is also significantly lower than that in NGT group, decline of ISI composite is stronger in IFG than in isolated IGT by 0.55 of difference (2.10 versus 1.55).

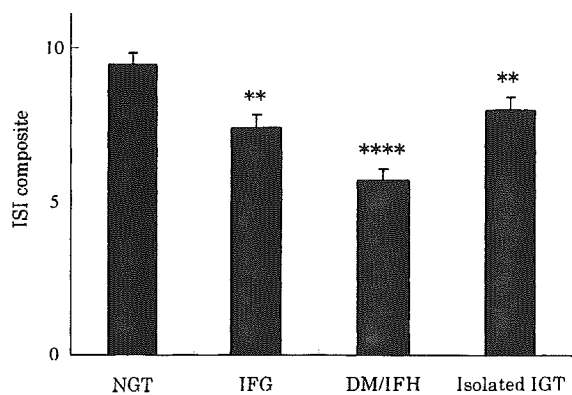


Fig. 2. ISI composite in subjects with NGT, IFG, DM/IFH, and isolated IGT. Data are mean  $\pm$  S.E. \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$  vs. NGT.

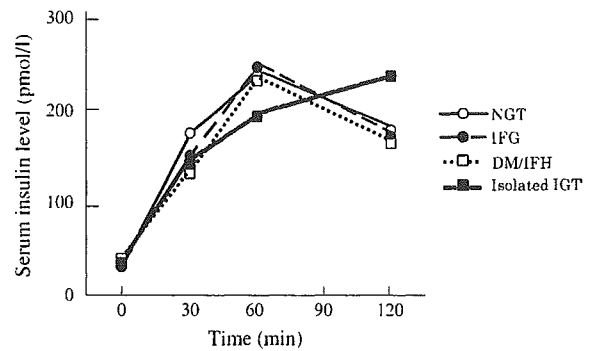


Fig. 3. Serum insulin levels during 75g OGTT in subjects with NGT, IFG, DM/IFH, and isolated IGT. 0 min:  $P < 0.05$  for DM/IFH vs. IFG;  $P < 0.01$  for DM/IFH vs. NGT. 30 min:  $P < 0.05$  for DM/IFH vs. NGT;  $P < 0.01$  for isolated IGT vs. NGT. 60 min:  $P < 0.05$  for isolated IGT vs. IFG;  $P < 0.01$  for isolated IGT vs. NGT. 120 min:  $P < 0.01$  for isolated IGT vs. DM/IFH;  $P < 0.001$  for isolated IGT vs. IFG;  $P < 0.0001$  for isolated IGT vs. NGT.

Analysis of the pattern of insulin secretion is shown in Fig. 3. An insulin peak is seen at 60 min in NGT, IFG, and DM/IFH subjects. There are no significant differences in peak values at 60 min among these three groups. On the other hand, isolated IGT shows no insulin peak during 120 min, and the insulin level at 60 min is significantly lower than that in NGT and IFG. Insulin levels at 2 h in NGT, IFG, and DM/IFH are significantly lower than that in isolated IGT.

#### 4. Discussion

In the present study, we have clarified the characteristics of IFG and DM/IFH regarding insulin secretion and insulin sensitivity in Japanese males by analysis of 75g OGTT results. Insulinogenic index was significantly lower in IFG and DM/IFH than in NGT. This result shows that insulin secretion in IFG and DM/IFH are distinctly impaired in this population. IFG showed a significantly lower value of ISI composite than NGT, indicating impaired insulin sensitivity. Clearly, loss of both insulin secretion and insulin sensitivity plays an important role in decreased glucose tolerance in these IFG and DM/IFH subjects.

Tripathy et al. reported in the Botnia study that the major determinant of IFG is the defective insulin action with compensative elevation of insulin secretion, rather than impaired  $\beta$ -cell function as in Caucasian subjects [19]. The present study shows

that Japanese IFG subjects (and also DM/IFH) exhibit both impaired insulin secretion and decreased insulin sensitivity. This discordance could be due to ethnic differences in  $\beta$ -cell capacity, as several reports suggest that insulin secretion is lower in Japanese than in other ethnic populations [12,13]. We have reported lower insulin secretory capacity in Japanese NGT subjects [20] that would be unlikely to compensate for even a slight decline in insulin sensitivity.

Our previous study found that Japanese subjects with impaired glucose tolerance (IGT: FPG level  $<7.0$  mmol/l and 2-h PG level of 7.8–11.1 mmol/l) or isolated postchallenge hyperglycemia (IPH: FPG level  $<7.0$  mmol/l and 2-h PG level  $\geq 11.1$  mmol/l), having elevated the 2-h PG levels with mild or no elevation of FPG levels in contrast to IFG and DM/IFH subjects, exhibit distinctly impaired insulin secretion, especially early-phase insulin secretion represented by insulinogenic index, and only mild insulin resistance. This indicates that the reduced early-phase insulin secretion is the primary determinant in development from NGT via IGT to IPH [21]. In this study, the subjects with isolated IGT show lower insulin secretion as those with IFG do. In regarding the insulin sensitivity in isolated IGT, although they show statistically lower ISI composite than NGT as IFG subjects do, the absolute value is apparently higher in isolated IGT than in IFG. To make the matter clear, we compared DM/IFH: showed higher FPG level than IFG, with IPH: showed higher 2-h PG level than isolated IGT, in regard to insulinogenic index and ISI composite. Insulinogenic index in IPH subjects is significantly lower than that in DM/IFH subjects and ISI composite in IPH subjects is significantly higher than that in DM/IFH subjects, according to our analysis (data not shown). These also suggest that the primary determinant of 2-h PG level in the Japanese population is early-phase insulin secretion. On the other hand, the subjects with IFG and DM/IFH exhibited impaired insulin sensitivity in addition to the impaired early-phase insulin secretion.

Two-hour PG levels seem to be better controlled in IFG and DM/IFH subjects than in IGT and IPH subjects. Because IFG subjects exhibit an elevated FPG levels with normal 2-h PG levels and isolated IGT subjects exhibit elevated 2-h PG levels with normal FPG levels, we used isolated IGT subjects rather than IGT subjects for comparison of insulin

secretory patterns. The insulin secretion peak occurred 60 min after the glucose administration in IFG and DM/IFH subjects, as in NGT subjects, and there was no significant difference in peak insulin values among these three groups, indicating no impairment of insulin secretion at 60 min in IFG and DM/IFH. The 2-h PG level in subjects with IFG and DM/IFH may be better regulated, at least partly, by this secretory peak at 60 min. On the other hand, no insulin peak occurred during 120 min of 75g OGTT in isolated IGT, and the insulin level at 60 min was significantly lower in isolated IGT than in NGT and IFG. Similar results regarding patterns of insulin secretion in IFG and isolated IGT were reported by Hanefeld et al. [22]. It is clear that IFG and DM/IFH differ from isolated IGT and IPH in both the capacity and the pattern of insulin secretion.

It is generally accepted that IFG and IGT subjects are more obese than NGT subjects at least in Caucasian subjects, so obesity is considered as a possible risk factor to progress NGT to IFG and IGT, further to diabetes. In contrast, our results show no significant BMI differences among NGT, IFG, and isolated IGT groups (Table 1), they are similar to that in Japanese subjects in a population-based study: NGT 23.1 ( $\text{kg}/\text{m}^2$ ), IGT 24.4, and diabetes 24.3 [23] and several other studies performed in Japan or other Asian countries exhibited almost identical results [21,24–27]. As we recently have mentioned, this tendency is a common feature of Asian population [28]. Ethnic differences between Caucasian and Asian populations could explain the contradiction, as well as the difference of insulin secretory capacity aforementioned.

It is also well known that the family history is a risk factor for type 2 diabetes; however, it is unclear whether the genetic factor contribute to the incidence of IFG and IGT. Jensen et al. indicated that generic factor is one of the contributors to the decreased insulin secretion [29], and it is the possible analyses on family history and genetic factor reveal the factors responsible for the ethnic differences of glucose intolerance. Unfortunately we were unable to statistically elucidate this question and further investigation is needed to clear it.

While IGT and IPH are recognized as important risk factors for cardiovascular events [2], it is unclear whether or not IFG is a risk factor for atherosclerosis

[3,4]. Recently, an association between chronic low-grade inflammation of vascular endothelium and both atherosclerosis [30] and insulin resistance [31–33] have been documented. Other reports [21,22] and our present results indicate that IFG is associated with insulin resistance more than IGT is. The relationships among insulin resistance, chronic low-grade inflammation of vascular endothelium, and atherosclerosis require further elucidation.

In conclusion, both impaired insulin secretion and decreased insulin sensitivity may contribute to progression from NGT via IFG to DM/IFH in the development of type 2 diabetes in these subjects, in contrast to the development from NGT via IGT to IPH in which impaired early-phase insulin secretion is the primary contributor. In addition, the peak of insulin secretion at 60 min plays a potential role in the regulation of 2-h PG levels in these subjects. This profile of glucose intolerance subgroups clarifies the process of deterioration from NGT to type 2 diabetes as well as the mechanisms of regulation of FPG and 2-h PG levels.

### Acknowledgements

This study was supported in part by a Grant-in-Aid for Creative Basic Research (10NP0201) and for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan; a grant from “Research for the Future” Program from the Japan Society for the Promotion of Science (JSPS-RFTF97I00201); by Health Sciences Research Grants for Comprehensive Research on Aging and Health, Research on Health Technology Assessment, and Research on Human Genome, Tissue Engineering and Food Biotechnology from the Ministry of Health, Labour and Welfare. We thank Dr. O. Kikuchi, Use Techno Corporation, Takeda Chemical Industries Ltd. and Dainippon Pharmaceutical Co. Ltd. for their help.

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## Gastric inhibitory polypeptide modulates adiposity and fat oxidation under diminished insulin action

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Received 27 July 2005

Available online 9 August 2005

### Abstract

Gut hormone gastric inhibitory polypeptide (GIP) stimulates insulin secretion from pancreatic  $\beta$ -cells upon ingestion of nutrients. Inhibition of GIP signaling prevents the onset of obesity and consequent insulin resistance induced by high-fat diet. In this study, we investigated the role of GIP in accumulation of triglycerides into adipocytes and in fat oxidation peripherally using insulin receptor substrate (IRS)-1-deficient mice and revealed that *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice exhibited both reduced adiposity and ameliorated insulin resistance. Furthermore, increased gene expression of CD36 and UCP2 in liver, and increased expression and enzyme activity of 3-hydroxyacyl-CoA dehydrogenase in skeletal muscle of *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice might contribute to the lower respiratory quotient and the higher fat oxidation in light phase. These results suggest that GIP plays a crucial role in switching from fat oxidation to fat accumulation under the diminished insulin action as a potential target for secondary prevention of insulin resistance.

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**Keywords:** GIP; IRS-1; PPAR $\alpha$ ; Energy expenditure

Glucose homeostasis is tightly controlled in vivo by the levels of insulin secretion and peripheral insulin sensitivity. Insulin is released from pancreatic  $\beta$ -cells in proportion to increasing concentrations of glucose, lowering blood glucose levels by stimulating glucose uptake into skeletal muscle and adipose tissue and by decreasing glucose production by the liver. Type 2 diabetes is characterized by both decreased insulin sensitivity and impaired insulin secretion, which results in persistent hyperglycemia of sufficient magnitude to

produce diabetic complications such as retinopathy, nephropathy, and neuropathy [1,2].

Glucose is metabolized in pancreatic  $\beta$ -cells, resulting in an increase in the ATP/ADP ratio, closing the ATP-sensitive potassium ( $K_{ATP}$ ) channels, causing plasma membrane depolarization, influx of  $Ca^{2+}$ , and insulin exocytosis [3,4]. In addition to this direct effect of glucose, hormonal factor(s) called incretins transmit signals from the gut to the pancreatic  $\beta$ -cells and play an important role in glucose homeostasis by promoting insulin secretion immediately upon meal ingestion [5,6].

Gastric inhibitory polypeptide (GIP), an incretin also called glucose-dependent insulinotropic polypeptide, is released from duodenal endocrine K cells to stimulate

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insulin secretion from pancreatic  $\beta$ -cells upon absorption of nutrients [7]. Homozygous mice lacking GIP receptor ( $GIPR^{-/-}$ ) exhibit impaired initial insulin secretion and hyperglycemia after oral glucose loading [8].

The GIP receptor is expressed in various tissues including the pancreatic islets, adipose tissue, and brain [9]. In adipocytes, GIP stimulates cellular uptake of 2-deoxy-D-glucose and increases heparin-releasable lipoprotein lipase activity in the presence of insulin [10,11]. However,  $GIPR^{-/-}$  mice have similar body weight [8] and the physiological role of GIP is, therefore, little in fat uptake into adipocytes under the normal diet.

Insulin signaling is responsible for fat uptake both into adipocytes where fat is stored, and into liver and skeletal muscle where fat is utilized as energy. We have previously shown that high-fat feeding in mice with normal insulin sensitivity results in extreme visceral and subcutaneous fat deposition and insulin resistance, and inhibition of GIP signaling protects from such obesity and insulin resistance [11]. As plasma GIP levels are exaggerated by high-fat diet, increased GIP signaling promotes fat accumulation into adipocytes rather than fat oxidation in such a situation. Therefore, GIP signaling is a potential target for primary prevention of obesity and consequent insulin resistance induced by high-fat diet.

In the present study, we investigated the role of GIP in the accumulation of triglycerides into adipocytes under diminished insulin action using insulin receptor substrate (IRS)-1-deficient knockout mice [12] and revealed that GIP plays a crucial role in switching from fat oxidation to fat accumulation in the state with the diminished insulin action and that GIP signaling is a potential target for secondary prevention of insulin resistance.

## Materials and methods

**Animals.** The generation of  $GIPR^{-/-}$  and  $IRS-1^{-/-}$  mice (C57BL/6 background) has been described previously [8,12]. Heterozygous mutants for the IRS-1 gene ( $IRS-1^{+/-}$ ) were crossed with heterozygous mutants for the GIPR gene ( $GIPR^{+/-}$ ), and then  $IRS-1^{+/-} GIPR^{+/-}$  mice were further bred. Genotyping was done by PCR. Only male  $IRS-1^{-/-} GIPR^{+/+}$  and  $IRS-1^{-/-} GIPR^{-/-}$  mice were used in the experiments. Mice were bred on normal chow and maintained using standard procedures. Animal care and procedures were approved by the Animal Care Committee of Kyoto University Graduate School of Medicine.

**Glucose tolerance test.** Male mice (10–20 weeks of age) were fasted for more than 16 h before the study. Glucose (2 g/kg body weight) was

then loaded orally. Blood samples were taken at the indicated times. Blood glucose levels were measured by the enzyme-electrode method. Plasma insulin levels were measured using an ELISA kit (Shibayagi, Gunma, Japan).

**Insulin tolerance test.** Male mice (10–20 weeks of age) were fed ad lib and intraperitoneally challenged with insulin (0.3 U/kg body weight). Blood samples were taken at the indicated times and measured as described above.

**Measurement of leptin and free fatty acid levels.** Blood samples were collected in heparinized capillary tubes from the tail vein and centrifuged at 3000 rpm. Plasma leptin levels were measured using an ELISA kit (Morinaga, Yokohama, Japan). Plasma free fatty acid levels were determined using a NEFA kit (Wako, Osaka, Japan).

**Energy expenditure.** Oxygen consumption and respiratory quotient were measured by indirect calorimetric system every 13 min for 24 h in mice under fed condition, as described previously [11,13]. Briefly, air from the room was pumped through the chamber, and the expired gas was filtered through thin cotton, which was dried and subjected to a gas analyzer (Alco System model RL-600, Chiba, Japan). The  $O_2$  and  $CO_2$  concentrations were measured and the respiratory quotient was calculated using software for analysis (Alco System). Fat oxidation was calculated in a formula of  $1.67 \times (1 - \text{respiratory quotient}) \times \text{oxygen consumption}$  as described previously [13].

**Quantitative RT-PCR.** Total RNA was isolated from liver and muscle with Trizol reagent (Invitrogen), and gene expression levels were corrected for GAPDH mRNA level and were examined by real time quantitative RT-PCR using a PE Applied Biosystems prism model 7000 sequence detection instrument. Primers and a TaqMan probe for each gene were designed using PRIMER EXPRESS software package (Applied Biosystems, CA) from gene sequences obtained from GenBank (Table 1).

**Activity of 3-hydroxyacyl-CoA dehydrogenase.** The muscle was quickly removed, weighed, and immediately homogenized in 20 volumes of ice-cold 5.4 M glycerol, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.02% bovine serum albumin, and 20 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 600g for 10 min, and the supernatant was used for enzyme assays. All homogenate operations were performed at 0–4 °C. The assay system of 3-hydroxyacyl-CoA dehydrogenase (HD) activity contained the following: 95 mM pyrophosphate buffer (pH 7.3), 0.2 mM NADH, 4.7 mM acetoacetyl-CoA, and supernatant of homogenate. The supernatant was added, and the rate of decrease in extinction at 339 nm was measured for 2 min at 25 °C [14].

**Statistical analyses.** Data are expressed as means  $\pm$  SE. Statistical analyses were performed by unpaired *t* test.

## Results

### Development of $IRS-1^{-/-} GIPR^{-/-}$ mice

$IRS-1^{-/-} GIPR^{+/+}$  and  $IRS-1^{-/-} GIPR^{-/-}$  mice were generated by intercrossing  $IRS-1^{+/-} GIPR^{+/-}$  mice. No significant difference in body weight was observed

Table 1  
Primers and probes for real time quantitative RT-PCR

Target	Forward primer	Reverse primer	Probe
CD36	GAGCCCACAGTTCGATCA	CTGGGAGTTGGCGAGAAAAC	CTCCTCGTGCAGCAGAATCAAGGGA
UCP2	TCCCTTGCCACTTCACTTCTG	TCTCGTCTTGACCACATCA ACAG	TTCTGCACCACCGTCATCGCCTC
ACO/AOX	TCACAGCAGTGGGATTCCAA	TCTGCAGCATCATAACAGTGTCTC	CACGTTTACCCCGGCTGCACC
HD	AGACAGGTAAGGGCTGGTATCAGT	AACTCAGAAAGCCAGGGATCAG	TGACAAGCCACTGGGTGCGATCC
CPT-1	TGGCAAAGGTCTTATCAAGAAGTG	CCTCTCGGAACATTCTGTGCATC	CAGAACTTGCCTTTGTCCCGGAAATG
PPAR $\alpha$	CCCTGAACATCGAGTTCGAA	TTGCAGCTCCGATCACACTT	TCGCCGAAAGAAGCCCTTACAGCCTT
PPAR $\gamma$	TGTCGGTTTCAGAAGTGCCTT	GCTCGCAGATCAGCAGACTCT	CCCAACCTGATGGCATTGTGAGACA

between *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice fed a normal diet, although both mice exhibited about 34% less body weight than wild-type (*IRS-1*<sup>+/+</sup> *GIPR*<sup>+/+</sup>) mice (data not shown), consistent with the previous finding [12].

The fasting blood glucose did not show a significant difference between *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice. After oral glucose loading, *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice had similar blood glucose levels compared to *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice, except for the early phase (15 min after glucose loading) (Fig. 1A). *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice exhibited significantly decreased plasma insulin levels in the early phase of glucose loading (Fig. 1B). These results indicate that GIP also functions as an insulinotropic signal under diminished insulin action.

#### Disruption of GIP signaling ameliorates insulin resistance

Intraperitoneal insulin tolerance test was performed to determine the influence of GIP signaling on insulin

resistance. Expectedly, *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice showed marked insulin resistance, compared with *IRS-1*<sup>+/+</sup> *GIPR*<sup>+/+</sup> mice. The drop in blood glucose levels in *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice was more obvious (Fig. 1C), showing that disruption of GIP signaling partially ameliorates the insulin resistance in IRS-1 knockout mice. These results indicate that GIP has the antithetical effects in glucose tolerance.

#### Fat used as the preferred energy substrate

We reported previously that interruption of the GIP signaling pathway prevents high-fat diet-induced obesity [11]. Dissection of the fat mass of *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice revealed that disruption of GIP signaling elicited a significant reduction in both visceral and subcutaneous fat mass even under the normal diet (Fig. 2A). Consistently, plasma leptin levels, which correlate well with body fat mass, were significantly lower in *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice than in *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice ( $2.23 \pm 0.29$  ng/ml vs  $6.64 \pm 1.49$  ng/ml,  $P < 0.05$ ).

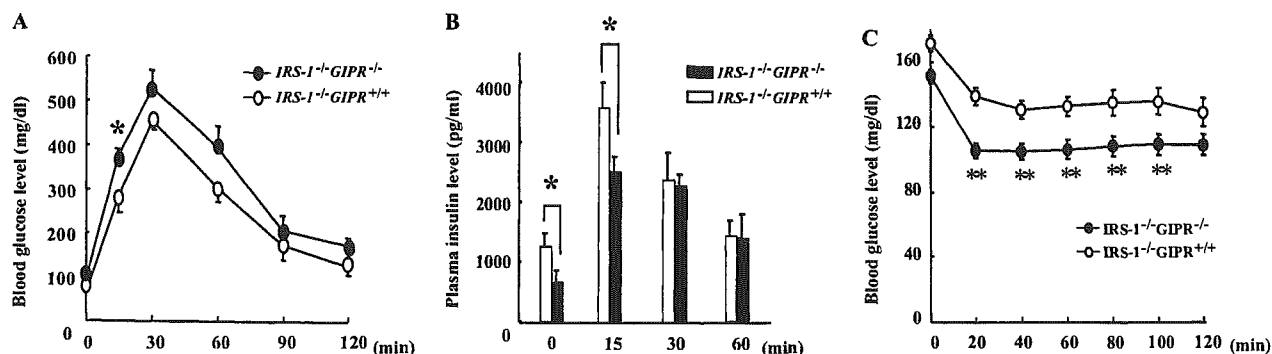


Fig. 1. Glucose tolerance test and insulin tolerance test. (A) Oral glucose tolerance test in age-matched *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> (open circle) and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice (filled circle). (B) Plasma insulin levels after oral glucose loading for age-matched *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> (open bars) and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice (filled bars). (C) Blood glucose levels during insulin tolerance test in *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> (open circle) and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> (filled circle) mice. Values are means  $\pm$  SE. \* $P < 0.05$  for *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice vs *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice. \*\* $P < 0.01$ .

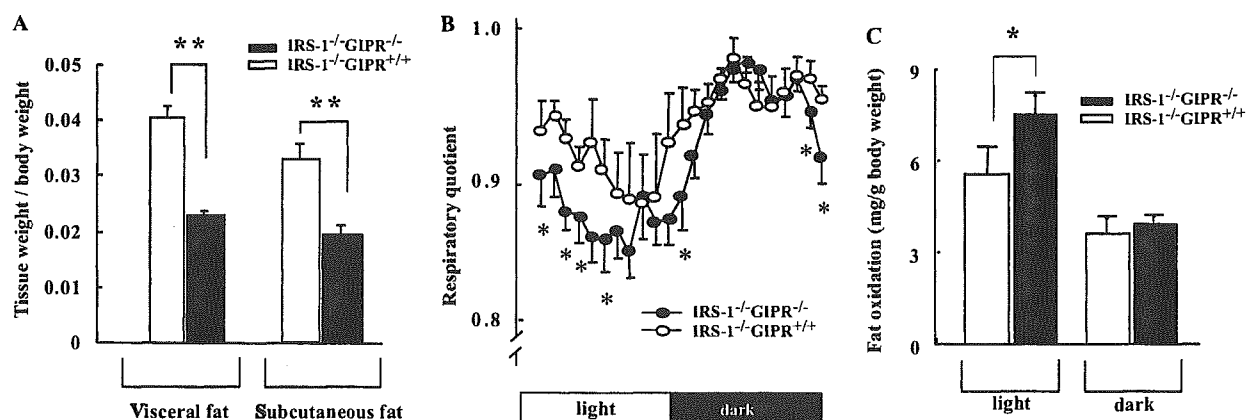


Fig. 2. Adiposity and energy expenditure. (A) Visceral and subcutaneous adipose tissues from *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> (open bar) and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> (filled bar) mice fed normal diet. Respiratory quotient (B) and calculated fat oxidation (C) under normal diet in *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> (open) and *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> (filled). \* $P < 0.05$ . \*\* $P < 0.01$ .

We then evaluated energy expenditure by measuring respiratory quotient and oxygen consumption by indirect calorimetry. Under the normal diet, respiratory quotients (Fig. 2B) of *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* and *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* mice were  $0.93 \pm 0.02$  and  $0.87 \pm 0.02$  in the light phase and  $0.98 \pm 0.02$  and  $0.98 \pm 0.01$  in the dark phase, respectively, and the calculated fat oxidation was increased in the light phase by disruption of the GIP signal (Fig. 2C).

#### Increased $\beta$ -oxidation in liver

We examined the expression levels of genes for fatty acid transport and  $\beta$ -oxidation in liver and found that the expression of CD36 and UCP2 was significantly increased in the liver of *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* mice compared to *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* mice (Figs. 3A and B). Gene expression of ACO/AOX, CPT-1, PPAR $\alpha$ , and PPAR $\gamma$  in liver did not differ significantly. We also found that the addition of fatty acids, which are natural ligands of PPAR $\alpha$  [15–18], increased the expression level of UCP2 using primary isolated hepatocytes (data not shown), consistent with the regulation of CD36 and UCP2 by PPAR $\alpha$  [19–21]. Although plasma free fatty acid levels were not significantly different between *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* and *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* mice ( $0.88 \pm 0.10$  mEq/L vs  $0.77 \pm 0.08$  mEq/L), an increase in the flow of fatty acids into hepatocytes may stimulate  $\beta$ -oxidation in liver through activation of PPAR $\alpha$ .

#### Increased $\beta$ -oxidation in skeletal muscle

We also examined the expression levels of genes for fatty acid transport and  $\beta$ -oxidation in skeletal muscle, and found that expression of HD was significantly increased in skeletal muscle of *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* compared to *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* mice (Fig. 3C). In contrast to the liver, expression of CD36 and UCP2 was not increased in skeletal muscle (data not shown).

Enzyme activities for skeletal muscle HD were also examined (Fig. 3D). HD activity in *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* mice ( $0.120 \pm 0.005$  U/mg) was significantly higher than in *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* mice ( $0.101 \pm 0.006$  U/mg).

#### Discussion

Impaired insulin secretion and insulin resistance are the major etiologic factors of type 2 diabetes. GIP receptor knockout mice showed mild glucose intolerance associated with impaired insulin secretion [8] and *IRS-1* knockout mice showed normal glucose tolerance associated with insulin resistance [12,22]. However, double knockout mice had increased blood glucose levels only in the early phase of oral glucose loading, indicating that GIP signaling plays another role in glucose tolerance.

Glucose and fat metabolism are closely related. In addition to its insulinotropic effect, GIP plays an impor-

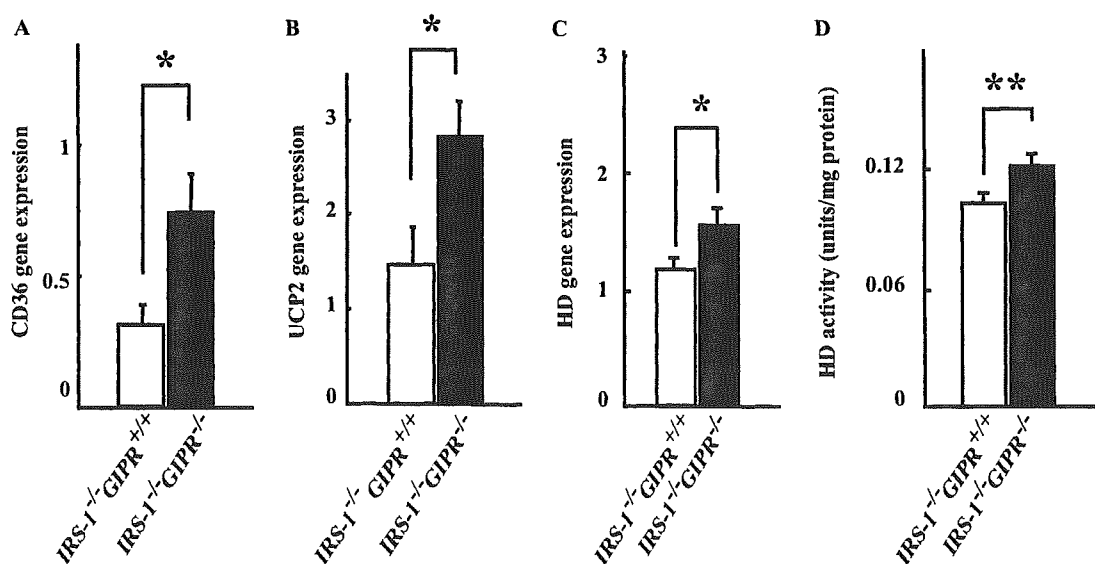


Fig. 3. Gene expression and enzyme activity Gene expression of CD36 (A) and UCP2 (B) in liver. *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* (open) and *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* (filled). Gene expression (C) and enzyme activity (D) of HD in skeletal muscle of *IRS-1<sup>-/-</sup> GIPR<sup>+/+</sup>* (open) and *IRS-1<sup>-/-</sup> GIPR<sup>-/-</sup>* (filled). \* $P < 0.05$ , \*\* $P < 0.01$ .

tant role in nutrient uptake into adipocytes. However, GIP signaling is little involved in fat accumulation into adipocytes under the normal insulin sensitivity, as *GIPR*<sup>-/-</sup> mice show similar adiposity to wild-type on control diet (Fig. 4A). In contrast, GIP signaling is required for effective accumulation of nutrients under high-fat diet, and inhibition of GIP signaling not only prevents obesity but also insulin resistance. Therefore, inhibition of GIP signaling primarily prevents the onset of obesity and consequent insulin resistance [11].

In this study, we have investigated the role of GIP signaling in triglyceride accumulation into adipocytes under normal dietary conditions under the diminished insulin action (Figs. 4B and C). Insulin receptor substrate (IRS) proteins are the major substrates of insulin receptor tyrosine kinase and mediate pleiotropic effects of insulin including regulation of cell metabolism, survival, growth, and differentiation [23]. Disruption of the IRS-1 gene results in insulin resistance [12,22]. It has been reported that insulin-induced phosphatidylinositol-3-kinase activity in the adipocytes of *IRS-1*<sup>-/-</sup> mice is about half that of wild-type mice [24]. *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice show significantly reduced adiposity compared to *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice, indicating that GIP plays an important role in fat accumulation into adipocytes under the diminished insulin action.

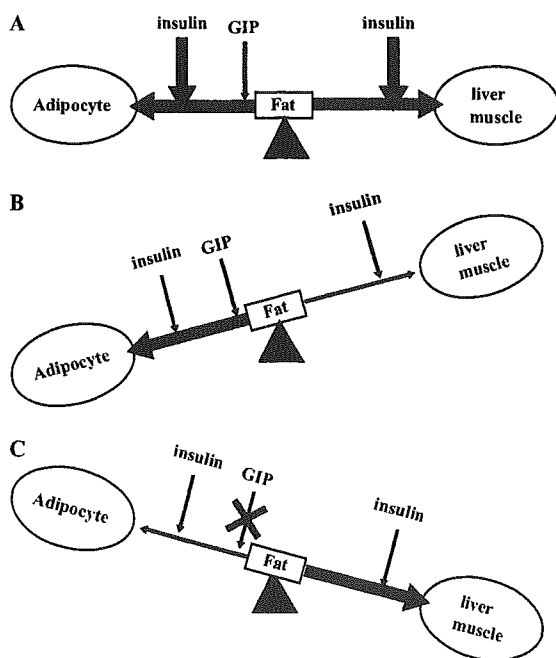


Fig. 4. Schematic models for GIP signaling, adiposity, and fat oxidation. (A) Insulin signaling is responsible for fat accumulation in adipocytes and fat oxidation in liver and skeletal muscle. The effect of GIP signaling is trivial. (B) Under the diminished insulin action, the effect of GIP signaling becomes relatively increased and fat moves toward accumulation in adipocytes. (C) Under the diminished insulin action and inhibition of GIP signaling, fat moves toward oxidation in liver and skeletal muscle.

Inhibition of fat oxidation is correlated with increased intracellular triglyceride content and decreased insulin action [25]. The *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice exhibited a lower respiratory quotient and a higher fat oxidation than *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice, especially in the light phase, with similar free fatty acid levels in serum, indicating that fat is not accumulating in adipocytes or in blood, but is being utilized as the preferred energy substrate (Figs. 4B and C). Liver and skeletal muscle where GIP receptors are not expressed are the major sites of whole body fat oxidation. CD36, also known as fatty acid translocase (FAT), is a multispecific integral membrane glycoprotein that has been identified as a facilitator of fatty acid uptake. UCP2 dissipates the proton electrochemical gradient by uncoupling fuel oxidation from ATP production. CD36 and UCP-2, both of which are transcriptionally regulated by PPAR $\alpha$  in liver, were increased in *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice compared to *IRS-1*<sup>-/-</sup> *GIPR*<sup>+/+</sup> mice. These results suggest that fatty acids activate PPAR $\alpha$  transcriptional activity and stimulate fat oxidation in the liver. In contrast to the liver, gene expression and enzyme activity of HD, the rate limiting enzyme of fat oxidation in peroxisomes, were increased in skeletal muscle. The molecular mechanism of such induction is unclear at present.

Overweight individuals frequently develop hypertension, dyslipidemia, or hyperglycemia. Clustering of these symptoms with insulin resistance constitutes metabolic syndrome [26]. Accordingly, improvement of obesity is crucial in treatment to prevent the vascular complications. Reduced adiposity in *IRS-1*<sup>-/-</sup> *GIPR*<sup>-/-</sup> mice should contribute to ameliorated insulin resistance and its related diseases associated with similar glucose intolerance and decreased postprandial insulin secretion.

The present study suggests that GIP plays a crucial role in switching from fat oxidation to fat accumulation under the diminished insulin action and inhibition of GIP signaling ameliorated insulin resistance. GIP signaling is a potential target for secondary prevention of insulin resistance.

#### Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by Health Sciences Research Grants for Comprehensive Research on Aging and Health from the Ministry of Health, Labor and Welfare, Japan.

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## Effects of thorough mastication on postprandial plasma glucose concentrations in nonobese Japanese subjects

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Received 4 December 2004; accepted 24 June 2005

### Abstract

Thorough mastication has the potential to affect postprandial plasma glucose concentrations by improving digestibility and absorption of nutrients. To evaluate the effects of mastication on postprandial plasma glucose concentration, we compared usual and thorough mastication in subjects with normal glucose tolerance (NGT group,  $n = 16$ ) and subjects predisposed to type 2 diabetes (first-degree relatives of type 2 diabetic patients, subjects with impaired glucose tolerance, and type 2 diabetic patients) (predisposed group,  $n = 10$ ) in a crossover trial of 52 test meals. Plasma glucose and serum insulin concentrations were measured for 3 hours postprandially, and the insulinogenic index (the ratio of incremental serum insulin to plasma glucose concentration during the first 30 minutes after meal) was calculated. In the NGT group, thorough mastication reduced the postprandial plasma glucose concentration at 90 minutes ( $5.8 \pm 0.3$  vs  $6.5 \pm 0.4$  mmol/L,  $P < .05$ ) and 120 minutes ( $5.4 \pm 0.2$  vs  $6.3 \pm 0.4$  mmol/L,  $P < .05$ ) and the area under the curve (AUC) from  $-15$  to 180 minutes ( $19.1 \pm 0.6$  vs  $20.6 \pm 0.8$  [mmol/L] · h,  $P < .05$ ) without an increase in the AUC for insulin. In the predisposed group, thorough mastication significantly augmented plasma glucose and serum insulin concentrations and the AUCs compared with usual mastication. Thorough mastication elicited a significantly higher insulinogenic index than usual mastication in the NGT group ( $205.0 \pm 27.6$  vs  $145.6 \pm 17.7$  pmol/mmol,  $P < .05$ ), whereas the predisposed group showed significantly less early-phase insulin secretion than the NGT group. In the NGT group the postprandial plasma glucose concentration upon thorough mastication of meal was significantly lower, most probably because of the potentiation of early-phase insulin secretion. In the subjects predisposed to type 2 diabetes, thorough mastication did not potentiate early-phase insulin secretion and elicited a higher postprandial plasma glucose concentration.

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### 1. Introduction

Fletcherism, the practice of chewing food slowly and thoroughly as an aid to digestion [1], was advocated by the American dietician Horace Fletcher (1849-1919). He found that prolonged mastication both inhibited overeating and contributed to reduced food intake [2]. Under laboratory conditions, it has been found that when people enjoy softer food, they masticate less and bite with less vigor [3]. Fast food such as hamburgers is highly palatable by clever seasoning and flavoring, but soft and airy and with a generally homogenous consistency, and is now so com-

monplace worldwide [4] that the physiological importance of thorough mastication is barely recognized.

The major physiological function of mastication is the mechanical disruption of food into small particles suitable for gastrointestinal absorption of nutrients [5]. Preabsorptive or cephalic-phase insulin release, a vagally mediated response, occurs within the first few minutes of food ingestion [6] and is thought to be required for normal postprandial glucose tolerance [7]. Thus, mastication plays a crucial role in determining the postprandial plasma glucose concentration. Modified sham feeding, in which food is chewed and tasted but not swallowed [8], has been shown to elicit cephalic-phase insulin release [9], but few studies have examined the relation between thorough mastication and postprandial plasma glucose concentra-

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tions. Read et al [10] found that thoroughly masticating food rather than merely swallowing it increased plasma glucose concentrations after the ingestion of 4 kinds of carbohydrate (sweet corn, potato, rice, and apple) in 6 healthy subjects, mostly because of improved digestibility and absorption. However, no variables other than the plasma glucose concentration during the early postprandial period were examined. The aim of the present study is to evaluate the effect of thorough mastication on postprandial plasma glucose concentrations. We used a mixed-nutrient meal of hamburger steak and rice as the test meal. Hamburger steak is a kind of processed meat, the frequent consumption of which is reported to increase the risk of type 2 diabetes [11]. The hamburger steak and rice used in this study were both readily swallowed without thorough mastication.

## 2. Subjects and methods

### 2.1. Subjects

A total of 26 volunteers (17 men and 9 women; mean age,  $38.9 \pm 11.5$  [SD] years [range, 25–71 years]; mean body mass index [BMI],  $21.8 \pm 2.8$  kg/m<sup>2</sup> [range, 16.8–26.8 kg/m<sup>2</sup>]) participated in the study. Sixteen had normal glucose tolerance (NGT), 6 were first-degree relatives of type 2 diabetic patients, 2 had impaired glucose tolerance (IGT), and 2 had mild type 2 diabetes mellitus without pharmacotherapy. None of the subjects were taking medication known to influence glucose concentration. Subjects were classified into 2 groups, one with NGT (NGT group) and the other with a predisposition to diabetes (predisposed group), which comprised IGT, mild type 2 diabetes, and first-degree relatives of type 2 diabetic patients, and underwent 1 session of each mastication procedure. Fourteen of the 16 NGT subjects and all 10 subjects in the predisposed group underwent 75-g oral glucose tolerance test (OGTT). Subjects with fasting plasma glucose (FPG) of less than 5.6 mmol/L and HbA<sub>1c</sub> of less than 5.0% and/or with FPG of less than 6.1 mmol/L and 2-hour

Table 1  
Clinical characteristics of the 26 subjects

	NGT group	Predisposed group
n (male/female)	16 (9/7)	10 (8/2)
Definition	16 NGT	6 First-degree relatives, 2 IGT, 2 type 2 diabetes
Age (y)	$35.6 \pm 2.1$	$44.1 \pm 4.4$
BMI (kg/m <sup>2</sup> )	$21.2 \pm 0.6$	$23.0 \pm 1.0$
HbA <sub>1c</sub> (%)	$4.6 \pm 0.1$	$5.4 \pm 0.4$
FPG (mmol/L)	$5.2 \pm 0.1$	$5.8 \pm 0.2$
Fasting insulin (pmol/L)	$57.4 \pm 5.0$	$49.5 \pm 4.3$
Total cholesterol (mmol/L)	$5.1 \pm 0.2$	$5.0 \pm 0.2$
HDL cholesterol (mmol/L)	$1.8 \pm 0.1$	$1.5 \pm 0.1$
Triglyceride (mmol/L)	$1.0 \pm 0.1$	$1.0 \pm 0.1$

Data are means (of 2 sessions)  $\pm$  SE. There were no significant differences between the NGT and the predisposed groups. HDL indicates high-density lipoprotein.

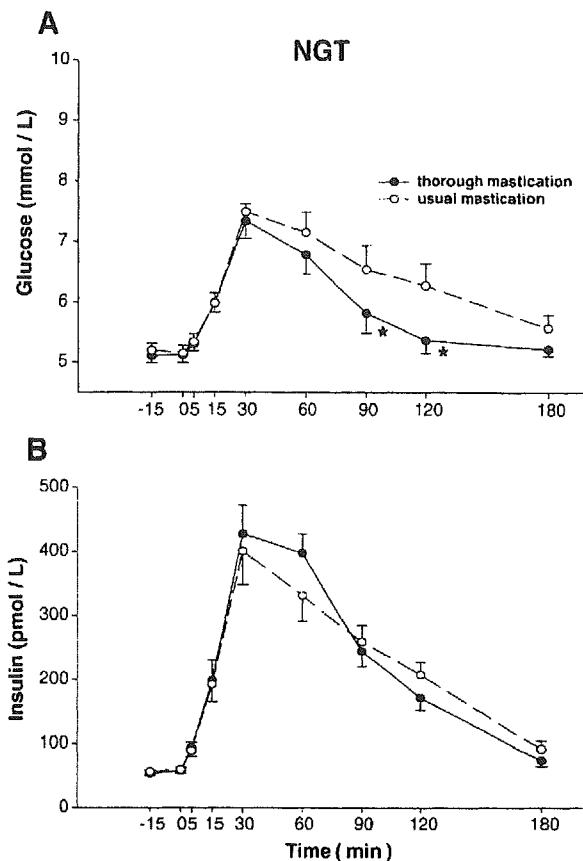


Fig. 1. Plasma glucose (A) and serum insulin (B) concentrations in the NGT group at various time points in usual and thorough mastication. Data are means  $\pm$  SE,  $n = 16$ , \* $P < .05$ .

glucose of less than 7.8 mmol/L in OGTT by 1998 World Health Organization diagnostic criteria [12] were classified as NGT. IGT and type 2 diabetes also were defined according to World Health Organization criteria. Table 1 shows the clinical characteristics of the NGT and the predisposed groups. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Kyoto University, and was conducted in accordance with the Declaration of Helsinki. All subjects gave written informed consent.

### 2.2. Experimental protocol

The study was a crossover experiment that included 52 sessions. After overnight fasting for at least 12 hours, the subjects began each eating session at 8:00 AM. A butterfly needle was inserted into an antecubital vein to draw blood samples at  $-15$  minutes and was kept open by a slow drip of physiological saline. Immediately after a blood sample was drawn at 0 minute, the test meal of 130 g hamburger steak of 962 kJ (230 kcal) (Tokiwa Kanpo Pharmaceutical, Osaka, Japan) and 100 g rice of 649 kJ (155 kcal) (Hagoromo Foods, Shizuoka, Japan), with a total energy content of 1611 kJ (385 kcal) comprising 51%, 15%, and 34% carbohydrate, protein, and fat, respectively, began. Each

food item was sealed in a retort pouch and heated in a standard microwave oven for 2 minutes before the meal. The hamburger steak and rice were divided into 8 equal portions. Each subject underwent both mastication procedures. In the “usual mastication” sessions, the subjects took 16 teaspoonfuls of food, chewing each teaspoonful for 10 seconds before swallowing. In the “thorough mastication” sessions, each teaspoonful was swallowed only after 30 seconds of chewing. The rate of mastication was maintained at about 1 cycle per second in each session. Thus, thorough mastication involved 3-fold more bites than usual mastication. As the difference in the time taken eating might be a confounding factor, the subjects in usual mastication paused for 20 seconds after every 10 seconds, during which they were permitted to drink nonenergetic water, equalizing the duration of all meals at 8 minutes. The succession of mastication procedure was randomized for each subject. The average duration of the experiment was 9.9 days for male subjects. Female subjects participated only during the follicular phase of the menstrual cycle, with the interval fixed at 4 weeks to reduce variations in insulin sensitivity [13].

Blood samples for glucose and insulin were withdrawn at –15 and 0 minute before each meal and at 5, 15, 30, 60, 90, 120, and 180 minutes after each meal.

### 2.3. Analytical methods

Plasma glucose was measured by the glucose oxidase method using a Hitachi Automatic Analyzer 7170 (Hitachi, Tokyo, Japan). Serum insulin was measured in duplicate using LS regand Eiken insulin (Eiken, Tokyo, Japan) by automatic chemiluminescence enzyme immunoassay analyzer BCS 600 (SRL, Tokyo, Japan). The cross-reactivity to proinsulin, C-peptide, and split insulin was 0.01%, 0%, and 0%, respectively.

### 2.4. Data analysis

Values are expressed as mean  $\pm$  SE unless otherwise noted. Statistical analysis was performed using StatView 5.0 (Abacus Concepts, Berkeley, CA). The area under the

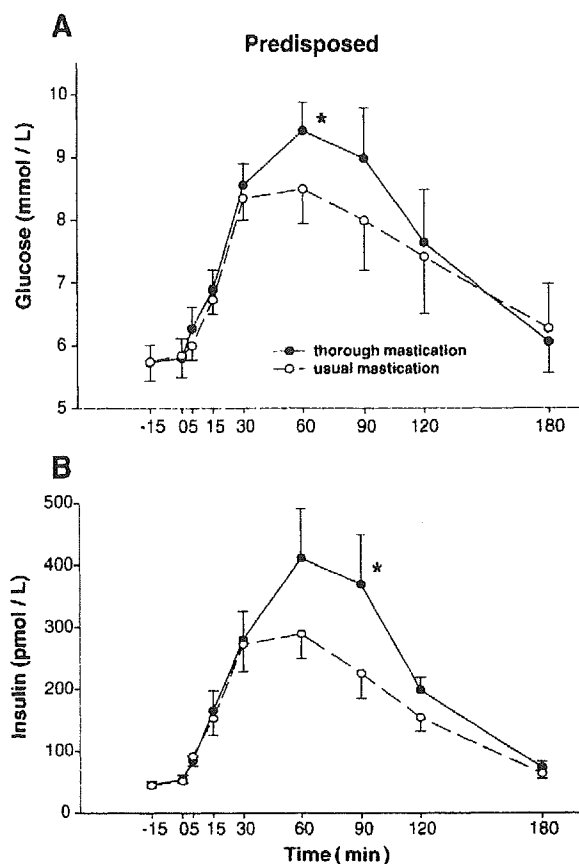


Fig. 2. Plasma glucose (A) and serum insulin (B) concentrations in the predisposed group at various time points in usual and thorough mastication. Data are means  $\pm$  SE,  $n = 10$ ,  $*P < .05$ .

curve (AUC) was calculated according to the trapezoid rule. FPG and fasting serum insulin concentrations are the average of 2 premeal values (–15 and 0 minute). The insulinogenic index (II) [14], the ratio of the incremental serum insulin to plasma glucose concentration during the first 30 minutes after glucose ingestion calculated by OGTT ( $II_{OGTT}$ ), has been commonly used as a measure of early-phase insulin secretion [15–18] since it was proposed by Seltzer et al [19] in 1967. In this study, the II during the first 30 minutes after meal tolerance test (MTT) ( $II_{MTT}$ ) was calculated as the serum insulin concentration (30 – 0 minute)/plasma glucose concentration (30 – 0 minute) (pmol/mmol), and  $II_{MTT}$  and  $II_{OGTT}$  were compared. To estimate differences between 2 means, Student paired  $t$  test was performed with paired variates. To compare unpaired variates, Student unpaired  $t$  test with equal variances or Welch test with unequal variances was used. Multiple comparisons between differences among individual time points were done by analysis of variance (repeated measures) followed by Student  $t$  test with Bonferroni correction. Pearson  $r$  was used to evaluate univariate correlations.  $P < .05$  was considered statistically significant.

Table 2

Comparison of the total AUCs (–15 to 180 minutes) for glucose and insulin in the NGT and the predisposed groups

	Glucose AUC ([mmol/L] · h) (–15 to 180 min)	Insulin AUC ([pmol/L] · h) (–15 to 180 min)
NGT group		
Usual mastication	20.6 $\pm$ 0.8	722.5 $\pm$ 60.3
Thorough mastication	19.1 $\pm$ 0.6*	722.5 $\pm$ 50.9
Predisposed group		
Usual mastication	23.7 $\pm$ 1.7	574.7 $\pm$ 65.3
Thorough mastication	24.9 $\pm$ 1.5**	755.5 $\pm$ 91.8**

Data are means  $\pm$  SE.

\*  $P < .05$ , significantly different from usual mastication in each group.

\*\*  $P < .01$ , significantly different from usual mastication in each group.

### 3. Results

All 26 subjects completed all sessions of the test meals. Fig. 1 shows the plasma glucose and serum insulin concentrations in the NGT group in usual and thorough mastication. The plasma glucose concentration in both masticatory procedures increased in the first 30 minutes to 7.4 and 7.3 mmol/L, respectively. On the other hand, plasma glucose in thorough mastication decreased more rapidly than in usual mastication and was significantly reduced at 90 and 120 minutes (90 minutes,  $5.8 \pm 0.3$  vs  $6.5 \pm 0.4$  mmol/L,  $P < .05$ ; 120 minutes,  $5.4 \pm 0.2$  vs  $6.3 \pm 0.4$  mmol/L,  $P < .05$ ) (Fig. 1A). The AUC for glucose in the NGT group from  $-15$  to 180 minutes was significantly less in thorough mastication than in usual mastication ( $P = .017$ ) (Table 2). Insulin secretion was increased in thorough mastication from 5 minutes to nearly 90 minutes (the major difference occurring at 60 minutes:  $397.5 \pm 29.4$  vs  $332.2 \pm 39.5$  pmol/L) (Fig. 1B). The AUC for insulin in the NGT group from 90 to 180 minutes was significantly less in thorough mastication than in usual mastication ( $228.9 \pm 20.1$  vs  $268.3 \pm 23.0$  [pmol/L] · h,  $P = .044$ ). The total AUCs for insulin in the 2 mastication procedures were the same (Table 2).

The data on the predisposed group are shown in Fig. 2. During the first 30 minutes, both plasma glucose and serum insulin concentrations showed a similar pattern in the 2 mastication procedures. At 60 minutes, there was a significantly higher glucose response in thorough mastication

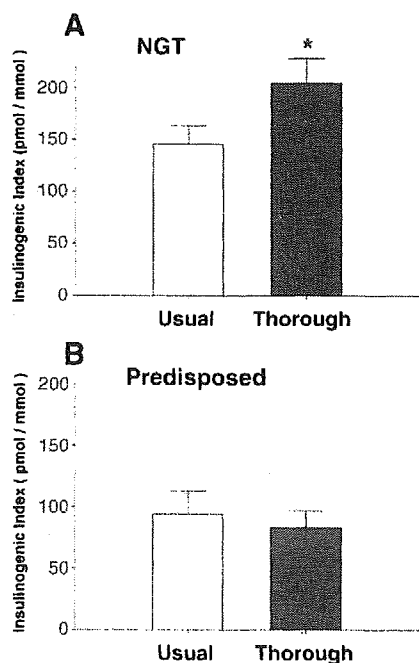


Fig. 3. II<sub>MTT</sub> in the NGT group ( $n = 16$ ) (A) and the predisposed group ( $n = 10$ ) (B) during the first 30 minutes after meal in usual and thorough mastication. Data are means  $\pm$  SE. \* $P < .05$ .

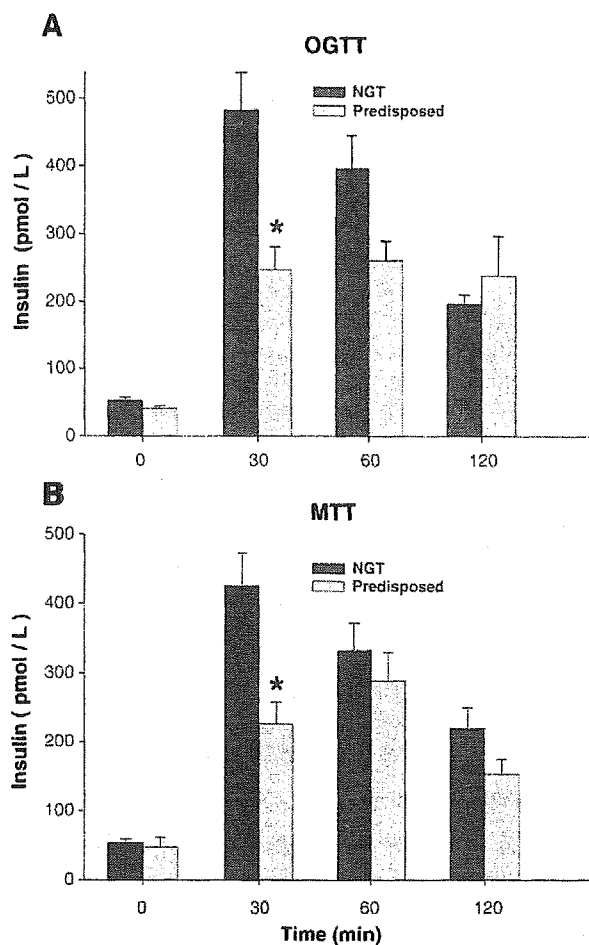


Fig. 4. Serum insulin concentrations in the NGT group (black bars,  $n = 14$ ) and the predisposed group (gray bars,  $n = 10$ ) measured by OGTT (A) and MTT in usual mastication (B) at 4 time points. Data are means  $\pm$  SE. \* $P < .05$ .

tion than in usual mastication ( $9.4 \pm 0.45$  vs  $8.4 \pm 0.55$  mmol/L,  $P < .05$ ) (A), as well as a significantly higher insulin response at 90 minutes ( $370.9 \pm 78.9$  vs  $226.0 \pm 40.2$  pmol/L,  $P < .05$ ) (B). The AUCs for both glucose ( $P = .008$ ) and insulin ( $P = .002$ ) in the predisposed group were increased significantly in thorough mastication compared with usual mastication (Table 2).

Fig. 3 shows the II measured by MTT for the 2 mastication procedures in the NGT (A) and the predisposed group (B). In the NGT group, II<sub>MTT</sub> in thorough mastication was significantly higher than in usual mastication ( $205.0 \pm 27.6$  vs  $145.6 \pm 17.7$  pmol/mmol,  $P = .02$ ) (Fig. 3A). On the other hand, there was no significant difference in II<sub>MTT</sub> between the 2 mastication procedures in the predisposed group (B).

Fig. 4 shows the serum insulin concentrations at 4 time points in OGTT (A) and MTT in usual mastication (B) in the NGT group ( $n = 14$ ) and the predisposed group ( $n = 10$ ) in subjects who underwent both OGTT and MTT. The

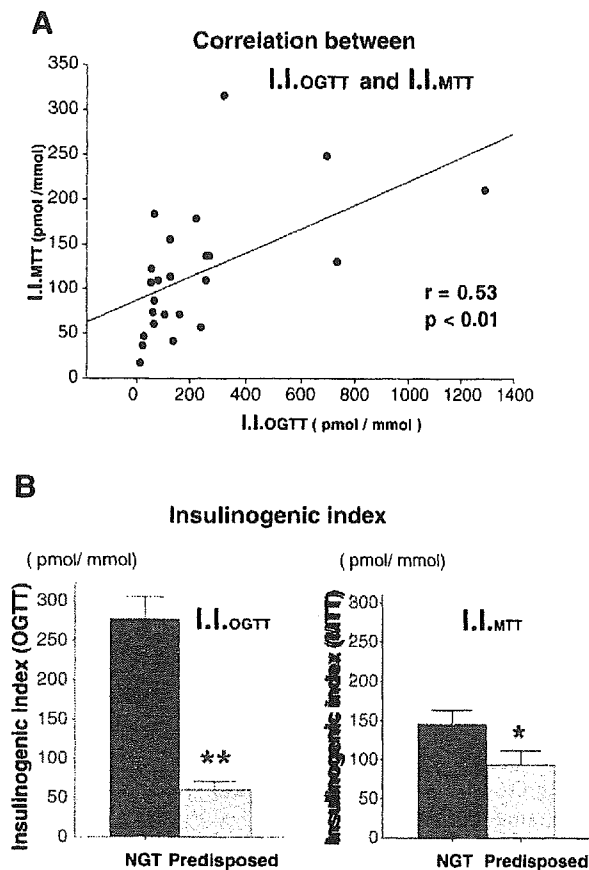


Fig. 5. A, Correlation between the II estimated from OGTT and MTT in usual mastication in 24 subjects who underwent both OGTT and MTT. B,  $II_{OGTT}$  (II during the first 30 minutes after OGTT) and  $II_{MTT}$  (II during the first 30 minutes after MTT) in usual mastication in the NGT group ( $n = 14$ ) and the predisposed group ( $n = 10$ ). Data are means  $\pm$  SE. \* $P < .05$ , \*\* $P < .01$ .

serum insulin concentrations in the NGT and the predisposed groups showed a general similarity in both OGTT and MTT. The fasting serum insulin concentrations in both groups were similar in both tests. The peak serum insulin concentration occurred at 30 minutes in the NGT group and at 60 minutes in the predisposed group in both tests. The serum insulin concentration at 30 minutes was significantly higher in the NGT group than in the predisposed group in both tests (OGTT,  $482.9 \pm 55.2$  vs  $246.8 \pm 34.4$  pmol/L,  $P < .05$ ; MTT,  $426.2 \pm 48.1$  vs  $227.4 \pm 30.9$  pmol/L,  $P < .05$ ). There was no significant difference in the serum insulin concentration between the 2 groups at 60 and 120 minutes in both tests.

Fig. 5 shows the correlation between  $II_{OGTT}$  and  $II_{MTT}$  in usual mastication (A) and the IIs (B).  $II_{MTT}$  was significantly correlated with  $II_{OGTT}$  in the 24 subjects who underwent both tests ( $r = 0.53$ ,  $P < .01$ ) (A).  $II_{OGTT}$  ( $276.6 \pm 28.0$  vs  $60.1 \pm 11.3$  pmol/mmole,  $P = .004$ ) and  $II_{MTT}$  ( $145.8 \pm 17.8$  vs  $94.0 \pm 17.6$  pmol/mmole,  $P = .046$ ) were significantly higher in the NGT group than in the predisposed group (B).

#### 4. Discussion

In this study, we compared the effects of thorough mastication on postprandial glucose and insulin secretion in subjects with NGT and subjects predisposed to type 2 diabetes. Thorough mastication was especially effective in reducing the postprandial plasma glucose concentrations in the NGT group, probably because of greater early-phase insulin secretion.

Surprisingly, the AUC for glucose was significantly less in thorough mastication compared with usual mastication, without an increase in the AUC for insulin. Mastication breaks food into small pieces, stimulates salivation, and mixes food with salivary enzymes, improving hydrolysis of carbohydrates in the mouth and stomach [10] and enhancing glycemic and insulinemic responses. Thus, thorough mastication should be expected to increase both postprandial plasma glucose and serum insulin concentrations. However, regardless of the mastication procedure, the plasma glucose and serum insulin concentration reached a peak at 30 minutes in the NGT group. In addition,  $II_{MTT}$  in thorough mastication was significantly higher than in usual mastication. Apparently, NGT subjects have sufficient early-phase insulin secretory capacity to lower the plasma glucose concentration after the more rapid absorption of glucose in thorough mastication. Thorough mastication was especially effective in NGT subjects in potentiating insulin secretion from 5 minutes (the cephalic-phase) to 90 minutes, resulting in lower plasma glucose concentrations after 30 minutes. Insulin secretion from 90 to 180 minutes was thus reduced, resulting in the same AUC for insulin in the 2 mastication procedures in the NGT group. Thus, the present study suggests that people with NGT can reduce postprandial plasma glucose concentrations by masticating food thoroughly.

In contrast to the NGT group, thorough mastication was not effective in reducing the postprandial plasma glucose concentration in the predisposed group. Compared with usual mastication, the glycemic response in thorough mastication was significantly enhanced at 60 minutes, and the insulinemic response was enhanced at 90 minutes. In addition, the AUCs for both glucose and insulin in thorough mastication were significantly greater than in usual mastication. In addition, in contrast to the NGT group, there was no significant difference in the II between the 2 mastication procedures in the predisposed group. The fact that thorough mastication did not potentiate insulin secretion in the predisposed group during the first 30 minutes suggests inability of the beta-cells to respond promptly to glucose stimulation. Thorough mastication might be expected to promote satiation with reduced food intake in ordinary life. Food intake can be reduced by a number of monoamines acting on noradrenaline, serotonin, dopamine, and histamine receptors within the hypothalamus [20]. The rate of 40 masticating cycles per minute has been shown to increase the firing rate of serotonergic neurons in cats [21]. Moreover, thorough mastication enhances satiation

independently of energy expenditure by activating neuronal histamine in the hypothalamus [22]. Accordingly, thoroughly masticating food might also benefit similarly predisposed individuals in daily life.

Early-phase insulin secretion is known to be disturbed in patients with type 2 diabetes, IGT, and normoglycemic first-degree relatives of patients with type 2 diabetes [14,23–27], so first-degree relatives of type 2 diabetic patients were included in the predisposed group. Early-phase insulin secretion in both OGTT and MTT was significantly less in the predisposed group than in the NGT group. Thus, the correlation between  $I_{\text{MTT}}$  and  $I_{\text{OGTT}}$  observed in the present study suggests that  $I_{\text{MTT}}$  calculated by the same formula as  $I_{\text{OGTT}}$  can be used as an index of early-phase insulin secretion. The  $I_{\text{MTT}}$  in the NGT and the predisposed groups of the present study were clearly different, most probably because of the difference in early-phase insulin secretion, which may underlie the altered postprandial plasma glucose concentrations. Early-phase insulin secretion is commonly referred to in both OGTT and MTT analyses [15–18]. Although the relation between the first-phase insulin response to intravenous glucose challenge and the early insulin response to oral glucose has been investigated recently [28–30], further studies are required to distinguish first- and second-phase insulin secretion sufficiently for in vivo comparison of mastication procedures. Because the plasma glucose concentrations increased gradually in this study, we used the term *early-phase insulin secretion*.

Thorough mastication was found to reduce the postprandial plasma glucose concentration mainly in the NGT group. Although the most important substance in physiological regulation of insulin release is glucose, incretin hormones (gastric inhibitory peptide [GIP] and glucagon-like peptide 1 [GLP-1]) also play important roles in postprandial insulin secretion in healthy subjects [7]. GIP and GLP-1 are released from the gut to the portal vein and are diluted when entering the systemic circulation. Only 10% to 15% of GLP-1 reaches systemic circulation and the pancreas in the intact form [31,32]. In the present study, thorough mastication elicited at most a 1.2-fold increase in the peripheral serum insulin concentration at 60 minutes in the NGT group compared with usual mastication. In a rodent study, intraportal injection of a pharmacological dose of GLP-1 was reported to evoke a peak of only a 2-fold increase in the peripheral insulin response to portal glucose compared with the control condition [33]. Accordingly, the slight change in GIP and GLP-1 in systemic circulation that may correspond to the difference in peripheral serum insulin concentrations in the NGT group would be difficult to detect by peripheral blood sampling. Further studies are required to determine whether differences in the rate of mastication affect incretin concentrations. In addition, other hormones, including glucagon, growth hormone, and cortisol, and other nutrients (amino acids) are also involved in insulin secretion upon meal ingestion [30,34]. We also

measured the plasma arginine concentration. Insulin secretion is stimulated by amino acids after the digestion of protein in meal [35]. The AUC of incremental arginine from 0 to 120 minutes in thorough mastication was significantly greater than in usual mastication (data not shown). Thus, increased absorption of arginine in thorough mastication is at least partly responsible for the increased insulin secretion in NGT subjects.

The pancreas has rich innervation from both the sympathetic and parasympathetic nervous system. Sympathetic fibers are found primarily in the splanchnic nerve, whereas parasympathetic fibers are found in the vagus nerve [36]. According to the study of Rasmussen et al [37] and our previous report [38], neural factors play important roles in the normal pattern of insulin secretion. There are 2 neural stages before the late enteric stage when nutrients are absorbed [37]. During the cephalic phase in thorough mastication, the release of acetylcholine is considered to be stimulated more strongly than in usual mastication through activation of vagal-efferent fibers. Thus, during the early enteric phase, when the neurons of the enteric nervous system are activated by nutrients entering the intestine [37], thorough mastication may promote stronger release of cholecystokinin by augmenting gastric emptying. Accordingly, acetylcholine and cholecystokinin might contribute to potentiating early-phase insulin secretion in thorough mastication in the NGT group. In contrast, in the predisposed group, potentiation of early-phase insulin secretion in thorough mastication was not observed, most likely because of the poor response of the beta-cell to neural stimulation.

In addition, mastication mixes food particles with saliva. In studies comparing normal and diabetic subjects, the flow rate of saliva, the volume of saliva secreted per minute, is diminished significantly in diabetic patients [39]. Diabetic neuropathy may well account for this decrease [40]. Because the concentration of amylase in diabetic subjects has been reported to be lower [40], higher [39], and similar [41] to healthy subjects, whether improved exposure of food particles to amylase in saliva affects postprandial plasma glucose concentrations in persons with NGT remains to be determined.

Although the predisposed group was composed of 3 subgroups, first-degree relatives of type 2 diabetic patients, IGT, and type 2 diabetes, it was clearly distinguished from the NGT group in terms of early-phase insulin secretion. However, further investigation of the effect of thorough mastication in each of the subgroups with more samples would be informative.

In conclusion, in the present study, thorough mastication elicited lower postprandial plasma glucose concentrations than usual mastication in the NGT group, most probably because of the potentiation of early-phase insulin secretion. In contrast, in the predisposed group, thorough mastication did not potentiate early-phase insulin secretion and elicited higher postprandial plasma glucose concentrations.

## Acknowledgment

This study was supported in part by the Japan Arteriosclerosis Prevention Fund (JAPF).

We thank Tokiwa Kanpo Pharmaceutical, SRL, Use Techno, and Abbott Japan for assistance in this study.

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## Soluble TNF Receptors and Albuminuria in Non-obese Japanese Type 2 Diabetic Patients

### Abstract

The aim of this study was to investigate the relationships between albuminuria and tumor necrosis factor (TNF)- $\alpha$  or soluble TNF receptors (sTNF-R1, sTNF-R2) in eighty-eight non-obese Japanese type 2 diabetic patients stratified into two groups according to albuminuria status-microalbuminuria or normoalbuminuria. Patients with microalbuminuria were older and had significantly higher concentrations of sTNF-R1 and sTNF-R2 than those with normoalbuminuria. There was, however, no significant difference in sex, diabetes duration, smoking, BMI, systolic and diastolic blood pressure, HbA<sub>1c</sub>, serum creatinine, and lipid profile between the two groups. Although serum TNF- $\alpha$  was positively correlated to serum sTNF-R1 and sTNF-R2, serum TNF- $\alpha$  level did not differ with respect to albuminuria. Univariate re-

gression analysis showed that urinary albumin concentration was positively correlated to age ( $r = 0.380$ ,  $p < 0.001$ ), serum creatinine ( $r = 0.214$ ,  $p < 0.05$ ) and concentrations of sTNF-R1 ( $r = 0.364$ ,  $p < 0.001$ ) and sTNF-R2 ( $r = 0.342$ ,  $p < 0.005$ ). Other variables, including TNF- $\alpha$ , were not associated with albuminuria. Multiple regression analyses showed that urinary albumin concentration was independently predicted by the level of sTNF-R1 ( $F = 32.1$ ), which explained 26.3% of the variability of urinary albumin concentration. From these results, it can be concluded that serum soluble TNF receptor is an important independent factor associated with albuminuria in non-obese Japanese type 2 diabetic patients.

### Key words

sTNF receptors · TNF-alpha · Albuminuria · Diabetes

### Introduction

The major clinical consequence of type 2 diabetes is mortality and morbidity from atherosclerotic vascular disease, especially coronary heart disease (CHD). The risk of CHD appears to be similar in patients with type 2 diabetes and impaired glucose tolerance [1,2]. Thus, factors other than the level of glycemia seem to accelerate the development of CHD in type 2 diabetes. This idea is supported by the observation that duration of diabetes and level of glycemia are not risk factors for atherosclerosis including

CHD in type 2 diabetic patients [3,4]. Atherosclerosis can be evaluated by urinary albumin excretion rate. Increased urinary albumin excretion rate is not only used as an index of diabetic nephropathy but also as an independent risk factor for atherosclerosis, including cardiac disease, in type 2 diabetic patients. An association between microalbuminuria and cardiac disease in type 2 diabetic patients has been demonstrated [5].

A number of studies published have shown that age, smoking, blood pressure, blood glucose, and abnormalities in lipoprotein

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Received 17 December 2004 · Accepted after revision 7 April 2005

### Bibliography

Horm Metab Res 2005; 37: 617-621 © Georg Thieme Verlag KG Stuttgart · New York · DOI 10.1055/s-870536 · ISSN 0018-5043

particles may lead to the development of microalbuminuria. Inflammation has also been implicated in the evolution of albuminuria. Festa et al. [6] recently demonstrated that diabetic patients with microalbuminuria had significantly higher levels of C-reactive protein (CRP) and fibrinogen compared to those with normoalbuminuria. Both CRP and fibrinogen are considered to be inflammatory markers. Stehouwer et al. [7] also documented that increased urinary albumin excretion, endothelial dysfunction, and chronic inflammation are interrelated processes that develop in parallel, progress with time, and are strongly and independently associated with risk of death in type 2 diabetic patients.

Tumor necrosis factor (TNF) is a potent proinflammatory cytokine involved in the pathogenesis of atherosclerosis. TNF is known to induce a cascade of inflammatory reactions involving production of other cytokines, thus participating in the development of atherosclerosis. TNF binds two receptors so far identified referred to as TNF-R1 and TNF-R2. Both of these receptors exist in soluble forms. The two receptors share almost no homology outside the ligand binding domain, suggesting that they signal for different biological functions [8–10]. Elevated soluble TNF-R1 levels have recently been shown to be predictive of cardiovascular mortality in patients with chronic heart failure [11]. However, the studies investigating the relationship between soluble TNF receptors and vascular complications in diabetic patients are limited. Zoppini et al. [12] very recently demonstrated that soluble TNF-R1 level is higher in type 1 diabetic patients with microalbuminuria than those with normoalbuminuria. To the best of our knowledge, however, the relationships between urinary albumin excretion rate and the levels of the two soluble TNF receptors have not yet been examined in type 2 diabetic patients.

In this context, a major problem is that albuminuria itself has been associated with atherosclerotic vascular diseases such as renal failure, cerebral infarction and CHD. Moreover, overweight condition or hyperglycemia *per se* may affect albuminuria, TNF- $\alpha$ , and soluble TNF receptors concentrations in humans [13,14]. We therefore recruited non-obese, well-controlled unique Japanese type 2 diabetic patients without evidence of vascular complications including CHD, cerebral infarction, or renal failure, taking into account of body mass index and fasting glucose level. This is the first finding that albuminuria is independently associated with serum level of soluble TNF receptor in non-obese well-controlled unique Japanese type 2 diabetic patients.

## Subjects and Methods

After informed consent was obtained, forty-five diabetic patients with microalbuminuria (twenty-nine men and sixteen women) and forty-three patients with normoalbuminuria (thirty-four men and nine women) were enrolled in the present study. They all were non-obese (BMI < 27 kg/m<sup>2</sup>) Japanese type 2 diabetic patients [15]. Type 2 diabetes mellitus was diagnosed based on the WHO criteria [16]. All subjects had ingested at least 150 g of carbohydrate in the three days before the study. Forty-one patients (91%) with microalbuminuria and 37 patients (86%) with normoalbuminuria were taking sulfonylureas, respectively. The rests were treated by diet alone. They all have not received insulin ther-

apy. Blood pressure was measured according to a standard procedure and hypertension was defined as systolic blood pressure  $\geq$  140 mm Hg and/or diastolic blood pressure  $\geq$  90 mm Hg or current use of antihypertensive medication. Twenty-two patients (49%) (Ca antagonist 11, ACE-I 8, ARB 3) with microalbuminuria and fifteen patients (35%) (Ca antagonist 5, ACE-I 6, both 2, ARB 2) with normoalbuminuria were treated with antihypertensive drugs, respectively. Fourteen patients (31%) with microalbuminuria and seventeen patients (40%) with normoalbuminuria were treated with lipid lowering agents, respectively. Cigarette smoking was dichotomized into "never" and "ever" (including past and current) using a questionnaire. There was no significant difference in gender, smoking, or medication status between the patients with microalbuminuria and those with normoalbuminuria (Table 1). They did not consume alcohol or perform heavy exercise for at least one week before the study.

Blood was drawn in the morning after a 12 h fast. Plasma glucose was measured using the glucose oxidase method. Triglycerides, total cholesterol, and HDL cholesterol were also measured. The LDL cholesterol level was calculated using the Friedewald formula [17]. Serum insulin was measured using a two-site immunoradiometric assay (Insulin Riabead II, Dainabot, Japan). Coefficients of variation were 4% for insulin > 25  $\mu$ U/ml and 7% for insulin < 25  $\mu$ U/ml, respectively. Serum TNF- $\alpha$  concentrations were measured by enzyme immunoassay kit (Quantikine HS Human TNF- $\alpha$  immunoassay kit, R&D systems, Inc, Minneapolis, MN, USA) and serum concentrations of sTNF-R1 and sTNF-R2 were measured by enzyme-linked immunosorbent assay (ELISA; BIO-TRAK, Amersham Life Sciences, Uppsala, Sweden) as described previously [18]. The limits of sensitivity for TNF- $\alpha$ , sTNF-R1 and sTNF-R2 were 0.5 pg/ml, 25 pg/ml and 50 pg/ml, respectively.

Urinary albumin concentration was assessed in a morning spot urine sample using a commercial enzymatic immunoassay. Urinary albumin concentration was measured in duplicate and the mean of the two values was used for the study. Intraassay and interassay coefficients of variation were less than 7% (Orion, Espoo, Finland). Several reports have indicated that early morning spot urine is usually sufficient for detecting the presence of microalbuminuria [19,20]. In the present study, we calculated urinary albumin excretion rate as a ratio of urinary albumin and urinary creatinine that markedly enhances the accuracy of the single spot urine sample in the assessment of microalbuminuria [21]. Microalbuminuria was defined as a urinary albumin concentration greater than 30 mg/g creatinine but less than 300 mg/g creatinine. Normoalbuminuria was defined as urinary albumin concentration less than 30 mg/g creatinine [22].

## Statistical analysis

The statistical analysis was performed using the StatView 5 system (Statview, Berkeley, CA). The differences of mean were determined by the Mann-Whitney U-test. Data were expressed as the mean  $\pm$  SEM. Simple (Spearman's rank) correlation coefficients between urinary albumin concentration and measures of variables were calculated, and a stepwise multiple regression analysis was then used to evaluate the independent association of these variables with urinary albumin concentration. Values of  $p < 0.05$  were considered significant. In multivariate analysis,  $F \geq 4$  was considered significant.