Possible involvement of food texture in insulin resistance and energy metabolism in male rats

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Abstract

Food texture is known to affect energy metabolism. Although feeding with soft pellets (SP) or via a tube is known to cause increases in body weight, it is unclear how different food textures influence energy metabolism. In this study, we investigated the effects of two different food textures on energy balance and glucose and lipid metabolism in male Wistar rats. The rats were fed SP or control pellets (CP) on a 3-h restricted feeding schedule for 14 weeks and their energy intake, body weight, and energy expenditure were examined. The levels of gastrointestinal hormones, glucose and insulin, were investigated at pre-, mid, and post-feeding. Glucose tolerance and insulin tolerance tests were conducted, and the expressions of molecules involved in the insulin signaling system or lipogenesis in the liver were examined. Histological investigation of pancreatic islets was carried out using anti-insulin and anti-Ki-67 antibodies. Furthermore, the expression in the liver and circulating blood of microRNA-33 (miR-33), which regulates insulin receptor substance 2, was examined. There were no significant differences in energy intake, body weight, or gastrointestinal hormone levels between the SP and CP rats; however, the SP rats showed glucose intolerance and insulin resistance with disruption of insulin signaling. Increases in lipogenic factors and miR-33 expression were also found in the SP rats. The numbers of insulin-positive areas and Ki-67-positive cells of SP rats were significantly increased. This study shows that a soft food texture causes diabetes without obesity, so differences in food texture may be an important factor in type 2 diabetes.

Introduction

Insulin resistance is a major factor in the pathogenesis of type 2 diabetes arising from a prediabetic condition and an important complication of obesity (Shepherd & Kahn 1999). Although obesity is defined as extreme body fat and is usually caused by ingesting calories in excess of daily requirements (Hedley et al. 2004, Bray & Bellanger 2006), it is thought that obese people have different eating habits from normal-weight people, with respect to bite size, ingestion rate, number of chews per bite, and chewing speed. Furthermore, it has been shown that...
that lower ingestion rates are associated with obesity and that lower ingestion rates are associated with reductions in body weight (Spiegel et al. 1993, Sasaki et al. 2003, Otsuka et al. 2006, Maruyama et al. 2008). Indeed, long-term tube feeding has been shown to cause large increases in both body weight and body fat in both rats and humans (LeBlanc & Brondel 1985, LeBlanc & Diamond 1986). These findings imply a close relationship between eating habits, food texture, and obesity or obesity-related diseases.

Dietary factors such as food texture are thought to affect feeding behavior and energy metabolism. Oka et al. (2003) demonstrated that rats fed soft pellets (SP) show an increase in body weight and greater adiposity than rats fed control pellets (CP), because of lowered postprandial thermogenesis. To further investigate the effect of food texture on energy metabolism, we examined the responses of gastrointestinal hormones to food intake, glucose metabolism, and insulin signaling in male Wistar rats fed SP or CP on a 3-h restricted feeding schedule. We also evaluated energy intake, body weight, energy expenditure, and the amount of food in the stomach 5 and 10 h after feeding. We monitored plasma levels of the satiety signals cholecystokinin (CCK), glucagon-like peptide 1 (GLP1), and peptide YY (PYY), as well as the levels of glucose and insulin at pre-, mid-, and post-feeding. We carried out glucose tolerance testing (GTT) and insulin tolerance testing (ITT) in both groups of rats. To investigate the effects of food texture on the insulin signaling system, we evaluated the expression of insulin receptor substance 2 (IRS2) and the phosphorylation of AKT in the livers of the rats by using western blotting analysis. Furthermore, we examined the levels of micro-RNA-33 (miR-33), a regulator of insulin signaling, in the liver and circulating blood of the rats. To examine the effects of SP on lipogenesis, we evaluated the expression of sterol regulatory element-binding transcription factor 1 (SREBF1), carbohydrate-responsive element-binding protein (ChREBP; MLX1PL), and acetyl-CoA carboxylase (ACC) and the phosphorylation of ACC by using western blotting analysis, the level of fatty acid synthase (Fasnl) by using quantitative PCR, and the triacylglycerol content in the livers of the rats. We also carried out immunohistochemistry to evaluate the histological characteristics of pancreatic islets. Although the energy balance of SP rats did not differ from that of CP rats, SP rats showed insulin resistance and increased levels of factors involved in lipogenesis. Our results indicate that the texture of absorbable food such as SP contributes to the acquirement of insulin resistance by promoting the conversion of glucose to triacylglycerol, which drives type 2 diabetes.

Materials and methods

Animals

A total of 30 male Wistar rats (Charles River Japan, Shiga, Japan) of 7 weeks of age were used. Rats were individually housed in plastic cages at a constant room temperature under a 12 h light:12 h darkness cycle (light: 0800–2000 h). The rats were randomly divided into two groups (n = 15 per group) and fed either CP or SP for 14 weeks. The CP group was fed standard laboratory chow (4.6% fat, 51% carbohydrate, and 25% protein; CLEA Japan, Tokyo, Japan). The SP group was fed a mixture of standard laboratory chow and water (standard laboratory chow, 1 g; water, 1.4 ml). As we planned to examine the concentrations of gastrointestinal hormones, glucose, and insulin at pre-, mid-, and post-feeding, the rats were fed between 0900 and 1200 h on a 3-h restricted feeding schedule. All rats were allowed ad libitum access to water throughout the study period. Body weight and calorie intake were monitored once a week throughout the experiment. All procedures were carried out in accordance with the Japanese Physiological Society’s guidelines for animal care. The study protocol was approved by the Ethics Review Committee for Animal Experimentation of the Faculty of Medicine, University of Miyazaki.

Oxygen consumption, respiratory exchange ratio, locomotor activity, and body temperature

O2 consumption, respiratory exchange ratio (RER), and spontaneous locomotor activity of the SP and CP rats (aged 20 weeks; n = 4 per group) were measured by using an Oxymax apparatus (Columbus Instruments, Columbus, OH, USA) (Nakazato et al. 2000) and a rat locomotor activity recording system device (ACTIMO; Bio Research Center, Tokyo, Japan) respectively (Kitaoka et al. 2007). The body temperature of all rats was measured before they were killed; a sensor tip (measurement range: 25–50 °C; measurement error: ±0.02 °C) was inserted into the rectum and the digital signal was transferred to a thermometer (MT-1; Šenko, Tokyo, Japan).

Measurement of food remaining in the stomach

The stomachs of CP and SP rats (n = 4 per group) were removed 5 or 10 h after feeding and the wet weight of remaining food was determined.
Plasma sample preparation and analysis
Blood samples were drawn from the tail vein of CP and SP rats (17 weeks of age; n=6 per group) at 0, 30, 60, and 180 min during feeding and at 30 and 60 min afterward. The samples were collected in tubes containing a protease inhibitor cocktail (Roche), immediately centrifuged (2000 g at 4°C; 10 min), and then stored at −80°C until assay. Plasma CCK, GLP1, PYY, and insulin levels were measured by using ELISA kits (GLP1 and PYY: Yanaihara Institute, Inc., Shizuoka, Japan; CCK: Phoenix Pharmaceuticals, Belmont, CA, USA; insulin: Morinaga, Yokohama, Japan) at the six time points listed above. Blood glucose was measured by using a handheld glucometer (Bayer) at the six time points listed above.

GTT and ITT
GTT and ITT were carried out on the CP and SP rats (18 weeks of age; n=4 or 5 per group). Rats were fasted overnight for the GTT and then received intraperitoneal injections of 2 g/kg body weight glucose at 0900 h. Blood glucose and insulin levels in the blood drawn from the tail vein were measured immediately before injection (time point 0) and then at 15, 30, 60, 90, 120, and 180 min after injection. ITT was carried out 4 h after feeding. The rats received 1 U/kg body weight insulin (Novo Nordisk, Tokyo, Japan) at 1600 h. Blood glucose levels were measured immediately before injection (time point 0) and then at 30, 60, 90, 120, and 180 min after injection.

Western blotting analysis
The livers of CP and SP rats (n=5 or 6 per group) were removed 30 min after i.p. injection of saline or insulin (1 U/kg body weight) or after overnight fasting. About 30 μg total protein per sample were analyzed by using SDS-PAGE (8% acrylamide) and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Tokyo, Japan). The membranes were then probed overnight at 4°C by gentle shaking with primary antibodies against IRS2 (Cell Signaling Technology, Danvers, MA, USA), AKT (Cell Signaling Technology), phospho-AKT (Ser473; Cell Signaling Technology), SREBF1 (SREBP-1; Santa Cruz Biotechnology), ChREBP (Novus Biologicals, Littleton, CO, USA), ACC (Cell Signaling Technology), or phospho-ACC (Ser79; Cell Signaling Technology) and incubated for 1 h at room temperature with an HRP-labeled goat anti-rabbit IgG (H+L) antibody (Epitomics, Burlingame, CA, USA). Specific proteins were detected by using an enhanced chemiluminescence system (Bio-Rad) in accordance with manufacturer’s instructions.

Western blotting results for IRS2, SREBF1, ChREBP, and ACC were quantified by densitometry relative to β-actin by using the National Institutes of Health image processing software, Image J (NIH, Bethesda, MD, USA). Western blotting results for phospho-AKT and phospho-ACC were quantified by densitometry relative to total AKT and ACC, respectively, by using the Image J software.

Quantitative PCR
The livers of rats from each group (n=5 or 6 per group) were removed after overnight fasting, and total RNA was rapidly extracted with TRIzol Reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg total RNA by using the commercially available Superscript III First-Strand Synthesis System Kit (Invitrogen), and the resulting samples were subjected to quantitative PCR. Quantitative PCR for Fasn was conducted on a LightCycler system (Roche Diagnostics) by using the SYBR Premix Ex Taq mix system (Takara Bio, Inc., Shiga, Japan) and the following primer sets: Fasn: forward primer, 5’-AGGTCTGGGCAACCTCATGG-3′ and reverse primer, 5’-AGGCCGTC-GAACCTGGACAGAT-3′ and 36B4: forward primer, 5’-TCATTGTGGGAGCAGACATGTG-3′ and reverse primer, 5’-AGGTCTCCCTTGGTGAAACAAAA-3′. The relative abundances of all reaction products were normalized to the level of ribosomal protein 36B4 mRNA.

Hepatic triacylglycerol content
CP and SP rats (n=3 per group) were allowed to fast overnight and then anesthetized with 50 mg/kg pentobarbital administered intraperitoneally. For assay of the hepatic triacylglycerol content, the lipids from 25 mg tissue were extracted by treatment with 1 ml chloroform:methanol (2:1 (v/v)) mixture as described previously (Neschen et al. 2002), and triacylglycerol was quantified by using a triglyceride E kit (Wako Chemicals, Tokyo, Japan).

miR-33 levels in the liver and circulating blood
We examined the expression of miR-33, which regulates the genes involved in fatty acid metabolism and insulin signaling. We removed 25 mg of the liver of CP and SP rats for quantitative analysis of miR-33 (n=3 or 4 per group). Total RNA was extracted from tissues using the miRNeasy Mini Kit (Qiagen), and first-strand cDNA was synthesized from 1 μg total RNA and terminated by using a Mir-X miRNA
First-Strand Synthesis kit (Clontech Laboratories). Quantitative PCR for miR-33 was conducted on a LightCycler system (Roche Diagnostics) by using the SYBR qRT-PCR Kits (Clontech Laboratories). The expression of miR-33 was normalized by using the 2^{-\Delta\Delta CT} method relative to U6 small nuclear RNA (Livak & Schmittgen 2001). The \Delta CT value was the difference between the cycle threshold (CT) value of the specific miRNA and the CT value of U6 (\Delta CT = CT (miRNA) – CT (U6), \Delta\Delta CT = \Delta CT (soft diet) – \Delta CT (control), 2^{-\Delta\Delta CT} = relative expression). miR-33 expression in plasma samples (n = 3 per group) was measured by using a Toray Industries miRNA analysis system (Toray Industries, Tokyo, Japan).

Histological analysis
Pancreata from SP and CP rats (n = 3 per group) were fixed for 24 h with 3.7% formaldehyde at 4 °C, dehydrated, embedded in paraffin, and cut into 3-μm-thick sections. The sections were deated with paraffin with the use of xylene, and some sections were autoclaved to prepare them for the detection of Ki-67, which is a cell proliferation marker (Scholzen & Gerdes 2000). The sections were treated with 0.3% hydrogen peroxide for 1 h to inactivate endogenous peroxidases and then incubated for 2 days at 4 °C with an anti-insulin antiserum (Histofine; Nichirei, Tokyo, Japan) or an anti-Ki-67 antiserum (Dakocytomation, Glostrup, Denmark). After the sections had been washed for 30 min with PBS (pH 7.4), they were incubated for 2 h with a biotinylated secondary antibody solution (diluted 1:250) and then incubated for 2 h with an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The sections were stained for 10 min at room temperature with 0.02% 3,3′-diaminobenzidine and 0.05% hydrogen peroxide in 50 mM Tris buffer (pH 7.6). Islet size was determined by measuring the insulin-positive area in two different sections from each of the six pancreata by using BZ analyzer software (Keyence, Osaka, Japan).

Statistical analysis
Data were analyzed by using the Statistical Package for the Social Sciences (SPSS) software version 12.0 for Windows. All data are presented as mean ± S.E.M. and statistical significance was evaluated by using Student’s t-test and two-way ANOVA with a post hoc Tukey–Kramer test. P values <0.05 were considered significant (two-tailed tests). The area under the curve (AUC) was calculated using the trapezoid method (Matthews et al. 1990).

Results
Calorie intake, body weight, and energy expenditure of rats fed CP or SP
To investigate the influence of food texture on energy balance, we assessed the variations in intake energy and energy expenditure between the CP and SP groups. There were no significant differences in calorie intake, body weight, oxygen consumption, locomotor activity, and body temperature between the two groups; however, RER was significantly decreased (P<0.001) in the SP rats compared with the CP rats (Fig. 1A, B, C, D, E, and F).

Amount of food remaining in the stomach after feeding
The amount of food remaining in the stomachs of CP rats 5 h after feeding was 77.5 ± 6.4% of the food that they ate during the 3-h feeding period, whereas that in the stomach of SP rats was 44.3 ± 2.4% (Fig. 1G). The amount of food remaining in the stomach of CP rats 10 h after feeding was 32.4 ± 4.7% of the food that they ate during the 3-h feeding period, whereas that in the stomach of SP rats was 15.0 ± 0.6% (Fig. 1G).

Gastrointestinal hormones and glucose and insulin responses to feeding
CCK, PYY, and GLP1 responses to feeding are shown in Fig. 2A, B, and C. There were no significant differences in responses to feeding or the basal levels of CCK, PYY, and GLP1 between the two groups (Fig. 2A, B, C and Table 1). The AUC for CCK, PYY, or GLP1 did not differ between the two groups (Table 1).

The glucose and insulin responses to feeding are shown in Fig. 2D and E. Although the basal level of glucose and insulin did not differ between the groups (Table 1), the glucose levels of SP rats remained at the peak level after feeding (Fig. 2D). The AUC for glucose response in the SP group was significantly higher (P<0.05) than that in the CP group (Table 1). Furthermore, SP rats had significantly higher (P<0.05) insulin levels than CP rats at 60 min after feeding (Fig. 2E). The AUC for insulin response in the SP group was also higher (P<0.05) than that in the CP group (Table 1).

Effect of a soft-pellet diet on glucose tolerance and insulin sensitivity
GTT showed that both blood glucose and insulin levels in the SP group were significantly higher (P<0.05) at 15, 30,
60, and 120 min after glucose injection than those in the CP group (Fig. 3A and B). In the GTT, the AUCs for the glucose and insulin responses in the SP group were higher (P!0.05) than those in the CP group (Fig. 3A and B). ITT showed that blood glucose levels in the SP group after insulin injection were almost identical to those in the CP group except for at 15 min after injection. In the ITT, there were no significant differences in the AUC for glucose response between the SP and CP groups (Fig. 3C).

Expression of factors involved in insulin signaling and lipogenesis, and hepatic triacylglycerol content

To investigate whether insulin signaling in SP rats is disrupted, we examined the expression of IRS2 and the phosphorylation of AKT in the livers of the rats by using western blotting analysis. IRS2 expression in SP rats was downregulated (P<0.05) compared with that in CP rats (Fig. 4A). Phosphorylation of AKT in CP rats was significantly higher (P<0.01) after insulin injection than after saline injection, whereas there were no significant differences in the phosphorylation of AKT in SP rats between the insulin injection and saline injection groups (Fig. 4B).

We also investigated the expression of SREBF1, ChREBP, and ACC and the phosphorylation of ACC, which are important factors involved in lipogenesis, in the livers of the rats. SREBF1 (P<0.01) and ChREBP (P<0.05) expression in SP rats were significantly increased compared with those in CP rats (Fig. 4C and D). Although ACC expression in SP rats tended to increase, it did not differ significantly from that in CP rats (Fig. 4E). Phosphorylation of ACC was significantly decreased (P<0.05) in the SP group compared with the CP group (Fig. 4F).
In addition, the level of Fasn mRNA in the SP group was significantly increased \((P < 0.05)\) compared with that in the CP group (Fig. 4G). Corresponding to the increase in lipogenic factors in the livers of SP rats, the hepatic triacylglycerol content in the SP group was significantly higher \((P < 0.05)\) than that in the CP group (Fig. 4H).

### miR-33 levels in the circulating blood and liver

miR-33, which is located within the intronic sequence of the Sreb12 gene, is known to regulate IRS2 expression. miR-33 expression in the livers of SP rats was significantly increased \((P < 0.05)\) compared with that in the livers of CP rats (Fig. 4I). Furthermore, miR-33 expression in the circulating blood of SP rats was also significantly increased \((P < 0.05)\) compared with that in the circulating blood of CP rats (Fig. 4I).

### Histological analysis

In light of the apparent insulin resistance in the SP group, the islet \(\beta\)-cell response to the SP diet was quantified by measuring the area of insulin-positive cells in the pancreata of the rats. The area of insulin-positive cells in SP rats was significantly increased \((P < 0.05)\) compared with that in CP rats (Fig. 5A and B). In addition, the number of Ki-67-positive cells in the islets of the SP group was also significantly increased compared with the numbers for the CP group (Fig. 5C).

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**Figure 2**

Levels of gastrointestinal hormones, glucose, and insulin after feeding in rats fed soft pellets or control pellets (CP). (A) Alteration in plasma cholecystokinin (CCK) levels \((n = 6\) per group). (B) Alteration in plasma peptide YY (PYY) levels \((n = 6\) per group). (C) Alteration in plasma glucagon-like peptide 1 (GLP1) levels \((n = 6\) per group). (D) Alteration in blood glucose levels \((n = 6\) per group). (E) Alteration in plasma insulin levels \((n = 6\) per group). \(* P < 0.05\) vs rats fed CP.
Table 1  Comparison of the CCK, PYY, GLP1, glucose, and insulin response to feeding in rats fed control pellets (CP) or soft pellets (SP). Values are expressed as means ± S.E.M.

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<th>CP</th>
<th>SP</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>CCK</strong></td>
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<tr>
<td>Basal (ng/ml)</td>
<td>3.04±0.20</td>
<td>2.94±0.14</td>
<td>0.70</td>
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<tr>
<td>Incremental AUC (ng/ml×4 h)</td>
<td>834.35±18.83</td>
<td>815.04±9.90</td>
<td>0.39</td>
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<tr>
<td><strong>PYY</strong></td>
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<tr>
<td>Basal (ng/ml)</td>
<td>0.47±0.04</td>
<td>0.46±0.04</td>
<td>0.84</td>
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<tr>
<td>Incremental AUC (ng/ml×4 h)</td>
<td>122.76±5.92</td>
<td>118.61±4.65</td>
<td>0.59</td>
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<td><strong>GLP1</strong></td>
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<tr>
<td>Basal (ng/ml)</td>
<td>33.41±4.28</td>
<td>32.19±2.29</td>
<td>0.81</td>
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<tr>
<td>Incremental AUC (ng/ml×4 h)</td>
<td>8135.75±401.34</td>
<td>7759.35±404.80</td>
<td>0.55</td>
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<tr>
<td><strong>Glucose</strong></td>
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<tr>
<td>Basal (mg/dl)</td>
<td>97.58±1.87</td>
<td>100.11±2.25</td>
<td>0.53</td>
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<tr>
<td>Incremental AUC (mg/dl×4 h)</td>
<td>26 931.00±298.51</td>
<td>27 711.67±121.61</td>
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<tr>
<td><strong>Insulin</strong></td>
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<tr>
<td>Basal (ng/ml)</td>
<td>1.52±0.21</td>
<td>1.82±0.39</td>
<td>0.40</td>
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<td>Incremental AUC (ng/ml×4 h)</td>
<td>526.12±35.72</td>
<td>657.02±48.17</td>
<td>0.05</td>
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Discussion

In this study, we investigated the metabolic features of rats fed a SP diet. The body weight, calorie intake, oxygen consumption, locomotor activity, and body temperature of SP rats were similar to those of CP rats; however, RER, an indicator of the type of fuel metabolized for energy utilization, in SP rats was significantly lower than that in CP rats. These findings indicate that rats fed SP utilize fat for energy metabolism more than do rats fed CP. We have also preliminarily examined adiposity of the visceral or subcutaneous fat of SP and CP mice using computed tomography (CT) (Latheta LCT-200; Hitachi-Aloka Medical, Tokyo, Japan) and Latheta LCT-200 software. The mice of each group was scanned at 2-mm intervals between the proximal end of L1 and the distal end of L6; the visceral or subcutaneous fat tissue was identified automatically on the CT images. Our preliminary data showed that adiposity of either the visceral or subcutaneous fat of SP mice increased compared with that of CP mice (visceral fat: CP 10.0±0.59%, SP 14.6±1.23%, P<0.05; subcutaneous fat: CP 3.73±0.58%, SP 11.7±2.47%, P<0.05; n=3 or 4 per group), even though there were no significant differences in body weight or energy intake between the two groups (Hasegawa K, Akieda-Asai S, Kawasaki Y & Date Y, unpublished observations). To elucidate the relationship between food texture and energy metabolism, it will be necessary to investigate the body composition of SP and CP rats in terms of adiposity or muscle volume.

In this study, SP rats showed postprandial hyperglycemia, glucose intolerance, insulin resistance, and increased lipid synthesis, even though they did not show visible obesity. In general, glucose intolerance is present in prediabetic or diabetic states, and an increase in lipid synthesis is often caused by the disruption of insulin signaling (Farese et al. 2005). Considering that prediabetic or diabetic states might be induced by the chronic inflammatory state, we preliminarily examined the mRNA expression of cytokines such as TNFα, IL6, or IL1β in the visceral fat and liver of SP or CP rats; however, we did not find any significant differences in the expression of these cytokines between the two groups (data not shown). Therefore, to elucidate the mechanism of how SP induced postprandial hyperglycemia and insulin resistance, we investigated the speed that the nutrient load was eliminated from the stomach. The food remaining in the stomachs of SP rats was significantly less than that in the stomachs of CP rats, implying that in SP rats the nutrient load reaches the intestine faster than it does in CP rats. In humans, it is known that even when the food stuff itself is the same, postprandial glucose levels vary according to the manner in which the food was processed, e.g., boiled potatoes increase postprandial blood glucose compared with baked potatoes (Atkinson et al. 2008). These data indicate that foods that are quickly digested and absorbed cause postprandial hyperglycemia. Together, our results indicate that the texture of absorbable foods such as SP may considerably contribute to sustained hyperglycemia after feeding.

It is known that several gastrointestinal hormones such as CCK, PYY, or GLP1 function as anorectic signals or regulators for glucose metabolism. Therefore, in this study, we investigated these hormones’ profiles in CP and SP rats. As shown in Fig. 2, SP rats did not show abnormal...
releasing patterns of the anorectic gastrointestinal hormones CCK and GLP1, indicating that the profile of these hormones does not contribute to the metabolic disorders of rats fed SP. On the other hand, PYY levels in SP rats at 240 min increased. A study of the PYY profile in humans has shown that in response to the ingestion of nutrients, plasma PYY levels increase within 15 min, peak at around 60 min, and remain elevated for up to 6 h (Adrian et al. 1985). The initial increase is thought to be induced via neural or endocrine mechanisms (Batterham et al. 2003), and the sustained release is thought to be due to the direct effects of the gut contents on PYY-producing cells mainly located in the lower intestine (Imamura 2002). Together, PYY levels of SP and CP rats after 240 min may increase more. In any case, considering that in SP rats the nutrient load is rapidly eliminated from the stomach and then reaches the intestine faster than it does in CP rats, the increase in PYY level in SP rats at 240 min may be due to the fast speed of SP passing the lower intestine.

To further investigate the mechanism of glucose metabolism in SP rats, we examined insulin signaling in the liver. Western blotting analysis showed that the level of IRS2 in the livers of SP rats is significantly decreased and that the phosphorylation of AKT does not increase even after insulin administration. These findings indicate that rats raised on an SP diet for 14 weeks have already acquired defective insulin signaling. Insulin is a strong activator of the lipogenic pathway through the activation of lipogenic transcription factors such as SREBF1 and ChREBP (Capeau 2008). In this study, we showed that SREBF1 and ChREBP

Figure 3
Blood glucose and insulin levels as determined by glucose tolerance testing (GTT) and insulin tolerance testing (ITT) in rats fed soft pellets or control pellets (CP). (A) Blood glucose level assessed with GTT (n=4 or 5 per group). (B) Plasma insulin level assessed with GTT (n=4 or 5 per group). (C) Blood glucose level assessed with ITT (n=4 or 5 per group). *P<0.05 vs rats fed CP. AUC, area under the curve.
levels in SP rats are markedly increased compared with those in CP rats. SREBF1 and ChREBP are known major mediators of insulin and glucose signaling in the liver respectively (Foretz et al. 1999, Ishii et al. 2004). Furthermore, these mediators are known to regulate lipogenic gene expression (Foufelle & Ferre´ 2002). ACC, a lipogenetic enzyme, catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, which is used by Fasn for lipogenesis. Indeed, leptin-deficient ob/ob mice have greater hepatic triacylglyceride content and levels of lipogenesis markers, including ACC (Perfield et al. 2012).

In addition, phosphorylated ACC inhibits the catalysis of acetyl-CoA to malonyl-CoA. In this study, ACC expression tended to increase and the phosphorylation of ACC dramatically decreased in SP rats compared with that in CP rats, resulting in increases in the mRNA levels of Fasn and hepatic triacylglycerol content. These data indicate that postprandial hyperglycemia in SP rats induced hyperinsulinemia, resulting in insulin resistance and lipogenesis.

On the basis of our data, we considered SP rats to be a model of diabetes without visible obesity due to hyperphagia. Therefore, to investigate biomarkers involved in the prediabetic or diabetic condition without obesity, we screened miRNAs in the circulating blood of SP and CP rats (data not shown). In SP rats, the levels of miRNAs

Figure 4
Insulin signaling and lipogenesis in the livers of rats fed soft pellets or control pellets (CP). (A) Insulin receptor substance 2 (IRS2) levels assessed by western blotting analysis (n = 5 or 6 per group). (B) Phosphorylation levels of AKT assessed by western blotting analysis with or without insulin injection (n = 5 or 6 per group). (C) Sterol regulatory element-binding transcription factor 1 (SREBF1) levels assessed by western blotting analysis (n = 5 or 6 per group). (D) Carbohydrate responsive element-binding protein (ChREBP) levels assessed by western blotting (n = 5 or 6 per group). (E) Acetyl-CoA carboxylase (ACC) levels assessed by western blotting analysis (n = 5 or 6 per group). (F) Phosphorylation levels of ACC assessed by western blotting analysis (n = 5 or 6 per group). (G) Fatty acid synthase (Fasn) mRNA levels assessed by quantitative PCR (n = 5 or 6 per group). (H) Hepatic triacylglycerol levels. (I) Hepatic and peripheral blood miR-33 levels (n = 3 in each group). *P < 0.05 vs rats fed CP; **P < 0.01 vs rats fed CP (except B, **P < 0.01 vs rats fed CP without insulin injection).
related to insulin resistance, such as miR-122, miR-200a, and miR-223 (Yang et al. 2012, Zhuang et al. 2012, Crépin et al. 2014), did not differ from those of CP rats. However, the level of miR-33 was increased in SP rats. miR-33 has been reported to repress the genes involved in cholesterol export (Horie et al. 2010). Recent studies have also revealed that miR-33 has a binding site in the 3’-UTRs of *Irs2*, thereby decreasing *Irs2* expression and inhibiting the phosphorylation of AKT (Dávalos et al. 2011, Fernández-Hernando & Moore 2011). Our results show that miR-33 levels are significantly increased in both the liver and circulating blood of SP rats. This indicates that the disruption of insulin signaling in SP rats may be induced at least in part by an increase in miR-33. In addition, the finding that miR-33 in the circulating blood of SP rats is increased indicates that miR-33 may be a biomarker for diabetic patients without obesity. To clarify the role of miR-33 in the diabetic state, further investigations into the diabetic stage at which the increase in circulating miR-33 is detectable are needed.

Pancreatic β-cells operate to maintain blood glucose levels within a narrow range by secreting insulin in response to glucose. Food intake or glucose infusion increases β-cell replication and the β-cell mass is adaptively regulated in response to changes in insulin sensitivity (Bonner-Weir 1994, Alonso et al. 2007). Sustained hyperglycemia and hyperinsulinemia induce insulin resistance, which results in β-cell hyperplasia. Leptin-deficient *ob/ob* mice develop insulin resistance up to 6 months of age and show marked β-cell hyperplasia (Tomita et al. 1992, Bock et al. 2003). Our histological data show that the size of the pancreatic islets in the SP group was significantly increased compared with that in the CP group. Furthermore, immunoreactivity of the proliferative marker *Ki-67* was markedly increased in some islets in the SP group, indicating that β-cell replication in SP rats is dramatically increased. Even though rats fed SP never showed visible obesity, they had a diabetic state with insulin resistance and islet β-cell hyperplasia. These findings indicate that a soft food texture induces postprandial hyperglycemia, resulting in insulin resistance and islet β-cell hyperplasia. Further investigation is needed to clarify which molecular mechanisms act on islet hyperplasia and/or function when rats are fed a SP diet.

In summary, we demonstrate that a 14-week SP diet does not induce obesity in rats, but that it does cause hyperinsulinemia and insulin resistance, resulting from sustained high glucose levels after feeding. Different food textures may therefore play an important role in lifestyle related diseases. To elucidate the factors involved in the pathophysiology of SP-induced metabolic disorders, further investigation of the molecular and functional mechanisms that link the digestive system to glucose homeostasis is required. These advanced studies may lead
to appropriate interventions for non-obese patients with metabolic syndrome.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
C-R B and K H performed all the experiments. S A-A performed molecular biological analyzes and contributed to overseeing the experiments. Y K and K S were responsible for animal care and conducted the animal experiments. Y-S C provided advice on experimental design. Y D managed the experiments and experimental design. All authors contributed to writing the manuscript.

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