Trehalose Prevents Adipocyte Hypertrophy and Mitigates Insulin Resistance in Mice with Established Obesity

Chikako Arai, Masaki Miyake, Yohsuke Matsumoto, Akiko Mizote, Chiyo Yoshizane, Yohko Hanaya, Kazuhiro Koide, Mika Yamada, Toshiharu Hanaya, Shigeyuki Arai and Shigeharu Fukuda

R&D Center, Hayashibara Co. Ltd., 675–1 Fujisaki, Naka-ku, Okayama 702–8006, Japan
(Received December 26, 2012)

Summary Our group recently demonstrated that simultaneous administration of trehalose with a high-fat diet (HFD) suppresses adipocyte hypertrophy and mitigates insulin resistance in mice. For the present study, we hypothesized that similar effects of trehalose would be observed in mice with previously-established obesity. Obese mice were fed a HFD and drinking water containing 0.3 or 2.5% (weight/volume) trehalose or distilled water (DW) ad libitum for 8 wk. After 7 wk intake of a HFD and trehalose, fasting serum insulin levels and homeostasis model assessment-insulin resistance (HOMA-IR) in the 0.3% Tre/HFD group were significantly lower than those in the DW/HFD group ($p<0.05$). After 8 wk of treatment, mesenteric adipocytes in the 0.3% Tre/HFD group showed significantly less hypertrophy than those in the DW/HFD group. Mechanistic analysis indicated that levels of high molecular weight (HMW) adiponectin in the serum of the 0.3% Tre/HFD group were significantly higher than those in the DW/HFD group. The expression levels of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) messenger RNA (mRNA) in muscle were also significantly increased by trehalose intake. Our data therefore suggest that administration of trehalose to obese mice mitigates insulin resistance by suppressing adipocyte hypertrophy and increasing serum HMW adiponectin, resulting in upregulation of IRS-1, and IRS-2 expression in muscle. These results further suggest that trehalose is a functional saccharide that may be used to prevent the progression of insulin resistance.

Key Words obese, trehalose, adipocyte hypertrophy, insulin resistance, mice

In recent years, there has been explosive growth in the number of people developing features of metabolic syndrome. Visceral adipose tissue levels are well correlated with the metabolic syndrome that leads to the development of insulin resistance, type 2 diabetes, and cardiovascular disease (1). Adipose tissue is essential for appropriate glucose and lipid metabolism as well as energy homeostasis (2). However, in rats, adipocyte hypertrophy is strongly linked to peripheral insulin resistance, independent of total body fat (3). Thus, suppression of adipocyte hypertrophy is a key strategy for improvement of insulin resistance.

Trehalose is a non-reducing disaccharide composed of two d-glucose residues. Trehalose has been shown to stimulate insulin secretion less potently than glucose in oral saccharide tolerance tests in humans (4). Given this hypoinsulminemic effect of trehalose, we have previously shown that simultaneous intake of trehalose and a high-fat diet (HFD) suppresses adipocyte hypertrophy and mitigates insulin resistance in mice (5). These effects were not elicited by glucose, maltose, high-fructose corn syrup (HFCS), or fructose. Mechanistic analysis suggested that trehalose exhibits its suppressive effects by decreasing insulin secretion and down-regulating mRNA expression of monocyte chemoattractant protein-1 (MCP-1).

As the next step, we hypothesized that these beneficial effects of trehalose may be observed even in mice with established HFD-induced obesity. To test this hypothesis, we evaluated the effects of trehalose intake on adipocyte hypertrophy and insulin resistance using mice who developed obesity after feeding with a HFD. The experiments initially involved feeding mice with a HFD for 4 wk and then treating these obese mice with drinking water containing trehalose for 7–8 wk. As a result, we found that trehalose intake suppressed adipocyte hypertrophy and mitigated insulin resistance even after the development of obesity. These findings suggest that trehalose can be used as a unique functional saccharide providing a novel dietary approach for people with the metabolic syndrome.

MATERIALS AND METHODS

Animals. Six-week-old female C57BL/6N mice were obtained from Charles River (Yokohama, Japan) and fed a standard diet (STD, NMF; Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum for 1 wk. The mice were kept in a temperature-controlled room with a 12-h light cycle. This study was approved by the Laboratory Animal Care Committee of the Hayashibara Co., Ltd., and all animal experiments were conducted in accordance with the Guidelines for Care and Use of Labora-
The experimental design. The experimental protocol is illustrated in Fig. 1. A total of 30 mice were used in this study. After 1 wk of acclimatization, 7-wk-old female mice were divided randomly into 4 groups that were matched for body weight. Three groups of mice were fed a commercial HFD (HFD32, 56.7% calories from fat; CLEA Japan, Inc., Tokyo, Japan) for 4 wk to induce accumulation of visceral adipose tissue and insulin resistance as reported previously (6, 7). Control mice were fed a commercial low-fat diet (LFD, 10.1% calories from fat; CLEA Japan, Inc.). The ingredient composition of the experimental diets is shown in Table 1. All mice were given ad libitum access to drinking water. After 4 wk of HFD feeding, HFD mice were divided into 3 groups that were matched for homeostasis model assessment-insulin resistance (HOMA-IR) and body weight. Then, mice in the trehalose administration groups were given ad libitum access to drinking water containing 0.3 or 2.5% (weight/volume) trehalose (Tre, 98% purity, Anhydrors, Hayashibara Co., Ltd., Okayama, Japan) along with continued access to the HFD (n = 8, for each group). Two control groups of mice were given the HFD and distilled water (DW), or the LFD and DW (n = 7, for each group). Three to four mice were housed together in one cage. Food and water were replaced and their intake monitored every second day. Body weights were recorded weekly throughout the experimental period. After 7 wk of trehalose intake, the mice were fasted for 17 h, followed by performance of an oral glucose tolerance test (OGTT). Blood glucose and insulin levels were measured and used to calculate HOMA-IR.

After 8 wk of trehalose treatment, the mice were euthanized under ether anesthesia. The adipose tissues were weighed, and blood samples collected from the abdominal vena cava under non-fasting conditions using capiject tubes (CJ-AS, Terumo Corp., Tokyo, Japan), followed by centrifugation at 4°C for 5 min at 10,000 rpm. To measure PAI-1 and GLP-1, 120 μL of blood was collected into chilled tubes containing EDTA (capiject, CJ-NA, Terumo Corp) and aprotinin (500 kIU/mL; blood: Wako Pure Chemical Industries, Ltd., Tokyo, Japan) and then centrifuged at 4°C for 10 min at 3,000 rpm. The samples were stored at −80°C until measurement of lipids, incretin hormones, and adipocytokines. Serum triglycerides, non-esterified fatty acids (NEFA), and total cholesterol were measured using the Triglyceride E-test kit, NEFA C-test kit, and Cholesterol E-test kit (Wako Pure Chemical Industries, Ltd.), respectively. ELISA kits were used to measure peptides or proteins as follows: Serum total glucose-dependent insulinotropic polypeptide (GIP) (EZRMGIP-55K, Millipore, MA), serum monocyte chemoattractant protein-1 (MCP-1) (MJE00, R&D Systems, Inc., Minneapolis, MN), plasma total glucagon-like peptide 1 (GLP-1) (EZGLP1T-36K, Millipore, Billerica, MA), plasma total plasminogen activator inhibitor-1 (PAI-1) (IMPAK1T-TOT, INR, Innovative Research, Novi, MI), and serum high molecular weight.
Table 2. Summary of the sequences of gene-specific PCR primers and the length of production used in the experiments.

| Target   | GenBank accession no. | Primer sequence (5'-3') | Length (bp) |
|----------|-----------------------|-------------------------|-------------|
| CYP A    | NM_008907             | CGAGCTGTTTGACAGCAGAAAG  | 226         |
| IRS1     | NM_010570             | CAGCAAGACGAAATGGAACGCT  | 247         |
| IRS2     | NM_001081212         | GTAGTTCAGGCTGCCCTGTC    | 190         |
| GLUT4    | NM_009204             | CAGGACCTGTTTGACAGCAGAA  | 135         |
| UCP2     | NM_011671             | GCCAATCGTTGACCTGCT      | 181         |

CYP A indicates cyclophilin A.

Table 3. Body and adipose weights, and serum concentrations of lipids, adipocytokines and incretin in obese mice after 8 wk of trehalose intake.

|                | DW/LFD                        | DW/HFD                        | 0.3% Tre/HFD               | 2.5% Tre/HFD               |
|----------------|-------------------------------|-------------------------------|----------------------------|----------------------------|
| Body weight (g)| 27.5±0.7†                    | 42.4±1.0                      | 41.0±1.3                   | 40.1±0.8                   |
| Adipose weight/body weight (mg/g) |                               |                               |                           |                           |
| Mesenteric adipose| 15.8±1.4†                    | 25.8±1.2                      | 27.9±2.7                   | 26.6±1.8                   |
| Perirenal adipose| 28.2±1.7†                    | 45.7±2.7                      | 42.1±2.1                   | 40.9±2.4                   |
| Retroperitoneal adipose| 36.6±2.9†                   | 63.4±1.7                      | 65.4±0.8                   | 65.2±1.6                   |
| Visceral adipose| 81.5±5†                      | 135±2                         | 136±4                      | 133±2                      |
| Serum          |                               |                               |                           |                           |
| Triglyceride (mg/dL) | 76±5†                       | 98±9                          | 87±4                       | 107±5                      |
| Non-esterified fatty acid (mEq/L) | 0.611±0.028†                | 0.869±0.031                   | 0.771±0.017                | 0.881±0.037                |
| Total cholesterol (mg/dL) | 88±2†                       | 168±3                         | 153±6                      | 163±5                      |
| MCP-1 (pg/mL)  | 100±6                        | 99±7                          | 107±6                      | 98±3                       |
| GIP (pg/mL)    | 126±8†                       | 194±29                        | 150±14                     | 153±15                     |
| Plasma         |                               |                               |                           |                           |
| Total GLP-1 (pm) | 41.2±4.0                   | 23.8±5.3                      | 36.5±12.9                  | 48.1±7.5†                  |
| PAI-1 (ng/mL)  | 2.03±0.28†                   | 4.04±0.55                     | 4.26±0.55                  | 3.35±0.23                  |

Values are means±SE of 7–8 mice per group. Eight mice in the Tre/HFD groups and 7 mice in the DW/HFD and DW/LFD groups. Statistical analysis was performed by Dunnett’s post hoc test vs DW/HFD as the control. Values with † show statistically significant (p<0.05) difference, †† show statistically significant (p<0.01) compared with values in the DW/HFD groups.

weight adiponectin (HMW adiponectin) (AKMAN-011, Shibayagi, Gunma, Japan).

Calculation of energy intake. The energy densities of HFD, LFD, and drinking water containing 2.5% trehalose or 0.3% trehalose were 20.9, 15.2, 0.418, and 0.0503 kJ per gram, respectively. Based on these data, mean energy intake per mouse in each group was calculated using the formulas below.

Energy intake (kJ/mouse/d):
- Mice taking HFD and 2.5% trehalose: Food intake (g)×20.9 (kJ)+Water intake (g)×0.418 (kJ)
- Mice taking HFD and 0.3% trehalose: Food intake (g)×20.9 (kJ)+Water intake (g)×0.0503 (kJ)
- Mice taking HFD and DW: Food intake (g)×20.9 (kJ)+Water intake (g)×0 (kJ)
- Mice taking LFD and DW: Food intake (g)×15.2 (kJ)+Water intake (g)×0 (kJ)

Oral glucose tolerance test and HOMA-IR index. After 8 wk of HFD and trehalose treatment, the mice were fasted for 17 h. DW without trehalose was given during the fasting period. Blood was drawn from the eye-ground vein immediately prior to the glucose tolerance test. A glucose solution of 2 g/kg body weight was then administered by gastric intubation. Blood was collected 30, 60, and 120 min after glucose administration. Serum glucose and insulin levels were determined using a Glucose CII Test kit (Wako Pure Chemical Industries, Ltd.) and a Morinaga high sensitivity mouse insulin ELISA kit (Morinaga, Kanagawa, Japan), respectively. Fasting blood glucose and insulin levels were used to calculate the HOMA-IR index according to the following equation: fasting blood glucose (mg/dL)×fasting insulin (µU/mL)/405

Histological analysis of mesenteric adipocyte size. Mesenteric adipose tissue was fixed with 10% buffered formalin and embedded in paraffin. Sections were deparaaffinized in xylene, stained with hematoxylin and eosin, and then examined by light microscopy.

Photographs of mesenteric adipose tissue were taken of 5 random areas per sample at 200×magnification. The number of adipocytes per area (0.290 mm²) was counted. Mean adipocyte size was calculated from the number of adipocytes in each of the 5 areas for each sample. The average number of adipocytes in the DW/
LFD, DW/HFD, 0.3% Tre/HFD, and 2.5% Tre/HFD groups was 652, 312, 369, and 369 cells, respectively.

RNA extraction and quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from mesenteric adipose tissue and muscles along the femur using the RNeasy Lipid Tissue Mini kit (QIAGEN, Tokyo, Japan) and DNase (QIAGEN) according to the manufacturer’s instructions. First-strand complementary DNA (cDNA) synthesis from 0.5 μg RNA was then performed using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). PCR primers were designed using Primer 3, and synthesized by Sigma-Aldrich (Hokkaido, Japan). All PCR primer sequences are shown in Table 2. Synthesized cDNA was mixed with SYBR Green Master Mix (Roche, Mannheim, Germany) and gene-specific primers, and real-time PCR was performed using a LightCycler 480 system (Roche). The relative cDNA copy numbers were computed using data obtained from a serial dilution of a representative sample of each target gene. For quantitative precision, the expression of each gene was normalized to that of the housekeeping gene Cyclophilin A (CYP A). Changes in gene expression in trehalose-exposed mice were expressed as fold change relative to the mean values for DW/HFD mice.

Statistical analyses. The data were expressed as averages ± SE. Statistically significant effects of trehalose were determined using Dunnett’s post hoc test vs. DW/HFD as the control (JMP9.0: SAS, Cary, NC). Non-parametric data were analyzed by the Steel test (JMP9.0: SAS). p-values less than 0.05 were considered significant.

RESULTS

Effects of trehalose on energy intake, body weight, and tissue weight in mice fed a HFD

Energy intake in the DW/LFD, DW/HFD, 0.3% Tre/HFD, and 2.5% Tre/HFD groups during the experimen-
Trehalose Prevents Adipocyte Hypertrophy

The period was 41.4 ± 1.0, 56.9 ± 0.7, 56.9 ± 1.1, and 54.8 ± 1.2 kJ/mouse/d, while trehalose intake was 0, 0, 7.5 and 62.5 mg/mouse/d, respectively. There was no significant difference in energy intake between the HFD groups.

Mice fed a HFD for 12 wk became obese relative to LFD-fed mice, exhibiting significantly increased energy intake, body weight, and mesenteric, perirenal, and retroperitoneal adipose tissue weight (Table 3). Perirenal adipose tissue weights in the 2.5% Tre/HFD group tended to be lower than those in the DW/HFD group, but the differences were not statistically significant. Mesenteric adipose tissue weights in the Tre/HFD groups were comparable to those in the DW/HFD group.

Measurement of mesenteric adipocyte size

We next measured adipocyte size in mesenteric adipose tissue. It is well known that mesenteric adipocyte tissue has greater lipolytic activity than other adipose tissues (8). Moreover, Keno et al. reported a significant correlation between mesenteric fat volume and fasting glucose levels in rats fed a high-sucrose diet (9).

In our experiments, HFD feeding induced a significant increase (p < 0.01) in the size of mesenteric adipocytes relative to the DW/LFD group. Mesenteric adipocyte hypertrophy was observed in the DW/HFD group (average size 4.748 ± 208.0 μm²), and mesenteric adipocytes in this group were twice as large as in the DW/LFD group (2.277 ± 107.2 μm²). Administration of 0.3% trehalose significantly suppressed adipocyte hypertrophy (adipocyte size in the 0.3% Tre/HFD group was: 4.019 ± 169.5 μm², p < 0.05) compared with the DW/HFD group, and administration of 2.5% trehalose tended to suppress adipocyte hypertrophy (adipocyte size for 2.5% Tre/HFD group was: 4.098 ± 226.8 μm², p = 0.06) (Fig. 2A), although administration of trehalose did not affect total abdominal adipose tissue mass (Table 3). Trehalose-induced suppression of adipocyte hypertrophy was also confirmed by histological observation (Fig. 2B).

Oral glucose tolerance test

After 7 wk of trehalose intake, mice were subjected to an oral glucose tolerance test (OGTT) (2 g/kg body weight, Fig. 3). HFD feeding for 11 wk induced an elevated glycemic response to glucose compared with mice fed a LFD. All HFD groups had significantly higher fasting blood glucose levels than the DW/LFD group.

Fig. 3. Oral glucose tolerance test. After 7 wk of trehalose intake in mice with established obesity, an OGTT (2 g/kg) was conducted after a 17 h fast. The blood glucose levels 0, 30, 60, and 120 min after glucose administration are shown. Values represent the averages ± SE of 8 mice in the Tre/HFD groups and 7 mice in the DW/HFD and DW/LFD groups. Statistical analysis was performed using Dunnett’s post hoc test vs. the DW/HFD group as the control. Blood glucose levels 30 min after administration glucose were not homogeneously distributed, so statistical analysis was carried out using the non-parametric Steel test. Blood glucose levels 0 and 60 min after glucose administration were significantly lower in the DW/LFD group than the DW/HFD group (* p < 0.01).

Fig. 4. Serum insulin levels at 0, 30, 60, and 120 min during the OGTT. The effects of trehalose intake on serum insulin levels in HFD-fed mice were examined in an OGTT (2 g/kg) after a 17 h fast. The values represent the averages ± SE of 8 mice in the Tre/HFD groups and 7 mice in the DW/HFD and DW/LFD groups. Statistical analysis was performed using Dunnett’s post hoc test vs. the DW/HFD group as the control. At 0 min: 0.3% Tre/HFD group vs. the DW/HFD group, ** p < 0.01. At 120 min: 2.5% Tre/HFD group vs. the DW/HFD group, * p < 0.05.
Arai C et al.

398

(p<0.01), but blood glucose levels 30, 60, and 120 min after oral glucose loading were comparable among the HFD groups (Fig. 3). Blood glucose levels 60 min after oral glucose loading were 238.8±19.1, 327.7±23.6, 317.7±11.9, and 360.8±15.3 mg/dL in the DW/LFD, DW/HFD, 0.3% Tre/HFD, and 2.5% Tre/HFD groups, respectively. Although blood glucose levels in the 2.5% Tre/HFD group were slightly higher than those in the 0.3% Tre/HFD and DW/HFD groups, these differences were not statistically significant.

Serum insulin levels during OGTT

Groups consuming the HFD had significantly increased (p<0.01) insulin secretion in response to oral glucose intubation. Fasting serum insulin levels were 0.88±0.17, 2.82±0.47, 1.46±0.18, and 1.82±0.25 μU/mL in the DW/LFD, DW/HFD, 0.3% Tre/HFD, and 2.5% Tre/HFD groups, respectively. The 0.3% Tre/HFD group had significantly (p<0.01) lower fasting insulin levels than the DW/HFD group. Levels of fasting insulin in the 2.5% Tre/HFD group tended to be lower than those in the DW/HFD group (p=0.06) (Fig. 4). Furthermore, the kinetics of serum insulin levels in the Tre/HFD groups were lower than those in the DW/HFD group. As shown in Fig. 4, after 120 min of glucose loading, serum insulin levels in the DW/LFD, DW/HFD, 0.3% Tre/HFD, and 2.5% Tre/HFD groups were 1.46±0.36, 3.66±0.46, 2.43±0.22, and 2.16±0.22 ng/mL, respectively. Insulin levels in the 2.5% Tre/HFD group were significantly (p<0.05) lower than those in the DW/HFD group.

Homeostasis model assessment-insulin resistance

HOMA-IR was calculated as: fasting blood glucose (mg/dL)×fasting insulin (μU/mL)/405. After 4 wk of HFD feeding, the HFD group was divided into 3 groups matched for HOMA-IR and body weight. HOMA-IR was assessed after 7 wk of HFD and trehalose intake. Values represent data from each mouse. There were 8 mice in the Tre/HFD groups and 7 mice in the DW/HFD and DW/LFD groups. Column descriptions: pre: HOMA-IR levels calculated after 4 wk of HFD feeding before initiation of trehalose administration. 7W: HOMA-IR indexes calculated after 7 wk of a HFD and trehalose intake, determined by the OGTT. Statistical analysis was performed using Dunnett’s post hoc test vs. the DW/HFD group as the control. The HOMA-IR index was significantly lower in the 0.3% Tre/HFD group than in the DW/HFD group at 7 wk (p<0.05).

Effects of trehalose intake on serum HMW adiponectin.

After 8 wk of HFD and trehalose intake mice with established obesity had their serum levels of HMW adiponectin measured by ELISA. Values represent data from each mouse. There were 8 mice in the Tre/HFD groups and 7 mice in the DW/HFD and DW/LFD groups. As the values were not distributed homogeneously, statistical analysis was performed using the non-parametric Steel test. HMW adiponectin levels were significantly higher in the 0.3% Tre/HFD group than in the DW/HFD group (p<0.01).

HOMA-IR. However, administration of trehalose suppressed this elevation of HOMA-IR (Fig. 5). The HOMA-IR index in the 0.3% Tre/HFD group (11.4±1.9) was significantly lower than that in the DW/HFD group (24.9±6.7) (p<0.05), and the index in the 2.5% Tre/HFD group (16.4±3.2) tended to be lower than that in the DW/HFD group (Fig. 5). These results indicate that trehalose can mitigate insulin resistance in HFD-fed mice.

**Effects of trehalose on serum concentrations of lipids, HMW adiponectin, GIP, and GLP-1**

Mice fed a HFD for 12 wk exhibited significantly elevated circulating levels of NEFA compared to LFD-fed
Trehalose Prevents Adipocyte Hypertrophy

The dysfunction and mitochondrial stress (in adipocyte function, particularly endoplasmic reticulum dysfunctions and mitochondrial stress) can lead to the storage of excess energy in adipocytes, which is a very important factor for mitigating insulin resistance, without changing total adipose weight.

In this study, the 0.3% Tre/HFD group had significantly lower serum fasting insulin levels than the DW/HFD group. Moreover, the kinetics of serum insulin levels during OGTT in the Tre/HFD group remained low compared with those in the DW/HFD group. Based on increased expression of both IRS-1 and IRS-2 mRNA in muscles of the Tre/HFD groups, we consider that lower fasting insulin levels in the Tre/HFD group is due to reduction of insulin accumulation in bloods by mitigating insulin resistance in muscles. It seems likely that the reduction of insulin accumulation further resulted in suppression of adipocyte hypertrophy.

Furthermore, administration of trehalose suppressed the elevation of HOMA-IR. The HOMA-IR index in the 0.3% Tre/HFD group was significantly lower than that in the DW/HFD group (p<0.05), and the index in the 2.5% Tre/HFD group tended to be lower than that in the DW/HFD group.

These results were consistent with our previous observations that simultaneous administration of 2.5% trehalose with HFD inhibited adipocyte hypertrophy and mitigated insulin resistance (5). We therefore confirmed our hypothesis proposed in the introduction section and concluded that administration of trehalose, either before or after the development of obesity, prevents adipocyte hypertrophy.

**DISCUSSION**

An imbalance between energy intake and utilization can lead to storage of excess energy in adipocytes, resulting in adipocyte hypertrophy. The development of adipocyte hypertrophy is accompanied by abnormalities in adipocyte function, particularly endoplasmic reticulum dysfunction and mitochondrial stress (10). The resulting intracellular and systemic changes induce dysregulation of adipokine production such as tumor necrosis factor-α (TNF-α), PAI-1 (11), and MCP-1 (12), which result in interference with insulin signaling. Skurk et al. (13) investigated the effect of adipocyte size on adipokine production and secretion, and showed that secretion of proinflammatory adipokines, such as IL-6, IL-8, and MCP-1, was increased significantly in very large adipocytes compared with small adipocytes. Olefsky also reported that small adipocytes had greater uptake of glucose than large adipocytes (14). Suppression of adipocyte hypertrophy is therefore a very important strategy for mitigating insulin resistance.

In our study, intake of 0.3% trehalose for 8 wk caused significant suppression of adipocyte hypertrophy (p<0.05), while intake of 2.5% trehalose tended to suppress adipocyte hypertrophy in mice with established obesity (p=0.06). As there was no difference in the weight of total visceral fat between the Tre/HFD and DW/HFD groups, we estimated that the number of small adipocytes increased in the Tre/HFD group. A similar observation was reported by Okuno et al. (15) who showed that administration of thiazolidinediones did not change the total weight of white adipose tissue, but increased the number of small adipocytes and decreased the number of large adipocytes. These changes were shown to normalize the expression levels of FFA and TNF-α, and may have contributed to an improvement in insulin sensitivity. Although we did not measure serum TNF-α in our study, we showed that NEFA levels tended to be lower in the 0.3% Tre/HFD group compared with the DW/HFD group (p=0.06). We therefore consider that suppression of adipocyte hypertrophy is a very important factor for mitigating insulin resistance, without changing total adipose weight.

In our study, intake of 0.3% trehalose for 8 wk caused significant suppression of adipocyte hypertrophy (p<0.05), while intake of 2.5% trehalose tended to suppress adipocyte hypertrophy in mice with established obesity (p=0.06). As there was no difference in the weight of total visceral fat between the Tre/HFD and DW/HFD groups, we estimated that the number of small adipocytes increased in the Tre/HFD group. A similar observation was reported by Okuno et al. (15) who showed that administration of thiazolidinediones did not change the total weight of white adipose tissue, but increased the number of small adipocytes and decreased the number of large adipocytes. These changes were shown to normalize the expression levels of FFA and TNF-α, and may have contributed to an improvement in insulin sensitivity. Although we did not measure serum TNF-α in our study, we showed that NEFA levels tended to be lower in the 0.3% Tre/HFD group compared with the DW/HFD group (p=0.06). We therefore consider that suppression of adipocyte hypertrophy is a very important factor for mitigating insulin resistance, without changing total adipose weight.
promote glucose-homeostasis, and reduces insulin resistance in mice fed a HFD.

In order to elucidate the mechanism of the beneficial effects of trehalose we focused on HMW adiponectin. When 0.3% trehalose administration was started after establishment of obesity, it significantly increased serum HMW adiponectin compared with DW/HFD. It was reported that supplementation with physiological concentrations of adiponectin in obese mice resulted in mitigation of insulin resistance and dyslipidemia (16). Furthermore, homozygous adiponectin-deficient (adipo−/−) mice showed moderate insulin resistance with glucose intolerance and showed a 2-fold greater incidence of arteriosclerosis (17). It is also well known that HMW adiponectin promotes fatty acid oxidation in the liver and muscles via AMPK activation (18). These findings suggest that increased HMW adiponectin mitigates the symptoms of the metabolic syndrome, such as insulin resistance, glucose intolerance, and dyslipidemia. In our study, although the levels of HMW adiponectin were not decreased by a HFD, they increased significantly in the 0.3% Tre/HFD group compared with the DW/HFD group. We therefore reasoned that the increase in HMW adiponectin level in the 0.3% Tre/HFD group may have contributed to mitigation of insulin resistance. Mullen et al. demonstrated adiponectin resistance in muscles from HFD-fed rats (19), and suggested that the action of adiponectin was determined by receptor signaling events rather than by ligand concentration alone. In our study, the levels of HMW adiponectin were comparable in the DW/HFD and DW/LFD groups, whereas muscle IRS-1 levels were down-regulated in the DW/HFD group. We therefore inferred that the muscle adipor1 receptor became resistant to HMW adiponectin in the DW/HFD group compared with the DW/LFD group. As expression of the muscle adipor1 receptor was not examined in this study, further studies are required to determine whether trehalose regulates expression of this receptor.

To further analyze the mechanism of trehalose-mediated improvement of glucose homeostasis, we measured muscle expression of genes involved in the regulation of insulin resistance using RT-PCR. Both 0.3% and 2.5% trehalose intake significantly increased expression of IRS-1 and IRS-2 mRNA compared with the DW/HFD group. Both IRS-1 and IRS-2 are insulin receptor substrates, and both play a role in insulin signal transmission. Based on these results, we infer that up-regulation of IRS-1 and IRS-2 mRNA expression is one of the mechanisms by which trehalose mitigates HFD-induced insulin resistance. Recently, it has been shown that IRS proteins are regulated by a feedback mechanism via phosphorylation of serine/threonine residues in addition to canonical tyrosine phosphorylation pathways (20). Further studies are necessary to examine whether trehalose suppresses up-regulation of serine phosphorylation and down-regulation of tyrosine phosphorylation in IRS-1 and IRS-2.

Total GLP-1 levels were significantly higher in the 2.5% Tre/HFD group compared with the DW/HFD group. The effects of GLP-1 include promoting glucose-mediated insulin secretion, lowering plasma glucagon, and helping to maintain glucose homeostasis (21). Therefore, the higher levels of total GLP-1 observed in the trehalose-treated groups were a good indication for mechanistic analysis. However, we measured serum total GLP-1 under non-fasting conditions in this study. To clarify the involvement of GLP-1 in the effectiveness of trehalose, it will be necessary to determine whether GLP-1 is increased by trehalose intake itself.

One of the main aims of our study was to determine the effective dose of trehalose. It appeared that 2.5% trehalose was less effective than 0.3% trehalose in some of the experiments, especially on serum glucose levels in the OGTTs. Lower serum lipid levels may be one reason for the reduced hyperglycemia we observed in the 0.3% trehalose group. In fact, consumption of 0.3% trehalose tended to suppress the increase in circulating FFA compared with the levels measured in the DW/HFD group. As a result of the lower serum lipid levels, glucose uptake was promoted in the organs, resulting in reduced hyperglycemia. Duckworth et al. (22) reported that increasing the dose of glucose (10 g, 25 g, or 100 g) resulted in an increase in insulin secretion (1.8 U, 2.7 U and 7.2 U, respectively), with a simultaneous decrease in hepatic extraction (67%, 53%, 42%, respectively). A reduction in hepatic insulin extraction would be expected to lead to the development of an insulin-resistant state, and therefore we considered that 0.3% trehalose (10 g/50 kgBW/d) containing lower glucose, is a more suitable dose than 2.5% trehalose (80 g/50 kgBW/d) for use in humans. This dose is calculated based on the body weight of obese mice (40 g) and the volume of drinking water or trehalose solution consumed (2.5 mL/d/mouse).

In summary, we have shown that intake of trehalose suppresses adipocyte hypertrophy and mitigates insulin resistance in mice with established obesity. Mechanistic analysis suggests that trehalose exerts its effects on insulin resistance by suppressing adipocyte hypertrophy and by increasing the levels of serum HMW adiponectin. HMW adiponectin may then mitigate insulin resistance by up-regulating expression of IRS-1 and IRS-2 mRNA in muscle. These data suggest that trehalose is a functional saccharide that protects against metabolic syndrome in humans.

Acknowledgments
We thank Masayoshi Kibata and Norie Arai for valuable advice on diabetological analysis of our data and are grateful to Keizo Kohno and Shuji Nakamura for reviewing the manuscript.

REFERENCES
1) Wild S, Roglic G, Green A, Sicree R, King H. 2004. Global prevalence of diabetes: estimates for the year 2000 and projection for 2030. Diabetes Care 27: 1047–1053.
2) Rosen ED, Spiegelman BM. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444: 847–853.
3) Schneider BS, Faust IM, Hemmers R, Hirsch J. 1981. Effects of altered adipose tissue morphology on plasma...
Trehalose Prevents Adipocyte Hypertrophy

4) Oku T, Nakamura S. 2000. Estimation of intestinal trehalose activity from a laxative threshold of trehalose and lactulose on healthy female subjects. *Eur J Clin Nutr* **54**: 783–788.

5) Arai C, Arai N, Mizote A, Kohno K, Iwaki K, Hanaya T, Arai S, Ushio S, Fukuda S. 2010. Trehalose prevents adipocyte hypertrophy and mitigates insulin resistance. *Nutr Res* **30**: 840–848.

6) Koya-Miyata S, Arai N, Mizote A, Taniguchi Y, Ushio S, Iwaki K, Fukuda S. 2009. Propolis prevents diet-induced hyperlipidemia and mitigates weight gain in diet-induced obesity in mice. *Biol Pharm Bull* **32**: 2022–2028.

7) Hiwatashi K, Kosaka Y, Suzuki N, Hata K, Mukaiyama T, Sakamoto K, Shirakawa H, Komai M. 2010. Yambushitake mushroom (*Hericium erinaceus*) improved lipid metabolism in mice fed a high-fat diet. *Biosci Biotechnol Biochem* **74**: 1447–1451.

8) Ostman J, Arner P, Engfeldt P, Kager L. 1979. Regional differences in the control of lipolysis in human adipose tissue. *Metabolism* **28**: 1198–1205.

9) Keno Y, Matsuzawa Y, Tokunaga K, Fujioka S, Kawamoto T, Kobatake T, Tomita M, Fugroel P, Kadowaki T. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* **7**: 941–946.

10) Ferranti S, Mozaffarian D. 2008. The perfect storm: obesity, adipocyte dysfunction, and metabolic consequences. *Clin Chem* **54**: 945–955.

11) Inadera H. 2008. The usefulness of circulating adipokine levels for the assessment of obesity-related health problems. *Int J Med Sci* **5**: 248–262.

12) Tatemya S, Tamori Y, Kawaguchi T, Kanda H, Kasuga M. 2010. An increase in the circulating concentration of monocyte chemoattractant protein-1 elicits systemic insulin resistance irrespective of adipose tissue inflammation in mice. *Endocrinology* **151**: 971–979.

13) Skurk T, Alberti-Huber C, Herder C, Hauner H. 2007. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* **92**: 1023–1033.

14) Olefsky JM. 1976. The effects of spontaneous obesity on insulin binding, glucose transport, and glucose oxidation of isolated rat adipocytes. *J Clin Invest* **57**: 842–851.

15) Okuno A, Tanemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umesono K, Akanuma Y, Fujiiwara T, Horikoshi H, Yazuki Y, Kadowaki T. 1998. Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* **101**: 1354–1361.

16) Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Haru K, Mori Y, Ide T, Murakami K, Tsuboyama-kasaoaka N, Ezaki O, Akanuma Y, Gavriloa O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda H, Nakano Y, Tobe K, Nagai R, Kimura S, Torimoto M, Fugroel P, Kadowaki T. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* **7**: 941–946.

17) Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Fugroel P, Nagai R, Kimura S, Kadowaki T, Noda T. 2002. Disruption of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem* **277**: 25863–25866.

18) Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida H, Yamashita S, Noda M, Kita S, Ueki K, Tobe K, Akanuma Y, Fugroel P, Foufelle F, Ferre P, Curling D, Kimura S, Nagai R, Kahn BB, Kadowaki T. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* **8**: 1288–1295.

19) Mullen KL, Smith AC, Junkin KA, Dyck DJ. 2007. Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats. *Am J Physiol Endocrinol Metab* **293**: E83–E90.

20) Siddik K. 2011. Signalling by insulin and IGF receptors: supporting acts and new players. *J Mol Endocrinol* **47**: R1–R10.

21) Girard J. 2008. The incretins: From the concept to their use in the treatment of type 2 diabetes. Part A: Incretins: Concept and physiological functions. *Diabetes Metab* **34**: 550–559.

22) Duckworth WC, Bennett RG, Hamel FG. 1998. Insulin degradation: progress and potential. *Endocrine Rev* **19**: 608–624.