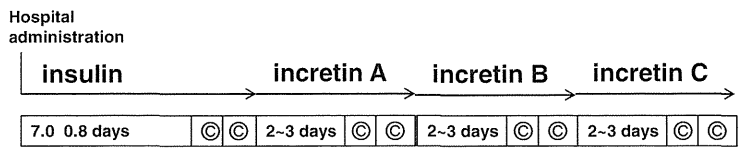

Figure 1 Study protocol. Incretin A-C indicates 0.3 mg liraglutide, 50 mg vildagliptin, or 6.25 mg alogliptin. ©: CGM monitoring day.

therapy to incretin therapy. After patients were admitted to hospital, blood glucose was controlled by insulin therapy targeting a fasting blood glucose level of <130 mg/dL and a 2 h post-prandial glucose level of <180 mg/dL without hypoglycemia for 7.0 ± 0.8 days (3–13 days). After insulin therapy, patients were treated with 0.3 mg liraglutide, 50 mg vildagliptin, and 6.25 mg alogliptin, in a randomized crossover design without a wash-out period.

Continuous glucose monitoring

CGM was performed during the last 2 days of insulin therapy as a baseline evaluation, and then patients were treated with incretin therapy. After at least 2 days of treatment with 0.3 mg liraglutide, 50 mg vildagliptin, or 6.25 mg alogliptin, CGM was performed on both the day of HD and the non-HD day for each incretin therapy. CGM was performed using a CGMS System GOLD system monitor (Medtronic MiniMed Inc., Northridge, CA, USA). Changes in glucose were monitored by CGM for 2 successive days, and injection of liraglutide and administration of vildagliptin or alogliptin once-daily began at least 36 h before CGM. Based on CGM data on the last 2 days, corresponding to the day of HD or non-HD day, the maximum glucose level, minimum glucose level, average and SD of 24 hours glucose, and duration of hyperglycemia (glucose level ≥200 mg/dL) and hypoglycemia (glucose level <70 mg/dL) were determined and compared the baseline on insulin with all therapies.

Statistical analysis

Summary statistics for continuous variables are presented as mean ± standard error. One-way analysis of variance and paired *t*-tests were performed to analyze differences between incretin therapies. A value of $p < 0.05$ was considered significant for all statistical tests.

Results

No severe hyperglycemia, ketosis, severe nausea, or other adverse effects were observed in patients at any time during incretin therapy. As shown in Figure 2A, maximum blood glucose level was approximately 200 mg/dL for all therapies (insulin; 213.9 ± 11.5 mg/dL (HD), 217.9 ± 15.5 mg/dL (non-HD), liraglutide; 198.2 ± 9.0 mg/dL (HD), 185.9 ± 131.1 mg/dL (non-HD), vildagliptin; 218.8 ± 17.1 mg/dL (HD), 213.2 ± 14.1 mg/dL (non-HD), alogliptin; 240.7 ± 18.2 mg/dL (HD), 233.0 ± 20.1 mg/dL (non-HD)). For incretin therapy, the maximum blood glucose level associated with liraglutide was significantly lower compared with treatment with alogliptin on both the day of HD and the non-HD day ($p < 0.05$), whereas there was no significant difference between liraglutide and vildagliptin. Conversely, there was no significant difference in minimum blood glucose level between the therapies (insulin; 81.8 ± 7.2 mg/dL (HD), 88.5 ± 6.2 mg/dL (non-HD), liraglutide; 81.9 ± 5.4 mg/dL (HD), 89.6 ± 9 mg/dL (non-HD), vildagliptin; 88.1 ± 6.3 mg/dL (HD), 95.6 ± 6.0 mg/dL (non-HD), alogliptin; 89.4 ± 7.1 mg/dL (HD), 92.4 ± 8.2 mg/dL (non-HD)).

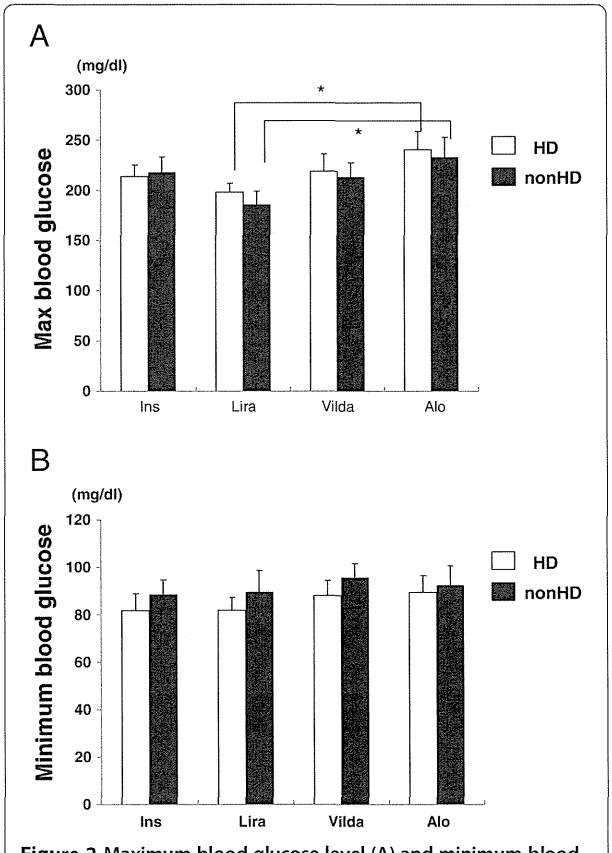

Figure 2 Maximum blood glucose level (A) and minimum blood glucose level (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

We next analyzed the average and standard deviation (SD), a magnitude of glucose fluctuation, measured using CGM. As shown in Figure 2A, the average blood glucose level associated with the therapies was 120–160 mg/dL (insulin; 134.3 ± 6.7 mg/dL (HD), 148.4 ± 10.2 mg/dL (non-HD), liraglutide; 129.0 ± 6.2 mg/dL (HD), 135.9 ± 6.8 mg/dL (non-HD), vildagliptin; 144.3 ± 7.7 mg/dL (HD), 149.6 ± 7.6 mg/dL (non-HD), alogliptin; 147.4 ± 7.3 mg/dL (HD), 149.7 ± 9.1 mg/dL (non-HD)). Compared with alogliptin, liraglutide significantly decreased the average blood glucose level on the day of HD, ($p < 0.05$). Furthermore, we compared the SD of insulin and incretin therapies. As shown in Figure 2B, the SD of liraglutide was lower in comparison to other treatments (insulin; 33.4 ± 4.2 mg/dL (HD), 33.3 ± 4.7 mg/dL (non-HD), liraglutide; 27.3 ± 1.9 mg/dL (HD), 21.5 ± 1.9 mg/dL (non-HD), vildagliptin; 34.7 ± 6.1 mg/dL (HD), 30.2 ± 4.5 mg/dL (non-HD), alogliptin; 38.0 ± 6.5 mg/dL (HD), 32.0 ± 5.1 mg/dL (non-HD)). On the day of HD, the SD of liraglutide was significantly lower compared with insulin and alogliptin treatment ($p < 0.05$), but not with vildagliptin (p=0.14), suggesting that liraglutide controlled blood glucose in patients undergoing HD with smaller glucose fluctuations.

Finally, we measured hyper- (blood glucose ≥200 mg/dL) and hypo-glycemic (blood glucose <70 mg/dL) periods associated with insulin and incretin therapy. As shown in Figure 3A, liraglutide was associated with a decreased hyperglycemic period compared with other treatments (insulin; 40.0 ± 15.0 min/day (HD), 117.9 ± 42.4 min/day (non-HD), liraglutide; 22.9 ± 23.9 min/day (HD), 33.3 ± 34.4 min/day (non-HD), vildagliptin; 87.1 ± 54.6 min/day (HD), 178.7 ± 95.0 min/day (non-HD), alogliptin; 104.1 ± 38.0 min/day (HD), 77.8 ± 26.9 min/day (non-HD). Both on the day of HD and the non-HD day, the hyperglycemic period associated with liraglutide treatment was significantly shorter compared with insulin and alogliptin ($p < 0.05$), but not with vildagliptin. Conversely, there was no significant difference in the hypoglycemic period between the therapies (Figure 3B, insulin; 16.3 ± 9.6 min/day (HD), 21.7 ± 28.6 min/day (non-HD), liraglutide; 49.5 ± 70.7 min/day (HD), 23.9 ± 23.5 min/day (non-HD), vildagliptin; 1.0 ± 1.4 min/day (HD), 1.9 ± 0.0 min/day (non-HD), alogliptin; 6.8 ± 5.7 min/day (HD), 8.0 ± 7.5 min/day (non-HD)). The frequencies of hypoglycemic periods were independent from the duration of hemodialysis.

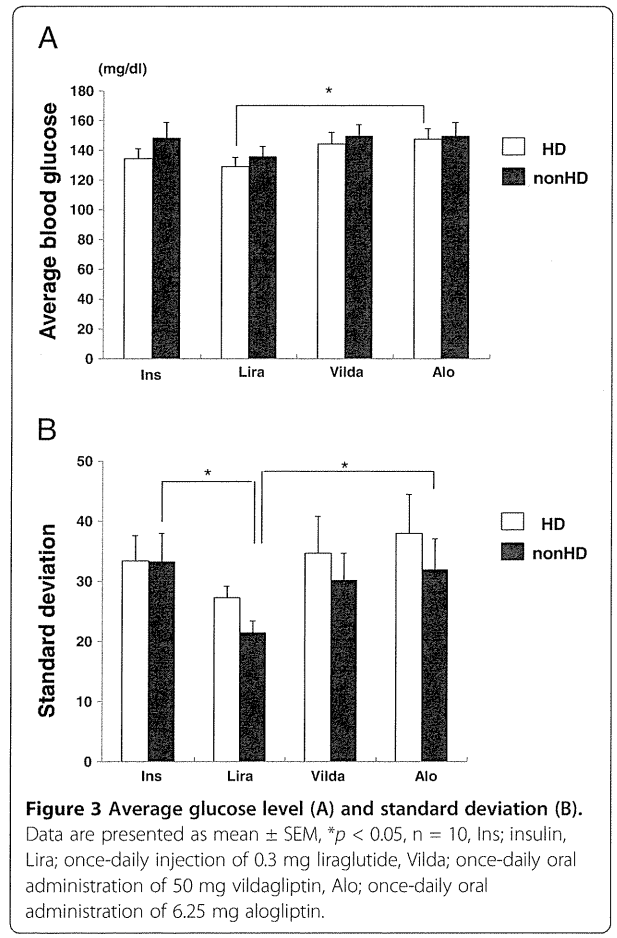

Figure 3 Average glucose level (A) and standard deviation (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

Discussion

In the present study, all 10 type 2 diabetic patients undergoing HD were able to terminate insulin therapy permanently, and were subsequently treated with incretin therapy, including a once-daily injection of 0.3 mg liraglutide, once-daily oral 50 mg vildagliptin, and 6.25 mg alogliptin until at least 3 months after the end of this study. Blood glucose data obtained from CGM suggested that switching from insulin therapy to incretin therapy was effective and well tolerated. After CGM monitoring with incretin therapies, no patient required insulin therapy, and subsequently, six patients continued to be treated with liraglutide, two patients with vildagliptin, and two patients with alogliptin. Their GA 3 months later with incretin therapies was 22.9 ± 0.3%, which was lower compared with the baseline GA, as shown in Table 1, suggesting long term tolerability of incretin therapy in diabetic patients undergoing HD. However, this study has some limitations, because of the small sample size and short-term nature of the study. In the present study, we have shown that it may be possible to use incretin therapy in type 2 diabetes patients undergoing HD, but further study with larger sample sizes over longer terms and including multiple regression analysis of contributing factors to glycemic control by incretin therapy are required to confirm the findings here.

Blood glucose control of diabetic patients undergoing HD is difficult, because of the risk of hypoglycemia. Additionally, therapeutic options for diabetic patients with renal impairments are limited, because reduced glomerular filtration leads to an accumulation of drugs and their metabolites [14]. Before the availability of incretin therapy, standard treatment of diabetic patients with ESRD was with insulin, some glinides, or α-glucosidase inhibitors. However, incretin therapy has emerged as another option for the treatment of diabetic patients with ESRD. Incretin therapy may be an ideal treatment for patients with diabetes and ESRD, because of the low risk of hypoglycemic events. Furthermore, as previously reported by us in an animal model, incretin may also have a vasoprotective effect [15,16]. Indeed, as renal impairment is one of the risk factors that can accelerate coronary artery disease [17]. In addition, incretin has received much attention for its effects on fatty liver [18] and bone [19]. In the present study, we measured liver enzymes and parathormone (PTH) level; however, no changes were observed in these markers after incretin therapy. The lipid lowering effect, as we previously reported [9], was not observed in the present study, probably because baseline lipid level was low in the present study.

In the present study, 0.3 mg liraglutide decreased blood glucose levels and fluctuations of blood glucose more compared with 50 mg vildagliptin and 6.25 mg alogliptin in diabetic patients undergoing HD. Liraglutide is a GLP-1 receptor agonist, which is available for diabetic patients with renal impairment, whereas exenatide, another GLP-1 receptor agonist, is not recommended for use by diabetic patients with severe renal impairment [20]. In a previous report, the safety and pharmacokinetics of liraglutide in subjects with varying stages of renal impairment was examined [21]. In this report, there was no significant difference in the pharmacokinetics and onset of adverse effects depending on the grade of renal impairment. However, there are no reports examining the safety of liraglutide at higher doses and over a longer term in patients with ESRD. In the present study, one patient was treated with liraglutide for over 9 months without adverse effects. However, further studies are required to confirm the safety and efficacy of liraglutide in diabetic patients undergoing HD. Theoretically, incretin therapy should not cause hypoglycemia. However, we observed hypoglycemic periods, and 0.3 mg liraglutide was associated with the highest frequency of hypoglycemia (Figure 4B). These data suggest that both hyperglycemia and hypoglycemia should be monitored when treating diabetic patients undergoing HD with incretin therapy. In the present study, the lowest hypoglycemic period, which was not significantly different from other therapies, was observed with vildagliptin.

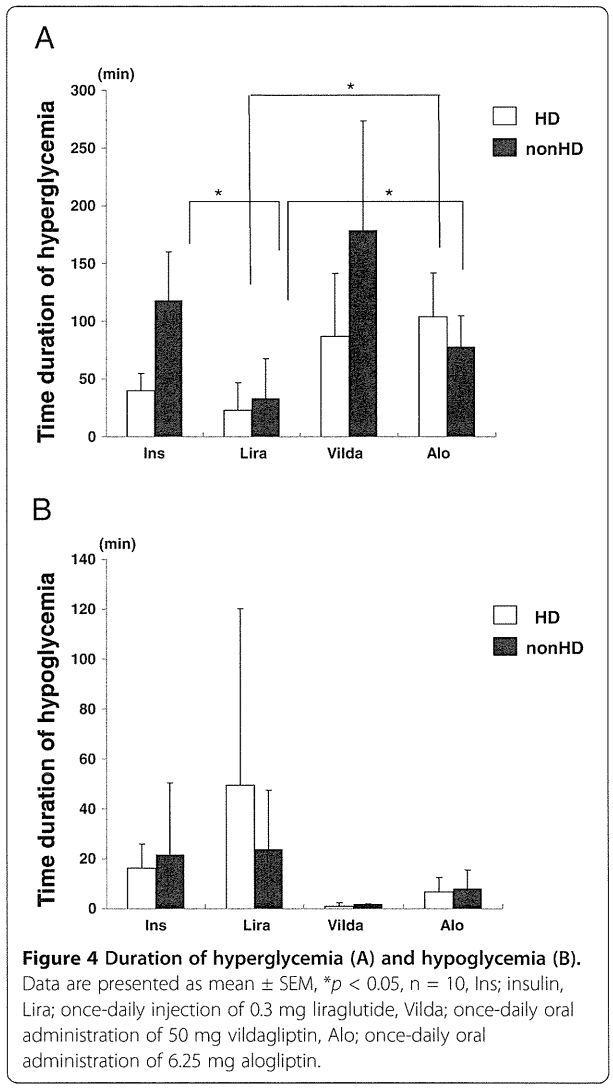

Figure 4 Duration of hyperglycemia (A) and hypoglycemia (B). Data are presented as mean ± SEM, *$p < 0.05$, n = 10, Ins; insulin, Lira; once-daily injection of 0.3 mg liraglutide, Vilda; once-daily oral administration of 50 mg vildagliptin, Alo; once-daily oral administration of 6.25 mg alogliptin.

Similar to our present study, other groups have previously demonstrated the efficacy and tolerability of the DPP-4 inhibitor, vildagliptin, in type 2 diabetic patients undergoing HD [22,23]. Kume et al. treated drug naïve type 2 diabetic patients undergoing HD with 50 mg vildagliptin once-daily for 24 weeks, and observed a significant reduction in postprandial glucose and GA from the baseline data [20]. Ito et al. treated type 2 diabetic patients undergoing HD with once-daily 50 mg or 100 mg vildagliptin for 24 weeks, and observed a significant reduction in HbA1c, GA, and postprandial glycemia [23]. Additionally, the efficacy of alogliptin in type 2 diabetic patients undergoing HD was also reported by another group [24]. Although these reports suggest efficacy and safety of DPP-4 inhibitors for the treatment of patients with type 2 diabetes undergoing HD, caution should still be exercised when treating patients undergoing HD.

Because 85% vildagliptin, 76% alogliptin, and 87% sitagliptin are excreted via the kidney [25], there is a risk that these compounds may accumulate during long-term use. Very recently, linagliptin, which is primarily excreted via bile acid, has become available [26]. According to CKD guidelines [11], linagliptin does not require dose reduction even in patients with ESRD, because of the stable pharmacokinetics of this compound in such patients. Unfortunately, we were not able to include linagliptin in the present study. However, it could potentially become a treatment option for diabetic patients undergoing HD in the future.

As described above, several reports have examined the efficacy of incretin therapy in type 2 diabetic patients undergoing HD. However, there have been no reports demonstrating a switch from insulin to incretin therapy in the treatment of diabetic patients undergoing HD. In the present study, we recruited patients who had S-CPR ≥2.0 ng/dL. Because the glucose lowering effect of incretin therapy depends on intrinsic insulin secretion, S-CPR needs to be high for incretin therapy to be effective. Baseline S-CPR of patients who completed the current study was 6.7 ± 1.3 ng/mL (2.25-16.3 ng/mL), suggesting that 2.0 ng/mL might be the borderline S-CPR concentration with which insulin therapy could be switched to incretin therapy in type 2 diabetic patients undergoing HD. In addition, we recruited patients who did not require insulin injections of >20 U/day to achieve good glycemic control, based on our preliminary experience. In the present study, baseline insulin injection dose was 11.6 ± 1.9 U/day (4–19 U/day).

Furthermore, this is the first report demonstrating CGM of incretin therapy in type 2 diabetic patients undergoing HD. CGM can monitor blood glucose levels for 24 hours, and detect the average and fluctuation range of blood glucose. Because incretin therapy can decrease not only the average blood glucose, but also fluctuations of glucose level, CGM is ideal for the evaluation of glycemic control in incretin therapy [27]. In the present study, incretin therapy, especially liraglutide, controlled both the average and fluctuation of glucose level in type 2 diabetic patients undergoing HD.

Conclusions

This study has shown that it is possible that insulin-treated type 2 diabetic patients undergoing HD might be able to switch from insulin to incretin therapy, if they have a serum C-peptide ≥2.0 ng/dL and an insulin injection dose <20 U/day. However, further studies with larger patient groups and over longer study periods are required to confirm the findings of the present study.

Abbreviations

HD: Hemodialysis; GLP-1: Glucagon-like peptide-1; DPP-4: Dipeptidyl peptidase-4; GIP: Glucose-dependent insulinotropic polypeptide; CKD: Chronic kidney disease; CGM: Continuous glucose monitoring; GA: Glycated albumin; S-CPR: Serum-C peptide; GAD: Glutamic acid dehydrogenase; ESRD: End-stage renal disease.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YU collected data and performed statistical analyses; TN wrote the manuscript and conceived of the research hypothesis; YA, HT, RN, YT, KM, HN, NH, KS, AT, KI, YA, YS, SO, HN, and TS reviewed and edited the manuscript and assisted in patient recruitment; TN assisted in conception of the research hypothesis and reviewed and edited the manuscript. All authors read and approved the final manuscript.

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LATE BREAKING CLINICAL TRIAL (JCS 2012)

Lifestyle Changes Through the Use of Delivered Meals and Dietary Counseling in a Single-Blind Study

– The STYLIST Study –

Keita Noda, MD, PhD; Bo Zhang, PhD; Atsushi Iwata, MD, PhD; Hiroaki Nishikawa, MD, PhD; Masahiro Ogawa, MD, PhD; Takashi Nomiyama, MD, PhD; Shin-ichiro Miura, MD, PhD; Hideto Sako, MD, PhD; Kunihiro Matsuo, MD, PhD; Eiji Yahiro, MD, PhD; Toshihiko Yanase, MD, PhD; Keijiro Saku, MD, PhD on behalf of the STYLIST Study Investigators

Background: Dietary habits are associated with obesity, and both are important contributing factors to lifestyle-related diseases. The STYLIST study examined the effects of dietary counseling by registered dietitians and the delivery of proper calorie-controlled meals (UMIN Registration No: 000006582).

Methods and Results: Two-hundred adult patients with hypertension and/or diabetes mellitus were randomly divided into 2 groups with/without dietary counseling and consumed an ordinary diet for 4 weeks. Each group was then subdivided into 2 groups with/without dietary counseling and received calorie-controlled lunch and dinner boxes for the next 4 weeks. The calories in the delivered meals were based on the subject's ideal body weight (BW) and physical activity level. BW, waist circumference, blood pressure, and laboratory data, including glycoalbumin, were measured at 0, 4, and 8 weeks. BW and the other parameters were significantly reduced during the study period in patients who received diet counseling in the ordinary diet period and/or delivered meal period but not in patients without dietary counseling, as assessed by linear mixed models for longitudinal data.

Conclusions: The combination of dietary counseling by dietitians and delivery of calorie-controlled meals was effective in reducing BW, as well as blood pressure and glycoalbumin, in patients with hypertension and/or diabetes mellitus. (*Circ J* 2012; **76:** 1335–1344)

Key Words: Body weight; Delivered meals; Dietary counseling; Registered dietitians; Single-blind study

Recently, the percentage of the population that could be considered obese has increased, in both developed countries,[1] including Japan,[2] and developing countries. Because obesity is related to the cardiovascular disease (CVD) burden and other metabolic disorders, including hypertension (HT), dyslipidemia, type 2 diabetes mellitus (DM), metabolic syndrome,[3–5] etc, body weight (BW) reduction entails both medical and economic considerations.

Editorial p1322

Although in many previous trials, calorie-restricted diets or formula food for the treatment of obesity, type 2 DM and HT were used with small to large populations[6–10] and dietary intervention (including dietary counseling) and exercise have been shown to lower the risk of CVD, there have been only a few reports on the use of delivered meals with/without dietary counseling in subjects with HT or type 2 DM.[11–13] In those

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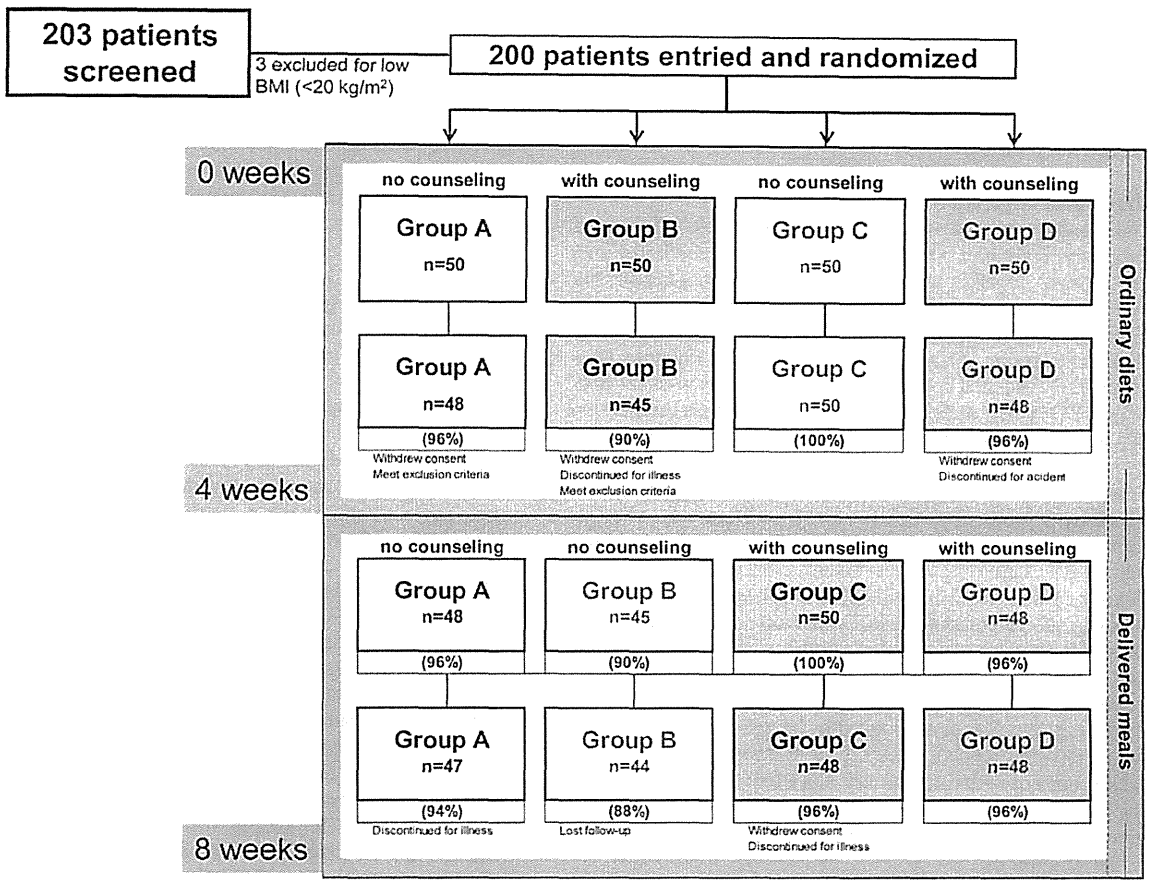

Figure 1. Flow chart of the participants from screening to the end of the study.

studies, meals were not delivered daily to the individual's home,[11,12] and no remarkable changes in BW were observed,[13] despite a long study period of 1 year. In addition, no clinical data were provided.[11,12] Our intension is to verify the effects of a combination of delivered meals with dietary counseling within a short period from a cost-benefit perspective. Therefore, our hypothesis was that the more frequent delivery of proper calorie-controlled meals for lunch and dinner in conjunction with dietary counseling could be effective for reducing BW in patients with HT/DM within a short period. This is the first, registered, multicenter, randomized, single-blind study from Japan of the use of dietary counseling together with delivered proper calorie-controlled meals in patients with HT/DM to note changes in BW.

Methods

Patients

The study subjects were recruited from among outpatients at Fukuoka University Hospital and Yuai Hospital in Fukuoka, Japan. The protocol was approved by the Independent Review Board (IRB) of Fukuoka University Hospital [No. 11-9-9], and registered under UMIN000006582. At the beginning of the trial, the Fukuoka University Extension Center contacted residents of Fukuoka City (Nanakuma area) to describe the research protocol, and the details of the trial. Of the 203 applicants that included residents of Fukuoka City, 3 were excluded due to low body mass index (BMI) (**Figure 1**). Each subject signed an informed consent form after the protocol was explained in detail. A subject was eligible for inclusion if all of the following criteria were met: type 2 DM, including impaired glucose tolerance and/or essential HT; aged ≥20 years, and able to eat meals regularly 3 times a day. A subject was not eligible for inclusion if any of the following exclusion criteria were met: allergic to common food; stroke or myocardial infarction within the past 3 months; on hemodialysis with end-stage renal disease; cancer or under cancer treatment; inability to ingest or digest, secondary obesity; HbA_{1c} >12% as defined by the Japan Diabetes Society (JDS) scale (because the regularly delivered meals would not be suitable for such patients); BMI<20 (lean individuals were excluded); height >1.8 m; patients who planned to change their lifestyle habits or reduce their BW during the study period; patients who planned to change their smoking habit during the study period; and women who were pregnant or lactating. The reason why we excluded patients with a height >1.8 m was that the maximum number of calories in the delivered meals was 1,800 kcal/day. More than this and the caloric deficit would have to be supplemented by additional ordinary diet, which we wanted to avoid. Finally, 200 subjects (97 males, 103 females, age 22–72 years)

were enrolled in this trial. In group A (see Protocol), 2 subjects dropped out at 4 weeks and 1 dropped out at 8 weeks; in group B, 5 dropped out at 4 weeks and 1 dropped out at 8 weeks; in group C, 2 subjects dropped out at 8 weeks; in group D, 2 subjects dropped out at 4 weeks (**Figure 1**). The participants withdrew because of adverse events that were not related to the delivered meals.

The Research Consortium

The present trial was supported by the Japanese Ministry of Economy, Trade and Industry and the Japan Research Institute, Ltd (Tokyo), together with a consortium of Nissin Healthcare Food Service Co Ltd (Tokyo), Kyudenko Co Ltd (Fukuoka), Yuai Hospital (Fukuoka, Japan), and the AIG Collaborative Research Institute of Cardiovascular Medicine, Fukuoka University, and included approximately 200 patients with HT and/or type 2 DM. The main focus of this consortium is the creation of a new service industry associated with medical care.

Protocol

The 2-by-2 protocol design is shown in **Figure 1**. After informed consent was given, physicians completed the screening forms. According to the information on the forms, the Fukuoka University Hospital Clinical Research Assist Center (CRAC, where computed randomization was performed, independent of the research consortium) randomly assigned the participants to 4 groups at the beginning of the ordinary diet period (0 week) as group A (no counseling during either study period), group B (counseling with the ordinary diet, but not with delivered meals), group C (counseling with delivered meals, but not with the ordinary diet), and group D (counseling for both study periods) (**Figure 1**). The ordinary diet was given for 4±1 weeks, and meals were delivered for an additional 4±1 weeks.

The calories in the delivered meals were based on each patient's ideal BW and physical activity. The ideal BW was the square of height (m)×22. The optimal number of calories/day was the ideal BW multiplied by physical activity. Physical activity (life intensity) was assigned 1 of 4 levels based on lifestyle and work-related strength.[14,15] For levels 1, 2, 3, and 4, the rates were 25–29.9, 30–34.9, 35–39.9, ≥40, respectively. Physical activity level was evaluated by physicians during a face-to-face interview regarding the intensity of daily activities at 0 weeks.[14,15] Three different delivered meals were prepared by Nissin Healthcare Food Service Co Ltd (Tokyo) to contain 400, 533 and 600 kcal/lunch or dinner, and, together with an ordinary breakfast, gave an estimated total daily caloric intake of 1,200, 1,600, and 1,800 kcal, respectively. The meals that each subject received were selected according to the calculated optimal number of calories/day. If the calculated optimal calories/day was more than 1,800, the caloric deficit was supplemented by additional ordinary diet. Each meal contained less than 3 g of salt. The chilled lunch and dinner boxes were delivered to the subjects or obtained by the subjects themselves from a convenience store (Family Mart. Co Ltd, Fukuoka, Japan) near their workplace or home from Monday to Friday, depending on their choice. On Saturday and Sunday, all subjects consumed an ordinary diet. The meals were heated before eating and no leftovers were stored.

Physicians

The physicians in charge of this trial were either in general practice, cardiologists, or diabetologists. They promoted a healthy lifestyle to the patients, as usual, but instructed the participants not to change their lifestyle throughout the study period. Changing their lifestyle was an exclusion criterion, no one was excluded from the study for this reason. The physicians were blinded to the randomization of dietary counseling and BW, but not blood pressure (BP).

Nutritional Counseling

All participants were interviewed by registered dietitians (RDs) to assess their dietary habits and exercise habits using a food and exercise frequency questionnaire (FEFQ) at week 0 before randomization. The FEFQ is a self-administered tool based on educational material regarding dietary counseling from the Ministry of Health Labour and Welfare of Japan.[16] The FEFQ comprises 28 food groups for staple food, side dish, oils, salts, sugar, and alcohol categories, and 4 exercise frequency groups (walking, jogging, gym, and other exercise). Food groups comprise 35 food items, including 10 quantity items (rice, bread, other staple food, vegetables, cooking sugar, sake, beer, shochu, whisky, and other alcohols), 3 preference items (fish: oily/usual/white; meat: high-fat/usual/low-fat; and flavor: strong/usual/thin), and 22 frequency items (tubers and roots, fruit, fish, meat, eggs, soybean and soybean products, milk, dairy products, seaweed, fried food, mayonnaise and dressing, pickles, salted food, processed food, instant food, miso soup, soup, noodle soup, coffee and black tea, fruit juice, confectionaries, and hol drinking). The FEFQ was completed by each patient under the supervision of a dietitian, who then checked the questionnaire before randomizing the patient into group A, B, C, or D; each patient completed a FEFQ before randomization. The participants in the counseling groups received individual face-to-face dietary counseling sessions for 30–60 min based on information from the FEFQ at the beginning of each study period with the ordinary diet for groups B and D, and the delivered meals for groups C and D. Counseling focused on principles of good nutrition and advice on meal planning, dietary calories, and alcohol consumption. In addition, a 10–20-min telephone counseling session was performed in the middle of each period, and the RDs checked dietary performance and advised the participant. Thus, the maximum number of counseling sessions was 0 for group A, 2 for groups B and C, and 4 for group D.

Nutritional Intervention and Adherence to the Intervention

Based on information from the FEFQ, dietary advice on the correct amounts of rice and bread (staple food), salt, vegetables, fruits, fish, meat, eggs, soy proteins, fiber, etc, during the ordinary diet period was given based on the Food Substitution Table for Diabetes Mellitus Diet Therapy 6th version of the Japan Diabetes Society.[17] During the study period with delivered meals, advice focused on breakfast and the RDs ensured that the delivered lunches and dinners were being consumed correctly. If the volume of staple food in the delivered meals exceeded the individual's usual volume, the subjects were advised that they did not need to eat it all. RDs also gathered and relayed information about the participants' tastes to the company responsible for meal production and delivery, and checked whether the subjects were complying with the dietary counseling. The RDs recorded anthropometric measurements, except BP, and coordinated the follow-up appointments. The participants documented all relevant information on daily diet report sheets at 4 and 8 weeks; for example, eating lunch or dinner at a restaurant (eating out), failure to eat a delivered meal, or the consumption of additional snacks, meals or alcohol, and the RDs could then estimate any unusual caloric intake for each participant.

Table 1. Baseline Characteristics of Patients

	Group A (n=50)	Group B (n=50)	Group C (n=50)	Group D (n=50)	P value**
Age, years	65.6±11.4	64.0±12.5	65.9±12.7	64.5±10.6	0.84
Sex, n, (%)					
Female	22 (44%)	29 (58%)	21 (42%)	31 (62%)	0.11
Male	28 (56%)	21 (42%)	29 (58%)	19 (38%)	0.11
Height, m	1.60±0.08	1.60±0.07	1.61±0.09	1.59±0.08	0.68
BMI (kg/m²)	25.9±3.4	27.3±4.8	26.0±3.6	25.6±3.5	0.17
Components of MetS, n (%)					
High WC	37 (74%)	38 (76%)	35 (70%)	34 (68%)	0.71
High TG	18 (36%)	12 (24%)	19 (38%)	16 (32%)	0.49
Low HDL-C	4 (8%)	1 (2%)	9 (18%)	4 (8%)	0.09
High BP	30 (60%)	32 (64%)	25 (50%)	34 (68%)	0.59
High blood glucose	23 (46%)	14 (28%)	21 (42%)	26 (52%)	0.18
Risk factors of CHD, n (%)					
HT	41 (82%)	41 (82%)	37 (74%)	38 (76%)	0.68
Type 2 DM	28 (56%)	21 (42%)	26 (52%)	22 (44%)	0.45
Smoking	6 (12%)	0 (0%)	6 (12%)	3 (6%)	0.06
Dyslipidemia, n (%)	28 (56%)	17 (34%)	25 (50%)	19 (38%)	0.09
Complications, n (%)					
CHD	15 (30%)	15 (30%)	12 (24%)	9 (18%)	0.46
Cerebrovascular disease	6 (12%)	3 (6%)	3 (6%)	4 (8%)	0.65

**Category and continuous variables were compared among groups by chi-square analysis and analysis of variance, respectively.

BMI, body mass index; MetS, metabolic syndrome; WC, waist circumference; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; CHD, coronary heart disease; HT, hypertension; DM, diabetes mellitus.

Primary and Secondary Endpoints

The primary endpoint was a change in BW during the ordinary diet period and delivered meals period with/without dietary counseling. Secondary endpoints were changes in waist circumference (WC), BP, blood sugar, glycoalbumin, HbA1c, and serum lipids, with/without dietary counseling. The BW and WC of the participants were measured at each visit, every 4 weeks during the trial period. WC was measured halfway between the lower rib and the iliac crest at the level of the navel. BP and pulse rate were measured every 4 weeks during the trial period.

Other Measurements

Blood cell counts, urinalysis and serum levels of triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose, HbA1c, AST, ALT, LDH, γ-GTP, creatinine, uric acid, sodium, potassium, chloride, calcium, glycoalbumin, and adiponectin were measured at the beginning (0 week) and end of each study period (at 4 and 8 weeks) by a central clinical laboratory (BML Corporation, Fukuoka, Japan), and the participants were requested to visit an out-patient clinic after overnight fasting at those times. After the physician ordered clinical laboratory tests, blood was drawn from the patient by technical staff in the Department of Clinical Laboratory, and sent to BML for measurement after separation.

Statistical Analysis

All the data analyses were performed using SAS (Statistical Analysis System Ver. 9.2, SAS Institute Inc, Cary, NC, USA) at Fukuoka University (Fukuoka, Japan), as described previously.[18,19] Baseline characteristics of patients were compared among groups for category and continuous variables by chi-square analysis and analysis of variance, respectively. Continuous variables during the study periods were presented as least-square means and standard error. Significant changes of continuous variables vs. baseline were examined by analysis of variance using linear mixed model, based on the intention-to-treat (ITT) principle, and differences among groups at baseline and at 4 and 8 weeks were examined by analysis of covariance after adjusting for stable variables including age, sex, HT, type 2 DM, smoking, and dyslipidemia. The combined effects of delivered meals and diet counseling on anthropometric measurements and blood glucose concentrations were examined by likelihood-based ignorable analyses using linear mixed models for longitudinal data. Type 3 tests of fixed effects are shown for group and group by period interaction and solutions for fixed effects are shown for group by period interaction. Sample size was calculated using SAS/STAT Power and Sample Size Application. To detect a mean difference of 1.5kg BW change between patients with and without diet counseling, assuming an unequal standard deviation of 2.5 and 5kg, a sample size of 174 was calculated with a 1-sided significance level of 0.05 and a power of 0.8. Therefore, 200 patients were recruited. The significance level was considered to be less than 0.05 unless indicated otherwise.

Results

Baseline Characteristics and Lifestyle of Participants

Table 1 shows the baseline characteristics of groups A, B, C, and D: there were no significant differences in age, sex, BMI, WC, or the prevalence of HT and DM, or risk factors, among the 4 groups at week 0. Among all of the patients, the prevalence of HT and type 2 DM were 78.5% (n=157) and 48.5% (n=97), respectively, and 27% (n=54) of the patients had both HT and type 2 DM. Components of the metabolic syndrome were categorized based on the criteria in Japan,[20] and there

Table 2. Lifestyles of Patients and Lifestyle Changes During the Periods of Ordinary Diets and Delivered Meals

	Group A (n=50)	Group B (n=50)	Group C (n=50)	Group D (n=50)	P value**
Lifestyle					
Physical activity level, n (%)					
I	31 (62%)	37 (74%)	33 (66%)	26 (52%)	0.14
II	18 (36%)	13 (26%)	17 (34%)	23 (46%)	0.22
III	1 (2%)	0 (0%)	0 (0%)	1 (2%)	0.57
Proper daily calorie intake range, kcal					
Lower calorie intake limit	1,534±231	1,481±199	1,525±219	1,537±247	0.57
Higher calorie intake limit	1,812±253	1,756±220	1,805±245	1,810±271	0.64
Estimated salt intake, n (%)					
>10g/day	42 (84%)	42 (84%)	41 (82%)	41 (82%)	0.99
Alcohol drinking, n (%)	12 (24%)	24 (48%)	13 (26%)	15 (30%)	0.04
<3days/week	4 (8%)	13 (26%)	4 (8%)	6 (12%)	0.02
≥3days/week	8 (16%)	11 (22%)	9 (18%)	9 (18%)	0.89
Exercise habit, n (%)	17 (34%)	15 (30%)	17 (34%)	15 (30%)	0.95
Walking	14 (28%)	11 (22%)	16 (32%)	12 (24%)	0.68
Jogging	2 (4%)	0 (0%)	0 (0%)	2 (4%)	0.25
Sport gym exercise	1 (2%)	5 (10%)	1 (2%)	1 (2%)	0.10
Target daily calorie control with delivered meals					
1,200 calories, n (%)	7 (14%)	6 (12%)	5 (10%)	8 (16%)	0.83
1,600 calories, n (%)	23 (46%)	31 (62%)	32 (64%)	21 (42%)	0.06
1,800 calories, n (%)	20 (40%)	13 (26%)	13 (26%)	21 (42%)	0.16
Average calories, kcal	1,624±196	1,604±174	1,612±164	1,620±207	0.95
Delivered meal intake rate, %	62±13%	66±12%	69±11%	64±13%	0.04
Lifestyle change					
During ordinary diet period, day/4 weeks					
Eating out	8±7	6±5	6±8	10±9	0.04
Snack	8±9	8±7	7±9	9±8	0.73
Drink less than usual	10±9	8±6	4±1	5±8	0.43
Drink more than usual	4±3	3±2	4±3	2±2	0.55
Exercise more than usual	5±5	3±2	7±7	6±6	0.30
Exercise less than usual	6±6	5±4	7±4	4±3	0.29
During delivered meal period, day/4 weeks					
Eating out	4±3*	3±3*	3±3*	4±3*	0.22
Snack	7±8	7±7	5±6	7±7	0.46
Drink less than usual	10±8	6±7	14±8	8±9	0.34
Drink more than usual	4±2	2±2	4±3	4±3	0.31
Exercise more than usual	7±7	4±3	4±3	7±9	0.63
Exercise less than usual	8±6	6±6	7±4	4±1	0.37

*P<0.001, delivered meal period vs. ordinary diet period, assessed by Wilcoxon signed rank test.
**Category and continuous variables were compared among Groups by chi-square analysis and analysis of variance, respectively.

were no significant differences among the 4 groups.

Table 2 shows the lifestyles of Groups A, B, C, and D: there were no significant difference in physical activity level, salt intake, and exercise habit among the 4 groups. Estimated salt intake >10 g/day was 84%, 84%, 82%, and 82%, in groups A, B, C, and D, respectively. The proportion of patients having an alcohol drinking habit for more than 3 days/week was not significantly different among the 4 groups (range, 16–22%), although group B patients had a higher proportion of patients with an alcohol drinking habit of less than 3 days/week (26%) compared to the other groups of patients (8–12%). In total, 68% of the patients did not have an exercise habit, and 26.5% patients walked for more than 30 min/day on more than 4 days/week.

Patients were recommended not to change their lifestyle during the study period. The number of days of alcohol drinking and exercising more than and less than usual were not significantly changed during the ordinary diet and delivered meal periods (**Table 2**). However, because meals were provided during the delivered meal period, the number of days of eating out was significantly reduced compared to the ordinary diet period (**Table 2**).

Primary and Secondary Endpoints

At baseline (week 0), BW, WC, BP, blood glucose concentrations, and serum lipid concentrations were not significantly different among the 4 groups, as assessed by analysis of covariance after adjusting for age, sex, HT, type 2 DM, smoking, and dyslipidemia (**Table 3**). During the study period, a significant reduction in BW at 8 weeks was observed in group B, C, and

Table 3. Anthropometric Measurements, Blood Glucose Concentrations, and Serum Lipid Concentrations During the Study Periods

	Group A	Group B	Group C	Group D	P value**
BW, kg					
0 weeks	66.8±1.5	69.6±1.9	67.6±1.7	64.8±1.5	0.41
4 weeks	67.0±1.5	69.7±1.9	67.6±1.7	64.9±1.5	0.90
8 weeks	66.8±1.5	69.0±1.9*	66.7±1.7*	64.4±1.5*	0.84
WC, cm					
0 weeks	92±1	94±2	92±1	93±1	0.69
4 weeks	92±1	95±2	91±1	92±1*	0.77
8 weeks	91±1	93±2	91±1*	91±1*	0.86
SBP, mmHg					
0 weeks	130±3	137±2	133±2	140±2	0.12
4 weeks	134±2	135±2	132±3	138±2	0.18
8 weeks	134±3	134±2	130±2	134±2*	0.62
DBP, mmHg					
0 weeks	78±2	80±1	75±1	83±1	0.16
4 weeks	78±2	79±1	76±2	82±1	0.08
8 weeks	79±2	78±1	75±1	79±1*	0.77
Pulse rate, /min					
0 weeks	72±1	69±1	72±2	73±2	0.39
4 weeks	72±1	70±1	73±2	71±2	0.29
8 weeks	71±1	71±1	74±2	73±2	0.35
Fasting blood glucose, mg/dl					
0 weeks	122±7	113±5	120±7	120±5	0.90
4 weeks	130±7	118±5	123±7	120±5	0.59
8 weeks	122±7	114±5	111±7	119±5	0.12
Glycoalbumin, %					
0 weeks	16.6±0.4	16.2±0.5	16.6±0.6	16.3±0.4	0.99
4 weeks	16.5±0.4	15.9±0.5	16.6±0.6	16.4±0.4	0.53
8 weeks	16.4±0.4	16.0±0.5	16.0±0.6*	16.2±0.4	0.65
HbA_{1c}, %					
0 weeks	6.2±0.1	6.1±0.2	6.1±0.1	6.0±0.1	0.86
4 weeks	6.2±0.1	6.1±0.2	6.1±0.1	6.0±0.1	0.99
8 weeks	6.1±0.1	6.0±0.2*	6.0±0.1*	6.0±0.1	0.94
Triglycerides, mg/dl					
0 weeks	149±12	122±8	159±12	140±12	0.23
4 weeks	142±12	113±8	141±12	137±13	0.46
8 weeks	145±12	112±8	134±13*	144±13	0.50
HDL-C, mg/dl					
0 weeks	55±2	60±2	55±3	61±2	0.94
4 weeks	55±2	58±2*	55±3	59±2	0.90
8 weeks	53±2*	58±2*	52±3*	59±2	0.84
LDL-C, mg/dl					
0 weeks	112±4	114±4	113±4	118±4	0.51
4 weeks	109±5	115±4	111±4	116±4	0.31
8 weeks	105±5*	110±4	105±4*	117±4	0.09

Data are presented as least-square means and standard deviation.
*P<0.05, vs. 0 weeks, assessed by an analysis of variance using linear mixed models.
**Continuous variables were compared among Groups by analysis of covariance after adjusting for age, sex, HT, type 2 DM, smoking, and dyslipidemia.
BW, body weight; SBP, systolic blood pressure; DBP, diastolic blood pressure. Other abbreviations as in Table 1.

D patients but not in group A, as assessed by analysis of variance using a linear mixed model for longitudinal data (**Table 3**). WC was reduced in group C at 8 weeks and in group D at both 4 and 8 weeks. None of the groups showed significant reductions in BP at 4 weeks. Only group D, in which participants received 8 weeks of dietary counseling, showed significant reductions in systolic and diastolic BPs at 8 weeks. Glycoalbumin was significantly decreased at 8 weeks in group C, but there were no changes in the glucose level in any of the groups. HbA_{1c} was significantly decreased at 8 weeks in groups B and C. Triglycerides were reduced at 8 weeks in group C patients. HDL-C was reduced at 8 weeks in groups A, B and C patients, and LDL-C was reduced at 8 weeks in groups A and C.

The effects of the combination of delivered meals and diet

Table 4. Effects of the Combination of Delivered Meals and Diet Counseling on Anthropometric Measurements and Blood Glucose Concentrations, as Assessed by Likelihood-Based Ignorable Analyses Using Linear Mixed Models

	Type 3 tests of fixed effects				Solution for fixed effects			
	Effect	Num DF	F value	P value	Effect	Group	Estimated SE	P value
BW, kg								
	Group	3	1.61	0.19	Group*period	A	−0.01±0.11	0.93
	Group*period	4	6.73	<0.001	Group*period	B	−0.33±0.12	0.01
					Group*period	C	−0.45±0.11	<0.001
					Group*period	D	−0.20±0.11	0.08
WC, cm								
	Group	3	0.43	0.73	Group*period	A	−0.24±0.25	0.34
	Group*period	4	4.57	0.001	Group*period	B	−0.15±0.26	0.57
					Group*period	C	−0.56±0.26	0.03
					Group*period	D	−0.90±0.26	<0.001
SBP, mmHg								
	Group	3	4.10	0.01	Group*period	A	2.02±1.31	0.12
	Group*period	4	2.73	0.03	Group*period	B	−1.65±1.31	0.21
					Group*period	C	−1.18±1.28	0.36
					Group*period	D	−3.14±1.27	0.01
DBP, mmHg								
	Group	3	5.29	0.001	Group*period	A	0.32±0.72	0.66
	Group*period	4	3.38	0.010	Group*period	B	−0.97±0.72	0.18
					Group*period	C	−0.17±0.71	0.81
					Group*period	D	−2.37±0.70	0.001
Glycoalbumin, %								
	Group	3	0.42	0.74	Group*period	A	−0.10±0.08	0.19
	Group*period	4	3.85	0.004	Group*period	B	−0.09±0.08	0.26
					Group*period	C	−0.27±0.08	<0.001
					Group*period	D	−0.05±0.08	0.50
BW, kg								
	Group	1	0.21	0.65	Group*period	A	−0.01±0.11	0.93
	Group*period	2	12.15	<0.001	Group*period	B+C+D	−0.33±0.07	<0.001
WC, cm								
	Group	1	0.63	0.43	Group*period	A	−0.24±0.25	0.34
	Group*period	2	7.03	0.001	Group*period	B+C+D	−0.54±0.15	<0.001
SBP, mmHg								
	Group	1	7.65	0.01	Group*period	A	2.03±1.31	0.12
	Group*period	2	4.89	0.008	Group*period	B+C+D	−2.02±0.74	0.007
DBP, mmHg								
	Group	1	2.1	0.15	Group*period	A	0.32±0.72	0.66
	Group*period	2	4.29	0.01	Group*period	B+C+D	−1.19±0.41	0.004
Glycoalbumin, %								
	Group	1	0.11	0.74	Group*period	A	−0.10±0.08	0.20
	Group*period	2	5.44	0.005	Group*period	B+C+D	−0.14±0.05	0.003

Num DF, numerator degrees of freedom; group*period, group by period interaction. Other abbreviations as in Table 3.

counseling were examined by likelihood-based ignorable analyses using mixed models for incomplete (unbalanced) data. As shown in **Table 4**, interaction effects between group and period (indicated by group*period) were significant for BW, WC, SBP, DBP, and glycoalbumin, indicating that the patterns of changes in these variables were different among the 4 groups of patients. Significance of the changes in these variables in each group was shown in the upper right panel of **Table 4**. As shown, the reductions in BW were significant in groups C and D patients and borderline significant in group D, but not significant in group A. Reductions in WC were significant in groups C and D, but not significant in group A and B patients. Reductions in systolic and diastolic BPs were significant in group D patients but not in groups A, B, and C patients. Reductions in glycoalbumin were significant in group C patients but not in groups A, B, and D patients.

Because groups B, C, and D patients received diet counseling during the ordinary diet period and/or delivered meal period, groups B, C, and D (group B+C+D) patients were combined and compared to group A for the pattern of changes in the variables. As shown in **Table 4**, the group by period interaction effects were highly significant. Group B+C+D patients had significant reductions in BW, WC, SBP, DBP, and glycoalbumin, whereas group A patients did not (**Table 4, Figure 2**).

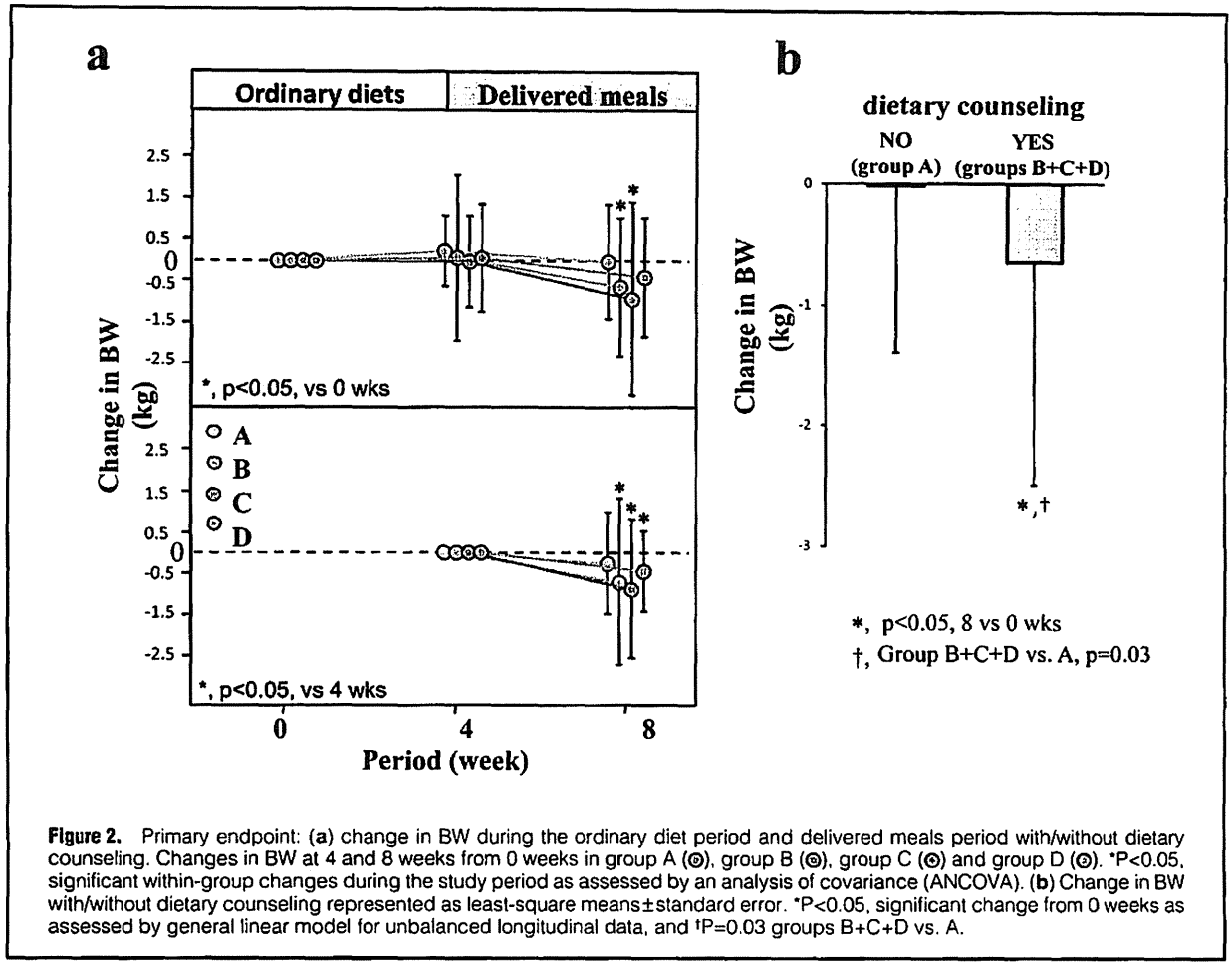

Figure 2. Primary endpoint: (a) change in BW during the ordinary diet period and delivered meals period with/without dietary counseling. Changes in BW at 4 and 8 weeks from 0 weeks in group A (◎), group B (◎), group C (◎) and group D (◎). *P<0.05, significant within-group changes during the study period as assessed by an analysis of covariance (ANCOVA). (b) Change in BW with/without dietary counseling represented as least-square means±standard error. *P<0.05, significant change from 0 weeks as assessed by general linear model for unbalanced longitudinal data, and †P=0.03 groups B+C+D vs. A.

Dietary Counseling and Delivered Meals

In groups B and D, 100% of the participants received face-to-face dietary counseling at week 0. In groups C and D, 100% of the participants received face-to-face dietary counseling at 4 weeks. The intake rate of delivered meals was 60%, 65%, 69%, and 63% for lunch, and 63%, 67%, 69%, and 65% for dinner, and the average intake rate was 62%, 66%, 69%, and 64%, in groups A, B, C, and D, respectively.

Safety

There were 6 adverse events that led to withdrawal during the study period; 2 of these events occurred in the delivered meal period, but none of the events was related to the delivered meals.

Discussion

There have been a few reports on the use of delivered meals with/without dietary counseling in patients with HT/DM.[11-13] One delivered meals, but patients were free to choose both the frequency and the period of delivery, and patients were encouraged to change their exercise habits.[13] Thus, that study examined the effects of the combination of delivered meals and exercise, and there were no changes in BMI during the course of the 1-year trial. The other 2 studies each lasted for 1 year, but the special meals consisted of 7 meals per week, which were delivered weekly, and there was no description of BW nor were clinical data provided.[11,12] In our study, the participants was advised not to change their lifestyle throughout the study period, and the combination of dietary counseling and proper calorie-controlled delivered meals was associated with reductions in BW (primary endpoint), and BP and glycoalbumin or HbA_{1c} (secondary endpoint) within 8 weeks.

BW did not decrease with dietary counseling for the 4 weeks of the ordinary diet period. However, our results regarding the reduction in overall BW in groups B, C, and D should be interpreted with caution, because these 3 groups received counseling from a dietitian either 2 or 4 times during the study period. Therefore, carry-over effects of counseling may have still been present even in group B (counseling with ordinary diet and no counseling with delivered meals). The lack of change in BW in group A and the change (reduction) in BW in the other groups suggest that dietary counseling, but not delivered meals alone, may have played a favorable role in reducing BW in this short-term study. The change in WC was likely to parallel that in BW. The effect of the combination of delivered meals and diet counseling on anthropometric measurements and other parameters was assessed by likelihood-based ignorable analyses using linear mixed models, and the patterns of the changes in these variables differed among the 4 groups of patients (**Table 4**). These results indicate that only group A patients, who did not receive diet counseling during either the

ordinary diet period or the delivered meal period, consistently had no significant changes in any of the variables.

A meta-analysis of aggregate data found that a decrease in BW by 1 kg, a modest weight loss, resulted in a reduction of systolic and diastolic BP by 1.2 and 1.0 mmHg, respectively,[21] while other reported weight loss strategies using dietary or behavioral interventions in type 2 DM produced small between-group improvements in BW.[22] From our data, an average −0.6 kg BW reduction decreased BP to a small extent, but significant reductions in both systolic and diastolic BPs were observed only after 8 weeks in group D, but not in group C. This indicates that dietary counseling for more than 4 weeks and delivered meals may be necessary to reduce BP. From the questionnaire sheet, 2%, 9%, 34%, 36% and 14% of the participants consumed salt ~7.9 g/day, 8.0–9.9 g/day, 10.0–11.9 g/day, 12.0–13.9 g/day, and ≥14 g/day, respectively, at week 0 (not tabulated). In addition, the number of days of eating out was significantly reduced compared to the ordinary diet period (**Table 2**). Thus, a low salt intake of ≤9 g/day and dietary counseling resulted in a modest reduction in BP in our study.

Mixed model data also showed that group B+C+D patients had significant reductions in not only BW but also BP, glycoalbumin, and HbA_{1c}, whereas group A patients did not (**Table 4**, **Figure 2**). This finding supports the notion that simultaneous interventions consisting of dietary counseling and calorie-controlled meals can promote BP reduction and glycemic control even in the short term. The reduction in triglyceride, HDL-C and LDL-C levels in all groups, including group A, indicated that balanced delivered meals may slow lipid absorption in these patients but did not relate to meaningful changes.

Changes in diet have been shown to modify the risk for coronary heart disease (CHD). For example, changes in dietary fatty acid improve lipoprotein profiles,[23] and the benefits of salt reduction are clear and consistent in human health.[24] According to the guidelines of the Japanese Society for the Study of Obesity,[25] obesity is defined as BMI ≥25. The criteria for pharmaceutical treatment are currently limited, and such treatment is associated with significant side effects.[26] Therefore, a strategic non-pharmacological promotion of health based on lifestyle changes should enhance public health, although it has been reported that there is not always a clear association between the intensity of health promotion in a community and the outcome.[27]

Dietary habits in this program were monitored by a dietitian only for participants who received dietary counseling. The investigators (physicians) instructed the subjects to avoid changing their lifestyle during the study period only at the onset of study and were blinded to the dietitian's counseling, the participant's dietary habits, and BW measurements. This study deign is important for evaluating the efficacy of dietary counseling. There is a paucity of evidence on the effectiveness of dietary counseling for such a short term (4 weeks of an ordinary diet and 4 weeks of delivered meals), but despite this, reductions in BW, BP and glycoalbumin were achieved. However, the use of delivered calorie-controlled meals for lunch and dinner on weekdays for 4 weeks did not achieve these reductions in the absence of dietary counseling. Dietary counseling might be important for BW reduction. Furthermore, although relatively long-term counseling might be required for BP reduction, short-term counseling might be enough to reduce the glycoalbumin level.

Study Limitations

In this 2×2 study design, randomization of cross-over for ordinary diet and calorie-controlled meals was not performed because of the limitations regarding meal production by the provider. Therefore, the participants in group B had a carry-over effect of dietary counseling during the delivered meal period, when no dietary counseling was provided. Because the exclusion criteria included BMI <20, non-obese participants were enrolled. Therefore, the change in BW might be different than that in a study that only includes obese patients. Although the sample size of 200 subjects is rather small and meals were delivered on weekdays for 4 weeks, to the best of our knowledge this is the first, single-blinded prospective comparative trial to use delivered meals in Japan or Asia. We used a self-administered FEFQ to assess the dietary and exercise habits of the subjects. Because only 1 questionnaire was administered at randomization, its reproducibility was not validated. However, the reproducibility and validity of food frequency questionnaires used for assessing dietary habits (patterns) in the Japanese population have been shown by other studies.[28,29] Most of the food groups and food items in our questionnaire was very similar to the food frequency questionnaires used in those studies. The follow-up period of dietary counseling was too short to reveal meaningful changes, especially with regard to changes in BW, HbA_{1c} and other lipoprotein parameters, and although dietary advice can confer modest benefits, the longer term effects (>10 months) are unknown.[30]

In conclusion, counseling by dietitians and delivery of proper calorie-controlled meals were effective for reducing BW, BP and glycoalbumin in patients with HT and/or type 2 DM. Both might be important non-pharmacological strategies for addressing lifestyle-related diseases.

Disclosures

Conflict of Interest: This work was supported by a grant-in-aid from the Japanese Ministry of Economy, Trade and Industry and by the Japan Research Institute, Ltd (Tokyo), together with a consortium of Nissin Healthcare Food Service Co Ltd (Tokyo), Kyudenko Co. Ltd (Fukuoka), Yuai Hospital (Fukuoka, Japan), and the AIG Collaborative Research Institute of Cardiovascular Medicine, Fukuoka University.

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Appendix

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Perilipin 5, a Lipid Droplet-binding Protein, Protects Heart from Oxidative Burden by Sequestering Fatty Acid from Excessive Oxidation*◆

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Background: Perilipin family proteins are important in determining the properties of lipid droplets (LDs).
Results: Perilipin 5-deficient mice lack detectable LDs, exhibit enhanced fatty acid oxidation, and suffer increased ROS production in the heart.
Conclusion: Perilipin 5 protects the heart from oxidative burden by sequestering fatty acid from excessive oxidation.
Significance: These findings may help to increase understanding of the functions of non-adipose LDs.

Lipid droplets (LDs) are ubiquitous organelles storing neutral lipids, including triacylglycerol (TAG) and cholesterol ester. The properties of LDs vary greatly among tissues, and LD-binding proteins, the perilipin family in particular, play critical roles in determining such diversity. Overaccumulation of TAG in LDs of non-adipose tissues may cause lipotoxicity, leading to diseases such as diabetes and cardiomyopathy. However, the physiological significance of non-adipose LDs in a normal state is poorly understood. To address this issue, we generated and characterized mice deficient in perilipin 5 (Plin5), a member of the perilipin family particularly abundant in the heart. The mutant mice lacked detectable LDs, containing significantly less TAG in the heart. Particulate structures containing another LD-binding protein, Plin2, but negative for lipid staining, remained in mutant mice hearts. LDs were recovered by perfusing the heart with an inhibitor of lipase. Cultured cardiomyocytes from *Plin5*-null mice more actively oxidized fatty acid than those of wild-type mice. Production of reactive oxygen species was increased in the mutant mice hearts, leading to a greater decline in heart function with age. This was, however, reduced by the administration of *N*-acetylcysteine, a precursor of an antioxidant, glutathione. Thus, we conclude that Plin5 is essential for maintaining LDs at detectable sizes in the heart, by antagonizing lipase(s). LDs in turn prevent excess reactive oxygen species production by sequestering fatty acid from oxidation and hence suppress oxidative burden to the heart.

Lipid droplets (LDs)[4] are cellular organelles storing neutral lipids, including triacylglycerol (TAG) and cholesterol ester. LDs are found in nearly all cell types, but their properties vary greatly among tissues. White adipose tissue (WAT) has large unilocular LDs that store an enormous amount of TAG in case of increased energy demand. LDs of other tissues, however, are usually much smaller. Their physiological significance is less well understood, although a possible role is in sequestering fatty acid (FA) in a chemically inert form, TAG, to circumvent the lipotoxicity of FA and its derivatives (1). However, excess accumulation of TAG, and hence aberrant development of LDs, often causes lipotoxicity, leading to diseases such as diabetes and cardiomyopathy.

LDs carry a defined set of surface-binding proteins whose compositions differ among cell types. The perilipin family, conventionally called the PAT family, is a representative group of LD-binding proteins composed of five members (2). The recently proposed unified nomenclature (3) names them PLIN1 (the classic perilipin), PLIN2 (also named ADRP, ADFP, or adipophilin), PLIN3 (Tip47, PP17, or M6PRBP), PLIN4 (S3–12),

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[4] The abbreviations used are: LD, lipid droplet; TAG, triacylglycerol; WAT, white adipose tissue; FA, fatty acid; BAT, brown adipose tissue; ATGL, adipose triacylglycerol lipase; HSL, hormone-sensitive lipase; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; ORO, Oil Red O; BEL bromoenol lactone; TBARS, thiobarbituric acid-reactive substance; LVID;d, left ventricular end-diastolic dimension; LVID;s, left ventricular end-systolic dimension; FS, left ventricular fractional shortening; ASM, acid-soluble material.

and PLIN5 (MLDP, OXPAT, LSDP5, or PAT1). These proteins have related amino acid sequences, particularly in the amino-terminal region called the PAT1 domain (2). Plin1 (the mouse homolog of human PLIN1) is highly abundant in WAT and brown adipose tissue (BAT). Plin2 and Plin3 are expressed in many cell types, whereas Plin4 is abundant in adipose tissue. In contrast, we (4) showed that Plin5 is highly concentrated in the heart, whereas other groups (5, 6) demonstrated that this protein is expressed in oxidative tissues, including the heart, BAT, skeletal muscle, and the liver.

Plin1 is the best characterized member of the perilipin family, playing a central function in both the storage and catecholamine-dependent mobilization of TAG in WAT (7, 8). Recent studies (9–15) have presented an elaborate model for the actions of Plin1 involving adipose triacylglycerol lipase (ATGL), comparative gene identification (CGI)-58 (also called α,β-hydrolase domain-containing 5), a coactivator of ATGL, and hormone-sensitive lipase (HSL) (for review see Ref. 16). The functions of other perilipin family members are less well understood, although the proteins were shown to protect TAG from attack by lipases (17). *Plin2*$^{-/-}$ mice are resistant to a high fat diet-induced hepatosteatosis (18), and Plin3 compensates for the defect of Plin2 in these mice (19).

Some 60–70% of the large energy demand of the heart for contractile function is fulfilled by FA (20). Thus, the heart has an extremely high capacity of lipid turnover and particularly small LDs. We presumed that Plin5 has a critical role in determining the properties and functions of LDs in the heart, and hence it is the key to understanding the physiological significance of heart LDs. Accordingly, we generated and characterized *Plin5* knock-out (*Plin5*$^{-/-}$) mice. To our surprise, *Plin5*$^{-/-}$ mice lacked detectable LDs in the heart, substantiating the essential role of Plin5 in maintaining heart LDs. These mice suffered from an accelerated decline in heart contractile function with age, probably due to increased production of reactive oxygen species (ROS). We propose that LDs contribute to suppressing oxidative stress in the heart by sequestering FA from excessive oxidative metabolism.

EXPERIMENTAL PROCEDURES

Mice—Plin5$^{-/-}$ mice were produced by using the standard gene disruption procedure (Fig. 1*A*). The *Plin5*$^{-/-}$ mice were backcrossed to the C57BL/6J strain (CLEA Japan, Inc.) for four generations. *Plin5*$^{+/+}$ and *Plin5*$^{-/-}$ mice were obtained by mating respective homozygous parents. Mice were housed under standard conditions at 24–26 °C with a 12:12 h light/dark cycle and given free access to standard chow (CLEA Japan, Inc.) and water. For some experiments, mice were fasted overnight by food deprivation. For the treatment with *N*-acetylcysteine (NAC), the chemical was dissolved in water at 1.88 mg/ml and given to the mice instead of water. The treatment was continued from 16 to 18 weeks of age to the day of experiment at 30–32 weeks of age, replacing the solution every 2–3 days. Assuming that mice 30 g in body weight drink 8 ml of water per day on average (21), this dose would correspond to 500 mg of NAC/kg/day. All procedures were performed in accordance with the guidelines established by University of Hyogo for the care and use of experimental animals.

Histological Analyses—Tissues were fixed with 10% formalin/PBS, incubated with 10% sucrose/PBS, and then with 20% sucrose/PBS, each overnight at 4 °C. The tissues were embedded in O.C.T. compound (Tissue-Tek) and sectioned 10 μm thick in a cryostat (Leica CM3050S-III). Sections were air-dried and stored at −80 °C until used. After removal of the O.C.T. compound by washing with water, sections were stained for lipid with 0.18% Oil Red O (ORO) in 60% isopropyl alcohol. Sections were washed with 60% isopropyl alcohol, counterstained with hematoxylin, and subjected to microscopic analysis.

Immunofluorescence Staining—Cryosections as prepared above were freed from the O.C.T. compound and permeabilized with methanol for 20 min at −20 °C. After washing with PBS three times for 5 min each at room temperature, sections were blocked with 2% BSA/PBS for 1 h at room temperature. Sections were then incubated with primary antibodies to Plin5 (raised in rabbit (4)) and Plin2 (raised in guinea pig) and diluted 1000-fold in 2% BSA/PBS overnight at 4 °C. Secondary antibodies, Alexa 488- and 594-conjugated anti-guinea pig and rabbit IgG (Molecular Probes), respectively, were used at a 500-fold dilution in 2% BSA/PBS for 1 h at room temperature. For double staining of Plin5 and lipid, permeabilization with methanol was omitted to avoid a loss of lipid. Because of freezing and thawing of the cryosections, antibodies were allowed to access intracellular compartments, although the fluorescence signals were inevitably weaker than those obtained by usual permeabilization. Sections were first stained for Plin5 and then with 100 μM Bodipy 493/503 (Molecular Probes). Samples were examined in a confocal laser microscope (Zeiss LSM510).

Electron Microscopy—Dissected tissues were prefixed with 2% glutaraldehyde/PBS at 4 °C. After being washed with PBS, tissues were fixed with 2% osmium tetroxide/PBS for 2 h at 4 °C. Specimens were embedded in Quetol-812 (Nissin EM), and then sectioned and observed in a JEM-1200EX (JEOL).

Biochemical Analyses—Lipids were extracted from tissues according to a standard procedure (22). TAG and FA levels were measured using triglyceride *E*-test (WAKO) and nonesterified fatty acid *C*-test (WAKO), respectively. Protein was determined with a protein assay kit (Bio-Rad), using BSA as a standard.

Immunoblot Analysis—Protein extracts were prepared from tissues by using lysis buffer (20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, protease inhibitor mixture (Roche Applied Science), and 1 mM EDTA). Protein concentrations were quantified, and the extracts were subjected to immunoblotting.

RT-PCR—Total RNA was prepared from cardiac ventricles using QIAzol reagent (Qiagen) and reverse-transcribed. Target genes were amplified with a SYBR qPCR kit (KAPA Biosystems) and quantified using an ABI PRISM7000 (Applied Biosystems).

Subcellular Fractionation—The hearts from fed and fasted wild-type and *Plin5*$^{-/-}$ mice were homogenized in 500 μl of 20 mM Hepes-NaOH (pH 7.4) containing protease inhibitor mixture (Roche Applied Science), using a Potter-Elvehjem homogenizer. After the removal of nuclei and cell debris by centrifugation at 700 × *g* for 10 min, 300 μl of the postnuclear

supernatant was mixed with an equal volume of 60% sucrose and placed below a 5-ml 0–30% (w/v) sucrose gradient in a centrifuge tube. Samples were centrifuged for 6 h at a maximal gravity of 140,000 × *g* at 4 °C in a swinging bucket rotor, S52ST, in a Hitachi CS100GXL ultracentrifuge. Samples were collected into 24 consecutive portions of 230 μl each from the top and combined into eight fractions by mixing three consecutive portions for subsequent analyses. Because a substantial amount of pellet was obtained at the bottom, it was resuspended in 230 μl of 30% sucrose, 10 mM Hepes-NaOH (pH 7.4), and numbered as fraction 9. For immunoblotting, a one-third volume of fraction 9 relative to those of fractions 1–8 was loaded onto gels. In another centrifugal experiment, 0–51.2% (w/v) sucrose gradient was employed.

Inhibition of ATGL and Related Lipases in the Heart—Mice fasted overnight were perfused with 10 ml of 50 μM bromoenol lactone (BEL) in saline for 6 min. The heart was then immediately isolated and analyzed. For the visualization of LDs, Nile Red staining was performed with frozen sections. *In vitro* lipase assay was performed as described (23).

Isolation of Cardiomyocytes—Ventricular myocytes were obtained as described previously (24). Briefly, ventricular tissues isolated 1.5–3 days after birth were cut into small pieces and digested to produce single cells with an enzyme solution (3:1 mixture of 1 mg/ml collagenase and 0.25% trypsin in PBS). The cells obtained were seeded into collagen-coated 35-mm dishes and incubated in 2 ml of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum. After incubation overnight, attached cells were subjected to an FA oxidation assay.

FA Oxidation Assay—In each experiment, cardiomyocytes obtained from 3 to 4 mice were seeded into six 35-mm dishes and incubated overnight, as described above. Cells were cultured for a further 2 days in DMEM, 10% fetal bovine serum supplemented with 200 μM oleic acid conjugated with BSA to allow most cells to start spontaneous beating. Cells were then treated with 1 ml of preincubation medium (DMEM, 200 μM oleic acid) with or without 40 μM etomoxir (three dishes each) for 1 h. Subsequently, cells were incubated in 1 ml of the assay medium (DMEM, 20 mM Hepes-NaOH (pH 7.4) containing 4 μM [1-^{14}C]palmitic acid conjugated with α-cyclodextrin), supplemented with or without 40 μM etomoxir for 1 h. During this procedure, each 35-mm dish was uncovered and put in a covered 10-cm dish containing a piece of filter paper soaked in 1 N NaOH to trap radioactive CO_2 ("1st filter paper"). After incubation, the culture supernatants were transferred to test tubes, and the cells were dissolved in 300 μl of cell lysis buffer (25 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% Triton X-100). Protein concentrations of the cell lysates were quantified for 50-μl aliquots. One hundred microliters of 10% BSA followed by 150 μl of 3 M perchloric acid were added to the remaining cell lysates, which were then mixed and centrifuged. The supernatants contained intracellular acid-soluble materials (ASM), representing active metabolic intermediates. The pellet was extracted with chloroform/methanol (2:1), and the organic layer was collected (intracellular acid-insoluble fraction, representing cellular lipids). The culture supernatants were also mixed with BSA and perchloric acid and left for 30 min at room temperature, during which time the tubes were covered with parafilm attached with NaOH-soaked filter paper to trap CO_2 forced out from the culture supernatants ("2nd filter paper"). The mixtures were then centrifuged and separated into acid-soluble (extracellular ASM, representing dead-end metabolites such as acetic acid) and acid-insoluble (extracellular acid-insoluble fraction, mostly representing unincorporated FA) fractions, as described above. The filter paper and soluble and insoluble fractions were subjected to scintillation counting. β-Oxidation activity was assessed by the radioactivity of CO_2 (sum of radioactivities of 1st and 2nd filter papers), intracellular as well as extracellular ASM, with the first two representing metabolites en route to complete oxidation in mitochondria. Incorporation of FA was evaluated from the sum of the radioactivity of CO_2, intracellular and extracellular ASM, and the intracellular acid-insoluble fraction. Values obtained for three culture dishes were averaged and used as a result of a single experiment.

Measurements of Blood Parameters—Blood glucose levels were determined by Glutest sensor (SKK). Serum ketone bodies were determined with a β-hydroxybutyrate assay kit (Cayman). Insulin was quantified using an insulin ELISA kit (Shibayagi).

Food Intake, Respiration, and Locomotion—Mice were put in cages individually and allowed to access food and water *ad libitum* during the experiment. Food intake was measured for 10 days, and average intake per day was calculated. The respiratory gas analysis was performed with an Arco-2000 (Arco System), as described previously (25). Locomotor activity was measured with a 14-channel DAS system (Neuroscience).

Thiobarbituric Acid-reactive Substance (TBARS) Assay—Heart tissue samples were homogenized in lysis buffer as described above. Protein concentrations were quantified, and the extracts were subjected to a TBARS analysis, according to a published procedure (26), with slight modifications.

Echocardiography—For age-matched mice under 1.5% isoflurane anesthesia, heart rate and rhythm were monitored using 4-limb-lead electrocardiography (TMH150, Visual Sonics). Left ventricular contractile forces were quantified by transthoracic echocardiography using Vevo2100 (Visual Sonics). The percentage of left ventricular fractional shortening (FS) was calculated as follows: ((LVID;d − LVID;s)/LVID;d) × 100, where LVID;d and LVID;s indicate left ventricular end-diastolic and end-systolic dimensions, respectively. When the animals were also subjected to a TBARS assay, the hearts were excised several days after the echocardiographic measurement.

Statistical Analysis—All data are shown as means ± S.E. Data were analyzed with Student's *t* test, and differences with $p < 0.05$ were considered statistically significant. The correlation between heart parameters and TBARS values was assessed by a regression analysis.

RESULTS

Generation of Plin5-deficient Mice—*Plin5*$^{-/-}$ mice were generated (Fig. 1, *A* and *B*), and the absence of Plin5 mRNA and protein in tissues of mutant mice was confirmed (Fig. 1, *C* and *D*). Expression of *Plin4*, a close neighbor of *Plin5* in the genome, was not affected (Fig. 1*C*). Intercrossing of *Plin5*$^{+/-}$ mice provided *Plin5*$^{-/-}$ offspring at a Mendelian ratio. Deletion of *Plin5* caused no abnormality in either cumulative body weight or

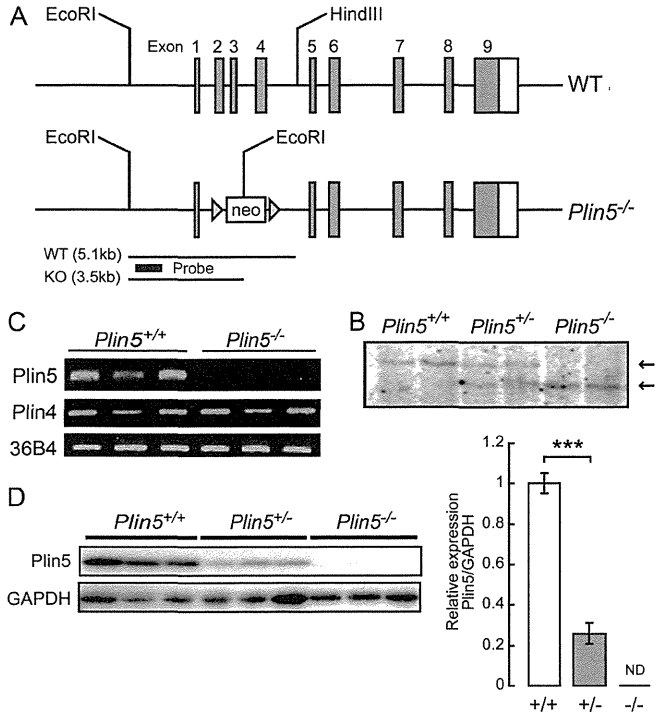

FIGURE 1. **Generation of Plin5 knock-out mice.** *A*, structures of wild-type and null Plin5 alleles. *B*, confirmation of *Plin5* disruption by Southern blotting with genomic DNA prepared from the tail. *Arrows* indicate wild-type (*WT*) and knock-out (*KO*) bands corresponding to the fragments depicted in *A*. *C*, RT-PCR to reveal the absence of Plin5 mRNA in the hearts of *Plin5*$^{-/-}$ mice. Plin4 mRNA was expressed at the normal level. *D*, confirmation of the absence of Plin5 protein in the hearts of *Plin5*$^{-/-}$ mice. The *graph* on the *right* shows the Plin5/GAPDH ratios of mice of the three genotypes. *ND*, not detectable; ***, $p < 0.001$.

body length (data not shown). Weights of tissues, including the heart, were not significantly different between the two genotypes of mice at 16–20 weeks of age (data not shown). Thus, *Plin5* deficiency does not cause apparent defects in growth and development. Further experiments were performed with male mice of age 16–20 weeks, unless otherwise noted.

Absence of LDs in the Hearts of Plin5$^{-/-}$ *Mice*—Based on the particularly abundant expression of Plin5 in the heart (4), we expected its deficiency to affect the properties of LDs most prominently in the heart. Accordingly, we first stained the heart sections of wild-type and *Plin5*$^{-/-}$ mice with ORO (Fig. 2*A*). In wild-type mice, small LDs were observed in a limited number of heart cells in the fed state. LDs were markedly augmented upon fasting as reported previously (27), due to increased delivery of FA from WAT. Surprisingly, no LDs were detected in the hearts of *Plin5*$^{-/-}$ mice by ORO staining either in the fed or fasted state. By electron microscopy, LDs were not observed in the hearts of fasted *Plin5*$^{-/-}$ mice (Fig. 2*B*). We also measured the content of TAG and FA in the heart. Consistent with the apparent absence of LDs, the TAG content in the hearts of *Plin5*$^{-/-}$ mice was lower than that in control mice (Fig. 3*A*, *panel a*), in both fed and fasted animals. The FA level was also lower in the hearts of *Plin5*$^{-/-}$ mice than that of wild-type mice (Fig. 3*B*, *panel a*).

Lipid and LD Contents of Other Tissues—Effect of Plin5 ablation on the contents of TAG and FA was also examined for other tissues. In soleus muscle, TAG content tended to be slightly lower in *Plin5*$^{-/-}$ mice than in control mice in the fed state, but it was not significantly different between them in the fasted state (Fig. 3*A*, *panel b*). FA content was not significantly different either between the two genotypes, although it was severely reduced by fasting (Fig. 3*B*, *panel b*). Upon ORO staining, LDs were detected in a small number of myotubes in both genotypes, when the mice were fasted (Fig. 2*C*). Their size and abundance were slightly greater in the wild-type mice. In the liver, levels of both TAG (Fig. 3*A*, *panel c*) and FA (Fig. 3*B*, *panel c*) were lower in *Plin5*$^{-/-}$ mice than wild-type mice when the animals were fed. In contrast, contents of TAG and FA were higher in *Plin5*$^{-/-}$ mice than wild-type mice when they were fasted. Thus, under fasting conditions, where Plin5 expression is abundant in the liver (4), the effect of Plin5 ablation on lipid accumulation was apparently opposite that in the heart. In BAT, TAG content was significantly lower for *Plin5*$^{-/-}$ mice than wild-type mice when they were fed (Fig. 3*A*, *panel d*). This difference was not observed when mice were fasted, due to the severe reduction in TAG that occurred only in wild-type mice. No difference was observed in the tolerance to cold exposure between the two genotypes (data not shown), despite the different TAG content in the fed state. Inguinal WAT contained indistinguishable levels of TAG in both genotypes of mice, although they were significantly decreased by fasting (Fig. 3*A*, *panel e*). Liver, BAT, and WAT contained abundant or large LDs in both genotypes (data not shown). Thus, the effect of Plin5 ablation was most prominently revealed as apparent lack of LDs only in the heart.

Blood Parameters and Other Systemic Effects of Plin5 Ablation—Blood nonesterified fatty acid levels were 1.4-fold higher in *Plin5*$^{-/-}$ mice than in wild-type mice in the fasted state (data not shown). This result, although the underlying mechanism is not clear, suggests that the absence of detectable LDs in the hearts of *Plin5*$^{-/-}$ mice is not due to decreased delivery of FA to the heart. No difference was noted between the two genotypes in blood concentrations of glucose, TAG, ketone body, and insulin (data not shown).

Food intake did not differ significantly between wild-type and *Plin5*$^{-/-}$ mice (Fig. 4*A*). *Plin5*$^{-/-}$ mice consumed 7% more oxygen than wild-type mice during the dark period (Fig. 4*B*). The respiratory quotient was similar between *Plin5*$^{-/-}$ and wild-type mice during either light or dark (Fig. 4*C*). The locomotor activity of *Plin5*$^{-/-}$ mice was higher than that of wild-type mice during the dark phase (Fig. 4*D*), being consistent with increased respiration. As shown in Fig. 9, fatty acid oxidation is enhanced in the hearts of *Plin5*$^{-/-}$ mice. This would yield more ATP in the hearts, hence apparently being consistent with the increased locomotor activity. The mechanism of behavioral change caused by the ablation of Plin5 needs to be addressed.

Intracellular Distribution of TAG and LD-binding Proteins in the Heart—Based on the marked effect of Plin5 ablation on heart LDs, we focused our further study on this organ. As shown in Fig. 3*A*, *panel a*, a substantial amount of TAG was contained in the hearts of *Plin5*$^{-/-}$ mice, despite the apparent lack of LDs. We asked where the remaining TAG was located in the heart cells, by subcellular fractionation employing sucrose-density gradient centrifugation. In wild-type samples, TAG was

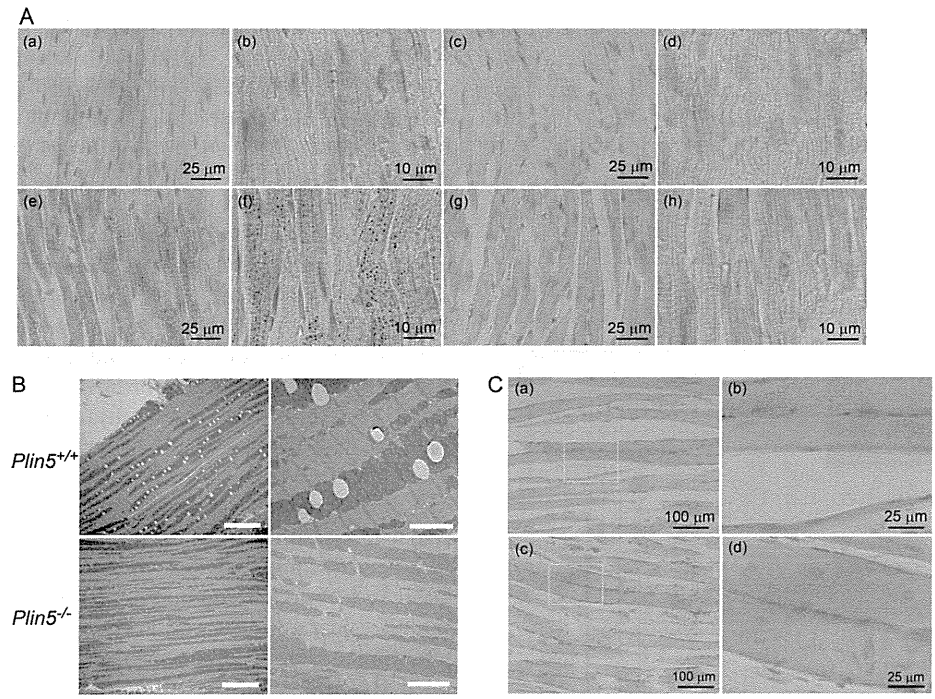

FIGURE 2. **Absence of LDs in the hearts of *Plin5*$^{-/-}$ mice.** *A*, ORO staining of heart cryosections of wild-type (*panels a*, *b*, *e*, and *f*) and *Plin5*$^{-/-}$ (*panels c*, *d*, *g*, and *h*) mice in the fed (*panels a–d*) and fasted (*panels e–h*) states. *Panels a*, *c*, *e*, and *g*, low magnification (*bar*, 25 μm). *Panels b*, *d*, *f*, and *h*, high magnification (*bar*, 10 μm). No LD was observed even by close inspection of the sections of *Plin5*$^{-/-}$ mice in either state. In wild-type mice, out of 20 randomly selected microscopic fields (each 86.4 × 60.5 μm), four in the fed state and all in the fasted state contained detectable LDs. The number of LDs in a field was 18.7 ± 11.0 in the fed mice and 966 ± 409 in the fasted mice, respectively ($p = 4.3 \times 10^{-9}$). The sizes of LDs were 0.22 ± 0.04 μm in the fed and 0.64 ± 0.11 μm in the fasted states, respectively ($p = 2.3 \times 10^{-7}$). *B*, representative electron microscopic images of the hearts of wild-type and *Plin5*$^{-/-}$ mice fasted overnight. *Left panels*, low magnification (*bar*, 10 μm), and *right panels*, high magnification (*bar*, 2 μm). Note that LDs are observed as *white ovals* only in wild-type mice. *C*, ORO staining of soleus muscle cryosections. *Panels a* and *b*, wild-type, and *panels c* and *d*, *Plin5*$^{-/-}$ mice, both fasted overnight. *Panels a* and *c*, low magnification. *Bar*, 100 μm. *Panels b* and *d*, high magnification of the area enclosed by a *rectangle*. *Bar*, 25 μm.

mostly recovered in the upper fraction under fasted conditions and also to a considerable extent under fed conditions (Fig. 5*A*). However, TAG was undetectable in the upper fraction in the hearts of *Plin5*$^{-/-}$ mice under both fed and fasted conditions, being consistent with the apparent lack of LDs. Instead, TAG was only found in the lower fractions, most abundantly in the pellet (fraction 9) in *Plin5*$^{-/-}$ mice. Even for wild-type mice, a substantial amount of TAG was recovered in the lower fractions, the pellet in particular, when they were fed.

In wild-type mice, although Plin5 was detectable in the upper fraction, it was found to a much larger extent in the lower fractions, particularly the pellet (Fig. 5*B*). Conversely, Plin2 was abundant in the upper fraction in the fasted state but was mostly recovered in the lower fractions particularly just above the pellet (fraction 8) in the fed state. Thus, the distribution of these two proteins was different in that Plin5 was less abundant in the upper fraction, although pelleted to a larger extent, as compared with Plin2. In *Plin5*$^{-/-}$ mice, Plin2 was recovered only in the lower fractions irrespective of feeding conditions. Proteins (Fig. 5*C*) as well as organellar and cytosolic markers (Fig. 5*D*) were also abundant in the lower fractions, with most concentrated just above the pellet (fraction 8). Actin was pelleted under the same conditions to a significant extent. These results suggest that TAG as well as perilipin proteins are mostly contained in denser structures, in the hearts of *Plin5*$^{-/-}$ mice and fed wild-type mice. These structures are probably different from major organelles such as mitochondria and endoplasmic reticulum, based on the different distribution in sucrose gradient.

To further characterize the TAG-containing dense structures, we performed another centrifugal experiment with a wider range of sucrose gradient. In both fed wild-type and *Plin5*$^{-/-}$ mice, TAG was distributed in the fractions in a density range of 1.18–1.22 (Fig. 5*E*), overlapping with the distribution of mitochondria and, to a lesser extent, endoplasmic reticulum (Fig. 5*F*). Plin5 and Plin2 were found in these fractions and also less dense fractions where endoplasmic reticulum was abundant. This result suggests that TAG is contained in structures with defined densities in the hearts of fed wild-type and *Plin5*$^{-/-}$ mice, although these structures were not separated from other organelles in this centrifugal condition.

We next examined the distribution of perilipin proteins in the heart cells by immunofluorescence microscopy. Plin5 and Plin2 coexisted in particulate structures in the hearts of wild-type mice (Fig. 6, *A–D*), consistent with previous observations (5, 28). Relative abundance of these proteins on individual particles was not necessarily constant, as judged by the different color tones of particles in merged views (Fig. 6, *C* and *D*). Even for fed mice, Plin5 and Plin2 extensively coexisted in particulate structures in most heart cells (Fig. 6*A*), although most of these particulates appeared significantly smaller than those in fasting mice (Fig. 6*B*). The abundance of these structures in fed mice was in contrast to the limited occurrence of LDs in a small population of cells revealed by ORO staining (Fig. 2*A*). For fast-