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Establishment and Pathophysiological Characterization of Type 2 Diabetic Mouse Model Produced by Streptozotocin and Nicotinamide

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    This study was performed in order to establish a mouse model that represents the non-obese type 2 diabetes reflecting a majority of diabetic patients among Asian races and to show its pathophysiological profiles. Streptozotocin (STZ) was administered to C57BL/6J mice with or without nicotinamide (120 or 240 mg/kg, STZ/NA120 or STZ/NA240), twice with an interval of 2 d, and plasma glucose concentration, body weight, water intake, insulin contents and insulin signal-related proteins were monitored. STZ-induced hyperglycemia (fasting and non-fasting), body weight loss and polyposia were significantly depressed by NA dose-dependently. In STZ/NA120 and STZ/NA240 mice, pancreatic insulin content was retained by 28 and 43% of normal control (10.5±0.93 μU/ml), respectively, and histological damage of pancreatic beta cells was also less severe than that observed in STZ mice. When given the calorie-controlled high fat diet, the STZ/NA mice caused hyperlipidemia, and significantly increased insulin resistance. These observations suggest that the combined administration of STZ and NA causes partial depletion of pancreatic insulin and that the high fat constituents lead to insulin resistance in this model. The present mouse model, therefore, well exhibits the recent diabetic pathophysiological characteristics of a majority of Asian patients.

    Key words   nicotinamide; streptozotocin; type 2 diabetes; model mouse; high-fat diet; insulin resistance

The number of patients with diabetes mellitus, who exhibit insulin resistance, is increasing recently all over the world.[1] The major causes have been suggested to be functional disorders in insulin secreting capacity and in carbohydrate metabolism deterioration with aging.[2,3] In the type 2 diabetic patients without obesity, who have been frequently found in Japanese, Chinese and Korean, the reduction in insulin secretion seems to be more important than the increase in insulin resistance.[4] These defects may be caused by long term hypersecretion of insulin in response to the intake of high calorie food owing to westernized food habits. The hypersecretion of insulin leads to depletion of the insulin stock and hence to the decline in insulin secretion from the pancreatic beta cells.

Several kinds of knock-out mice have been recently developed as animal models for diabetes. Although these models express several important molecular pathological features of diabetes, they carry some disadvantages in that they do not exactly express some of the symptoms of type 2 diabetes. Moreover, maintenance of these genetically-modified mice requires intensive managements in large-scaled facilities so that it needs so much cost. In order to overwhelm these disadvantages of genetically-modified model mice, we attempted to establish a type 2 diabetic model with non-obese features mouse by a non-genetic way.

Streptozotocin (STZ) is a widely used chemical inducer for type 1 diabetes.[5] STZ has been shown to produce free radicals in the body which specifically cut DNA chains in the pancreatic beta cells, resulting in disorder of the function of pancreatic beta cell and, at a later phase, destruction of the beta cells by necrosis.[6] These processes evoke activation of the poly ADP ribose synthase to repair the damaged DNA,[7] and a large amount of nicotinamide dinucleotide[8] is consumed for this restoration.[9] Here, consumption of NAD is effectively supplemented by intake of nicotinamide (NA). Meanwhile, in some clinical practice, a high dose of NA is occasionally administered to diabetic patients to prevent the progress of insulin-dependent diabetes (IDDM).[10] Type 2 diabetic model in rats with combined application of STZ and NA has been already reported.[11] Thus it was considered that the similar procedures would be applicable for the prevention from STZ-induced excessive destruction of beta cells in mice. We report here the establishment of a novel method for producing non-obese type 2 diabetic mouse model with a non-genetic way, as well as its pathophysiological characterization. We also show that the present mouse model is useful for searching drugs that are beneficial to treatment of the non-obese type 2 diabetes.

MATERIALS AND METHODS

Animals and Drugs   All animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH publication number 85-23, revised 1985) and the Guidelines of the Animal Investigation Committee, Chiba University, Japan.

Male C57BL/6J mice of 5—6 week old age were purchased from Japan SLC Inc. (Hamamatsu, Japan) and were kept on a 12 h light/dark cycle in a temperature-controlled vivarium. They were allowed to take food and water *ad libitum* before any food-related treatment started.

Reagents   Following chemicals, enzymes, and antibodies were used in this study; nicotinamide (NA), streptozotocin (STZ), insulin (Sigma-Aldrich Japan, Tokyo, Japan), rabbit polyclonal antibodies against insulin receptor substrate-1 (IRS-1), IRS-2 and glucose transporter-4 (GLUT-4), mouse

monoclonal antibody against phosphatidyl inositol-3 kinase p85α (PI-3K p85α) (Santa Cruz, Inc, CA, U.S.A., Code No. sc-559, sc-8299, sc-7938, sc-1637, respectively), test kits for a free fatty acid "NEFA C-test Wako," a triglyceride "Triglyceride G-test Wako," a total cholesterol "Cholesterol E-test Wako" and a HDL-cholesterol "HDL cholesterol E-test Wako" (Wako Chemicals, Osaka, Japan), a RIA kit insulin "EIKEN" (Eiken Kagaku, Tokyo, Japan), a Mercodia Ultrasensitive Mouse Insulin ELISA kit (Mercodia, Uppsala, Sweden). Krebs-Ringer bicarbonate (KRB) buffer consisted of 118 mM-NaCl, 4.9 mM-KCl, 1.75 mM-CaCl$_2$, 1.23 mM-MgSO$_4$, 1.23 mM-NaH$_2$PO$_4$, and 25 mM-NaHCO$_3$.

High-Fat Diet A high-fat diet and a chow diet were manufactured by Oriental Yeast Co. Ltd., Japan, according to our original recipe (Table 1A). The content of the calorie of fat in the high-fat diet was set to be twice as much as that in the chow diet. The fat in lard was used as the fat content in the high-fat diet since such animal fat is known to cause insulin resistance.[12] In contrast, soybean oil was used as the fat content in the chow diet. The contents of other nutrients, such as crude proteins, crude ash, and water were equivalent in both diets (Table 1B). Total calories of the high fat and chow diets were adjusted to be 341 and 342 kcal/100 g, respectively, and these diets were processed into solid tablets which had the same volume and stiffness with roughage and soluble nitrogen free extract (Table 1C). The high-fat diet was given to animals of 5 week old age.

Table 1. The Table of Constituents, Calorie and Nutrition in High-Fat Diet

A. Constituents of the diets

	Chow diet (%)	High fat diet (%)
Casein	25	25
Cystine	0.3	0.3
Soybean oil	6	1
Lard	—	13
α-Starch	10	10
β-Starch	22	5
Sucrose	20	20
AIN93G mineral	3.5	3.5
AIN93G vitamin	1	1
Choline ditartarate	0.25	0.25
Tributylhydroquinone	0.014	0.004
Cellulose powder	11.936	20.946

B. Calorie comparative table

	Chow diet (%)	High fat diet (%)
Protein	25	25
Lipid	16	34
Carbohydrate	59	41

C. Nutrition table

	Chow diet (%)	High fat diet (%)
Water content	7	7
Crude protein	21	21
Fat	6	13
Crude ash content	4.2	4.2
Roughage	11.1	19.5
Soluble nitrogen free extract	50.7	35.3
Total calorie (kcal/100 g-diet)	342	341

Induction of Diabetes in Mice with Streptozotocin and Nicotinamide In a preliminary experiment, we determined an optimum dosing and schedule for administering STZ and NA, with male C57/BL6J mice of 6 week old age. Mice were fasted for 16 h before receiving chemicals. STZ was dissolved in 50 mM-citric acid buffer at the time of use and was administered at 100 mg/kg, i.p. twice on days 0 and 2. NA was dissolved in saline and was injected at 60, 120, 240 and 480 mg/kg i.p. at 15 min before administration of STZ. The plasma glucose (PG) concentration and the body weight were measured on days 0, 7, 14, 28 and 35. Biochemical measurements and pathological examinations were performed on day 35. In another series of experiments, diabetes was induced by combined administration of STZ (100 mg/kg, i.p.) and NA (240 mg/kg, i.p.).

Plasma Glucose Concentration Blood (5—10 μl) was collected from the tail vein of mice every 7 d after starting of the induction of diabetes. PG concentration was measured 12 h before and 16 h after removal of food at 7 p.m. as a 'non-fasting' and a 'fasting' PG concentration, respectively, using Blood Glucose Test Meter (Sanwa Kagaku, Nagoya, Japan). Instantly after measurement of PG concentration, food was given to mice.

Body Weight Gain and Water Intake The body weight gain and the water intake were measured on day 35 after starting of the induction of diabetes. The water consumption of each animal group was estimated as the mean value per each group for 24 h.

Oral Glucose Tolerance Test (OGTT) Mice had been fasted for 16 h before oral administration of glucose solution (2.0 g/kg), and PG concentration was measured at −30 ("Pre"), 15, 30, 60 and 120 min after the glucose administration on day 35 after induction of diabetes.

Insulin Tolerance Test (ITT) Mice had been fasted for 16 h before injecting insulin (0.04 U/10 g of body weight, s.c.), and PG concentration was measured at −30 ("Pre"), 30, 60 and 120 min after the injection of insulin on day 35 after induction of diabetes. Insulin (Penfil R 100 U/ml) was diluted with the insulin dilution buffer (10 mM-HCl solution containing 0.1—0.25%-phenol and 1.4—1.8%-glycerin) according to the indications in the *Japanese Pharmacopoeia*.

Plasma Insulin Concentration in the Diabetic Model Mice On day 35 to 38 after starting of the induction of diabetes, animals were fasted for 16 h and then blood was collected from orbit. The plasma was obtained by centrifugation at 10000×g for 10 min at 4 °C, and 25 μl of it was assayed for insulin by ELISA with Mercodia Ultrasensitive Insulin ELISA Kit (Mercodia AB Uppsala, Sweden).

Pancreatic Insulin Content On day 35 to 38 after starting of the induction of diabetes, animals were fasted 16 h and then euthanized with CO$_2$. The pancreas was isolated immediately, and pancreatic insulin content was measured with a RIA kit insulin "EIKEN" (Eiken Kagaku, Tokyo, Japan), according to the method of Tokunaga *et al.*[13]

Histopathological Examination of Pancreatic Islets On day 35 to 38, pancreatic islets were examined immunohistochemically. The pancreas was dissected out and immediately fixed with 4%-paraformaldehyde in 0.1 M-phosphate buffer for 120 min at 4 °C, rinsed in 70%-ethanol, and embedded in paraffin. The thin sections of 10 μm thickness were deparaffinized, rehydrated and incubated with antibod-

ies against insulin (DAKOC Arpinteria, CA, 1/100 dilution), glucagon (DAKOC Arpinteria, CA, 1/100 dilution) or somatostatin antibodies (DAKOC Arpinteria, CA, 1/100 dilution) for 20 min at room temperature. Finally, the preparations were stained by the streptavidin–biotin complex-peroxidase method using an immunostaining kit (LSAB2 kit/HRP, DACO) to colorize with DAB.

Biochemical Analysis in Tissues from the Diabetic Model Mice In order to examine insulin resistance in the present mouse model, the insulin signaling-related proteins in the liver and adipose tissues on day 35 to 38 were detected by the following methods. Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and the liver was excised after consecutive perfusion through portal vein with (i) 1 ml of heparin (1000 U/ml in saline), (ii) 10 ml of Ca^{2+} free Krebs-Ringer bicarbonate (KRB) buffer, and (iii) 10 ml of KRB buffer containing Ca^{2+} (1 mM), 0.05%-collagenase, and 0.005%-trypsin inhibitor. The epididymal fat pad were also excised from the anesthetized mouse as adipose tissues. After measuring the wet weight, the tissues were minced in the lysis buffer, containing 50 mM-Tris–HCl, 150 mM-NaCl, 1%-Triton X-100, 10 μg/ml-leupeptin, 10 mM-β-glycerophosphate, 1 mM-Na_3VO_4 and 1 mM-PMSF, and 150 mg of the tissues were homogenized in 1 ml of the lysis buffer on ice. The tissues were centrifuged at $386000 \times \boldsymbol{g}$ for 60 min at 4 °C. The supernatant (cytosol fraction) was used for Western blotting with rabbit polyclonal antibodies against IRS-1 and IRS-2 and mouse monoclonal antibody against PI-3K p85α. The precipitate (crude membrane fraction) was examined for detection of GLUT-4, which was translocated to plasma membrane, with a rabbit polyclonal antibody against GLUT-4. For detection of the immunosignals, HRP-conjugated IgG was treated as the secondary antibody and the ECL signal was detected by image analyzer LAS 1000 plus (Fuji Film Co. Ltd., Tokyo, Japan). The tissue suspension were prepared according to the ordinary method, and used for Western blotting as mentioned above for liver. The data were expressed as a percent of normal control value, and an average from independent three experiments was obtained.

Effect of Buformin and Glibenclamide in OGTT Buformin (100 mg/kg) or glibenclamide (4 mg/kg) was orally administered to high-fat fed STZ/NA240 mice twice a day for 2 weeks before OGTT.

Data Analysis Data are expressed as mean±S.E.M. Statistical differences were analyzed by paired t-test and Tukey-Kramer's multiple-comparison test with Stat View version 5.0 (SAS Institute, U.S.A.). Differences with $p < 0.05$ were considered as statistically significant.

RESULTS

Establishment of Type 2 Diabetic Mouse Model with Combined Administration of Streptozotocin and Nicotinamide In a preliminary experiment, we determined the optimal concentration of streptozotocin (STZ) and nicotinamide (NA) and the schedule for administration of these chemicals. First, the following schedules were compared for STZ treatment; (A) 100 mg/kg, i.p., twice with an interval of 2 d, (B) 100 mg/kg, i.p., twice with an interval of 1 week, and (C) 40 mg/kg, i.p., twice with an interval of 5 d. In 5 weeks after starting of each schedule for STZ treatment, PG con-

centration was compared among the schedules at 60 min after administration of glucose in OGTT. As the result, the PG concentration showed a significant elevation in the protocols A, B and C, whereas no significant difference was observed among these protocols. However, the high level of PG was then maintained for 120 min only in the protocol A, which led us to decide to use the protocol A in the following experiments.

Secondly, the combined administration of STZ (100 mg/kg, i.p.) and NA (60, 120, 240, 480 mg/kg, i.p.) was examined in the protocol A. This treatment depressed the elevation of PG, the loss of body weight gain and the excess water intake in a dose-dependent manner. In particular, doses of 120 and 240 mg/kg NA significantly inhibited the increase in PG concentration, restored body weight gain and depressed the overeating. These observations let us to use the optimal protocol of STZ (100 mg/kg, i.p.) and NA (120, 240 mg/kg, i.p.), administered twice with an interval of 2 d, for the development of diabetes.

We further examined the strain difference in ddY and ICR mice by comparing the diabetic indices, and found that the data variance of each diabetic index in C57BL/6J mice was smaller than that in ddY and ICR mice (data not shown). Thus, C57BL/6J mice were considered to be most suitable for establishing the type 2 diabetic model. In fact, C57BL/6J mice are extensively used in a number of studies on diabetes such as insulin resistance, hyper-insulinemia and hyperglycemia induced by high-fat diet,[14,15] which is also harmonized with our decision.

Finally, we performed a series of experiments to examine if the optimal protocol procedures induce pathophysiological features of a type 2 diabetes in C57BL/6J mice. In the combined groups of STZ and NA (STZ/NA), all values of fasting PG concentration (Fig. 1A), body weight (Fig. 1B) and water intake (Fig. 1C) were intermediate between those of the normal control and the STZ group on days 21, 28 or 35 after induction of diabetes. In non-fasted animals, NA also inhibited the STZ-induced hyperglycemia in a dose-dependent manner (Fig. 2), as was shown in fasted animals.

OGTT and ITT In OGTT, the PG concentrations in both STZ/NA120 and STZ/NA240 groups ranged between those noted in the normal group and the STZ alone group. The recovery from hyperglycemia in STZ/NA120 and STZ/NA240 groups was not observed at 120 min after administration of glucose, although the PG concentration returned to the original level in the normal group (Fig. 3A).

In ITT, the STZ alone group showed an initial prompt reduction in PG during the first 30 min after the injection of insulin. Then, the PG concentration stopped the decline. It was sustained at the value of over 150 mg/dl. These PG levels were significantly higher than those in normal group ($p < 0.001$). In contrast, the PG levels of STZ/NA groups were maintained at the original levels prior to administration of insulin. These levels were comparable to those in normal group (Fig. 3B).

Pancreatic Insulin and Plasma Insulin Concentration Treatment with STZ alone greatly reduced the pancreatic insulin content; it fell to less than 5% of the control pancreatic insulin content (Fig. 4A). Correspondingly, plasma insulin level was also diminished in the STZ alone group to about 20% of the control level (Fig. 4B). In contrast, the loss of
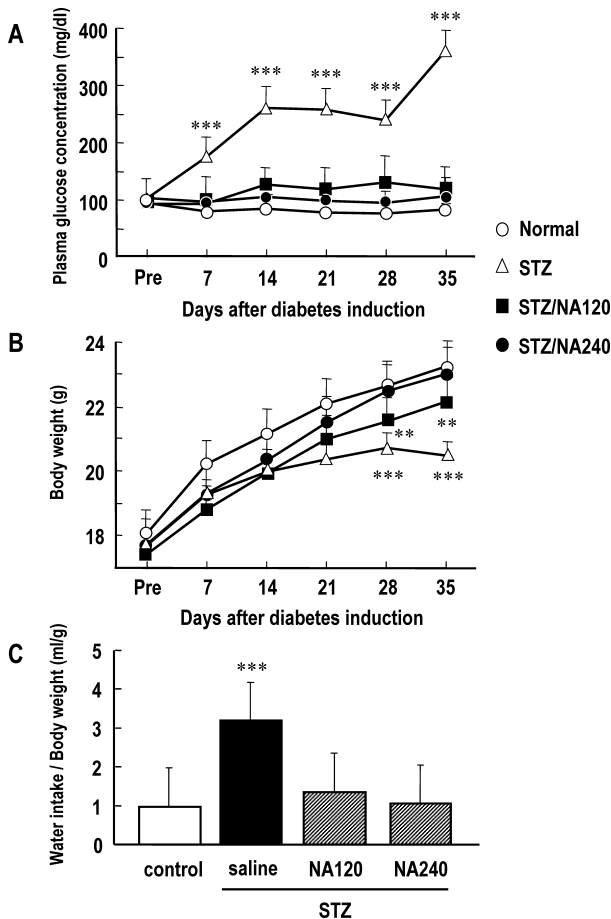
Fig. 1. Development of Diabetic Mouse Model with STZ and NA

Alteration of fasted PG concentration (panel A) and body weight gain (panel B) during 35 d after treating with STZ (100 mg/kg) and NA (120 or 240 mg/kg) in C57BL/6J mice. Mice treated with STZ (100 mg/kg) alone served as control. Saline was injected in the control group. The amount of total water intake was measured on day 35 after starting of induction of diabetes (panel C). Data are expressed as mean±S.E.M. ($n=7$—8). **, *** Significantly different ($p<0.01$, $p<0.001$, respectively) from the control group.
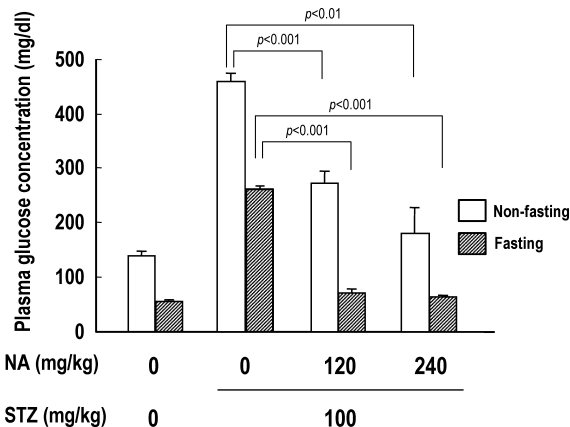
Fig. 2. Dose-Dependent Depression by NA of STZ-Induced Hyperglycemia

The non-fasting and fasting PG concentration were measured 12 h before and 16 h after removal of food at 7 p.m. on day 34 after starting of induction of diabetes, respectively. Data are expressed as mean±S.E.M. ($n=7$—8).

pancreatic insulin content was significantly prevented by treatment with NA, in a dose-dependent manner. The reduction in plasma insulin concentration was also restored by
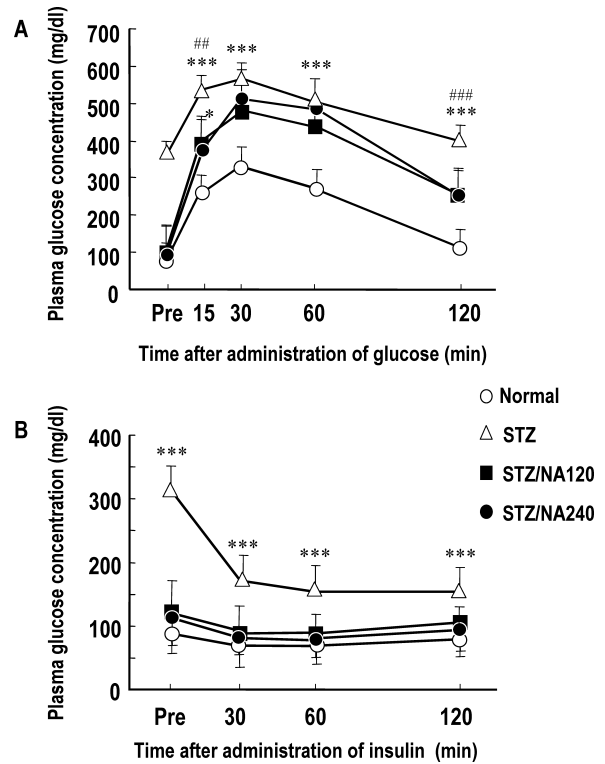

Fig. 3. Effect of NA on Glucose Tolerance and Insulin Tolerance in STZ-Treated C57BL/6J Mice

Glucose tolerance was evaluated from the time course of PG after administration of glucose solution (2 g/kg, p.o.) in OGTT (panel A). Insulin tolerance was evaluated from the time course of PG after administration of insulin (0.04 U/10 g-body weight, s.c.) in ITT (panel B). OGTT and ITT were performed on day 35 after induction of diabetes. Data are expressed as mean±S.E.M. ($n=7$—8). *, *** Significantly different ($p<0.05$, $p<0.001$, respectively) from the vehicle control. ##, ### Significantly different ($p<0.01$, $p<0.001$, respectively) from STZ/NA120 group.

NA120 and NA240, although not in a dose-dependent manner.

Histopathological Observations in the Pancreatic Islets The pancreatic beta cells were markedly and specifically lost in the tissue of the STZ alone group (Fig. 5). This morphological damage in the pancreatic beta cells was distinctly prevented in the groups of STZ/NA120 and STZ/NA240. The treatment with NA protected beta cells from destruction by STZ in a dose-dependent manner. In contrast, neither number of glucagon nor somatostatin positive cell was affected in number by treatment with STZ or STZ/NA.

Western Blotting of the Insulin Signaling Related Proteins The expressions of IRS-1, IRS-2 and PI3K in the liver of the STZ alone group were significantly reduced to 10—40% of the respective normal level (Fig. 6A), while that of IRS-2 in the adipose tissues of the STZ alone group was significantly diminished to about 60% of the normal level (Fig. 6B). On the other hand, the combined NA240 depressed the reduction of these molecular expressions in the liver or the adipose tissues, but the recovery did not reach the respective normal level. The amount of GLUT-4 in the adipose tissues was not significantly different among all groups (Fig. 6B).

Effect of High-Fat Diet on the Insulin Resistance First, we examined to see if the intake of high fat in the food affects the blood components in normal mice. When normal mice of 5 week old age had been raised with high-fat diet for
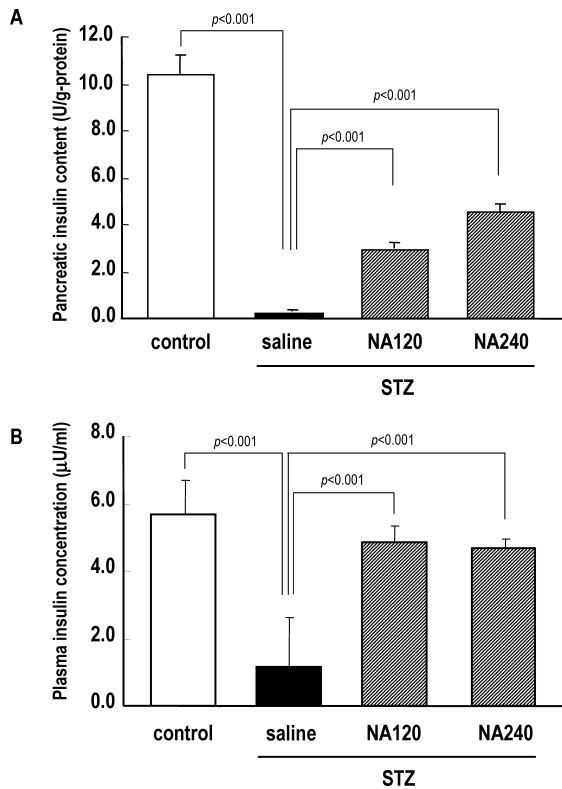
Fig. 4. Effect of NA on STZ-Induced Decrease in Insulin Level of Pancreas and Plasma in C57BL/6J Mice

The insulin levels of pancreas (panel A) and plasma (panel B) are shown. The measurements of insulin level were performed on day 35 to 38 after induction of diabetes. Data are expressed as mean±S.E.M. ($n=5$).
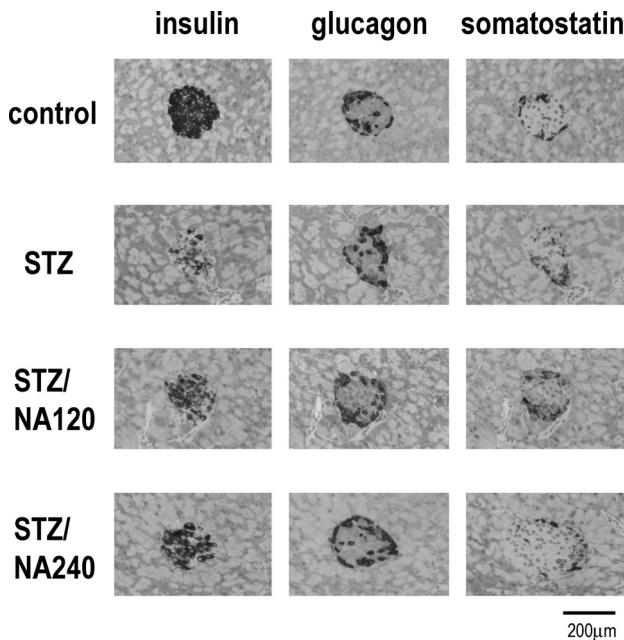

Fig. 6. Expression of Insulin Signaling-Related Proteins in the Liver and Adipose Tissues of C57BL/6J Mice

Protein expressions of IRS-1, IRS-2, PI3K or GLUT-4 were analyzed with Western blotting of the homogenates in the liver (panel A) and the adipose tissues (panel B). The tissue samples were excised on day 35 to 38 after induction of diabetes. Data are expressed as mean±S.E.M. ($n=3$).

Fig. 5. Effect of NA on STZ-Induced Histopathological Damage in the Pancreatic Islets of C57BL/6J Mice

Pancreatic alpha, beta and delta cells are immunohistochemically stained with the corresponding antibodies, as mentioned in the Materials and Method. Other portions of tissues were stained by Hematoxylin–Eosin. The pancreatic samples were excised on day 35 to 38 after induction of diabetes.
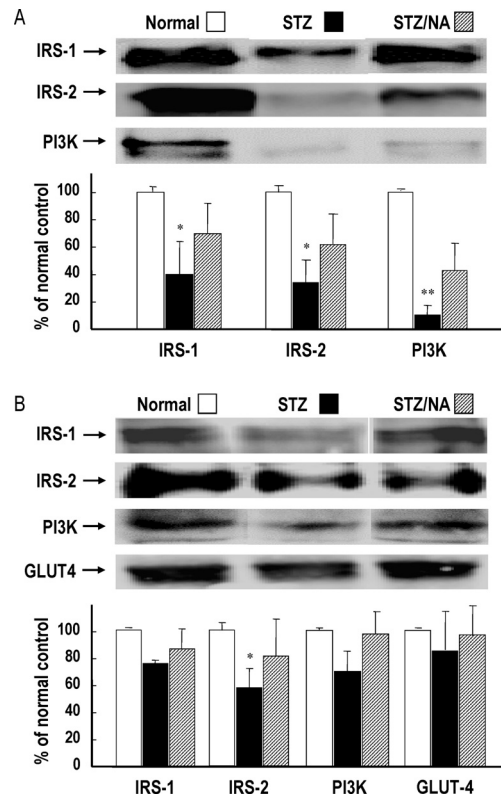
6 weeks, the fasting PG did not exhibit any significant change, as compared with that in the animals raised with chow-fed diet (Fig. 7A). Although the high-fat diet did not affect the free fatty acid content, it significantly elevated the triglyceride, total cholesterol and high density cholesterol HDL contents (Table 2). Body weight of the high-fat diet-fed group was heavier than that of the chow diet-fed group (Table 2). In the next step, we examined the effect of high-fat diet, given for 6 weeks, on the glucose tolerance in STZ/NA-induced diabetic mice. OGTT was performed 5 weeks after induction of diabetes in the animals fed with high-fat diet for 6 weeks. Significant deterioration in glucose tolerance was found in both chow-fed and fat-fed diabetic groups (Fig. 7B). At 60 min after administration of glucose, PG concentration of diabetic fat-fed group (372.3 ± 18.10 mg/dl, $p<0.001$ *vs.* normal chow-fed) was significantly increased, as compared with diabetic chow-fed group (277.2 ± 14.68 mg/dl, $p<0.01$ *vs.* STZ/NA240, fat-fed group). At 120 min after glucose administration, the same significant increase was still observed (Fig. 7B).

Insulin Tolerance Test in High-Fat Diet Mice   Insulin resistance induced by high-fat diet was evaluated with ITT in 7 weeks after taking high-fat diet, namely 6 weeks after induction of diabetes. Plasma glucose concentration was measured at 30 min after injection of insulin (0.4 U/kg, s.c.). The ratio of glucose disappearance was lowered in the high fat-fed STZ/NA240 group (Fig. 8). In particular, significant differences were shown between high fat-fed normal and dia-

Table 2. Effect of High-Fat Diet on the Body Weight Gain, Plasma Glucose Concentration, Triglyceride, Free Fatty Acid, Total Cholesterol and HDL-Cholesterol in the Mouse Model

	Chow diet		High fat diet	
	Normal	STZ/NA240	Normal	STZ/NA240
Body weight (g)	23.9 ± 1.4	23.0 ± 0.52	27.7 ± 0.51**	23.7 ± 0.65
Fasting plasma glucose (mg/dl)	76.0 ± 3.0	111 ± 11	80.0 ± 6.3	97.4 ± 11
Triglyceride (mg/dl)	113 ± 9.0	141 ± 11	137 ± 5.8	168 ± 13**
Free fatty acid (mEq/l)	2.50 ± 0.10	2.65 ± 0.20	2.60 ± 0.10	2.70 ± 0.10
Total cholesterol (mg/dl)	92.6 ± 5.3	103 ± 5.4	119 ± 11*	122 ± 4.0**
HDL-cholesterol (mg/dl)	72.8 ± 6.9	75.1 ± 3.0	93.7 ± 11	94.6 ± 3.2**

*$p < 0.05$ and **$p < 0.01$ *versus* the respective chow fed group. Data are expressed as the mean$\pm$S.E.M., $n = 9$—10.
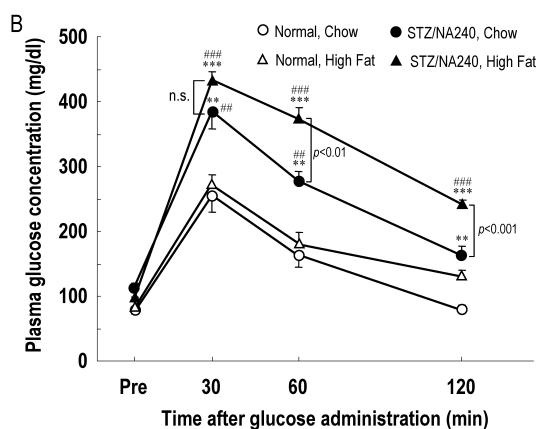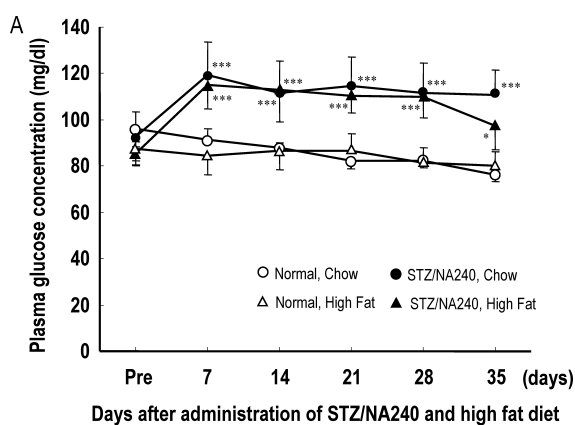

Fig. 7. Effect of Feeding with High-Fat Diet on the Development of Glucose Tolerance in STZ/NA240-Treated C57BL/6J Mice

Monitoring levels of PG after starting of induction of diabetes with STZ/NA240 (panel A) and OGTT on day 35 (panel B) are shown. The OGTT was performed by administering glucose solution (2 g/kg, *p.o.*) 16 h after removal of food. *, ** and *** Significantly different ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) from the chow diet-fed normal group, ##, ### Significantly different ($p < 0.01$, $p < 0.001$, respectively) from the chow diet-fed STZ/NA240 group, according to non-parametric Welch's *t*-test. Data are expressed as mean$\pm$S.E.M. ($n = 9$—10).

betic mice and also between diabetic chow-fed and high fat-fed mice, which indicates that this diabetic model got the insulin resistance by high-fat diet.

Abatement Effect of Buformin on the Insulin Resistance In OGTT with high fat-fed diabetic mice, buformin (100 mg/kg, *p.o.*) significantly inhibited the hyperglycemia 30 min after administration of glucose, although glibenclamide (4 mg/kg, *p.o.*) did not show that effect. (Fig. 9)
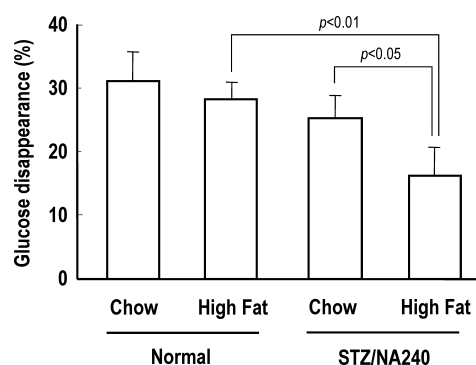

Fig. 8. Effect of High-Fat Diet on Fractional Glucose Disappearance in Response to Exogenously Applied Insulin in STZ/NA240-Induced Diabetic Mice

Blood was collected before and 30 min after injecting insulin (0.04 U/10 g body weight), and PG was measured. Fractional glucose disappearance was calculated as follows. Glucose disappearance (%)=(PG$_{(before)}$−PG concentration$_{(after)}$)/PG$_{(before)}$×100, where PG$_{(before)}$ and PG$_{(after)}$ indicate PG concentration before and 30 min after insulin injection, respectively. Data are expressed as mean$\pm$S.E.M. ($n = 4$).
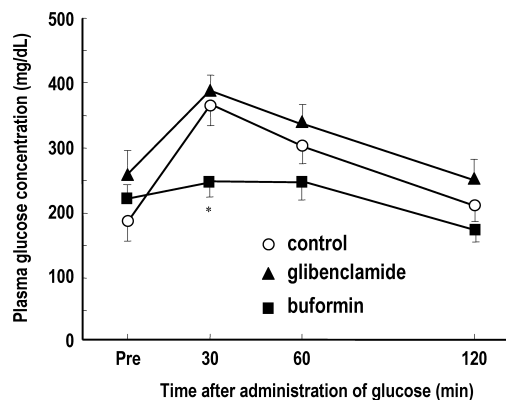

Fig. 9. Effect of Buformin and Glibenclamide on Glucose Tolerance in STZ/NA240-Induced Diabetic Mice Fed with High-Fat Diet

The STZ/NA240 mice, which had been fed with high-fat diet, were starved for 16 h before glucose solution was orally administered (2 g/kg) in OGTT. Buformin (100 mg/kg, *p.o.*) or glibenclamide (4 mg/kg, *p.o.*) was administered every day for 2 weeks before the day of OGTT. Data are expressed as mean$\pm$S.E.M. ($n = 9$). * Significantly different ($p < 0.05$) from the control group.

DISCUSSION

The present diabetic model, developed by STZ/NA, possesses characteristics quite similar to the type 2 non-obese diabetes which constitutes a majority of East Asian diabetic patients, as indicated from the following two important features. First, the present STZ/NA-induced diabetic mouse

model is the non-obese type, in which the glucose tolerance was mildly impaired. It showed almost normal fasting PG concentration, normal body weight gain and normal ability of insulin secretion, although having the diminished pancreatic insulin content. Here, the pancreatic insulin content was from 20 to 40% of normal animals. Second, the present STZ/NA-induced diabetic mice quite easily developed insulin resistance, when they were fed with high-fat diet. They showed normal insulin sensitivity when they were fed with chow diet. However, they retarded recovery of PG concentration, reduced insulin secretion in response to glucose loading and exhibited resistance to exogenously applied insulin, when fed with high-fat diet. The insulin resistance in the liver is considered to chiefly participate in the decreased sensitivity to insulin in case of high-fat diet ingestion.[16]

In previous studies, type 2 diabetic models have been made in mouse or rat by multiple administration of low dose (MLD) of STZ (40 mg/kg, i.p., every 5 d), aiming at adjusting the degree of destruction of beta cells.[17,18] The MLD-STZ model takes, however, at least twelve weeks for the diabetic symptoms to become stable. Moreover, the severity control of disease needs sophisticated adjustment of the dosage of STZ throughout its administration. Masiello and co-workers reported a type 2 diabetic model in rats with combined application of STZ and NA.[11] In this mouse model, only 5 weeks were needed for the establishment of diabetes, as compared with 12 weeks in previous STZ-alone rat models.[18] Meanwhile, using mouse as an experimental animal species may be more useful than using rats from several viewpoints. Mouse is more advantageous over rat in the low breeding cost. Mouse would be suitable for the screening of anti-diabetic drugs, if biological active substances isolated from natural resources are limited in its amount. These circumstances led us to establish a non-obese type 2 diabetic model in mice by modifying the method of Masiello in rats. In addition, the cost, time, and complexity for establishment of our mouse model would be much less than those possibly required for any genetically manipulated model.

The most important characteristic in this mouse model is that the insulin production was retained at about 30% of normal mice and the insulin secretion into the plasma was almost the same as that noted in normal mice. The ratio of remaining beta islets in STZ/NA120 and STZ/NA240 groups was suggested to reflect that of insulin production. These results correspond with previous findings on the transplantation therapy of the pancreas in humans and the experimental pancreatectomy in animals; the donors of 50 to 80% extirpated pancreas can keep the normal PG concentration and insulin secretion.[19—21] However, it has also been reported that the pancreatectomized patients result in a deterioration of insulin secretion and glucose tolerance because of suffering from stress against eating habits such as high glucose or high fat intake.[21]

In ITT, the PG concentration of STZ alone group was sustained at the value of over 150 mg/dl and it was significantly higher than that in normal control group at 120 min after injection of insulin (153.7 ± 37.9 mg/dl, $p < 0.001$). This fact indicates that the hypoglycemic activity of insulin may not be sufficient in STZ alone group, which suggests the alteration of insulin signal transduction. Then, we decided to examine the insulin signaling-related proteins in the liver and the adi-

pose tissues in the present model.

The expressions of IRS-1, IRS-2 and PI3K in the liver were reduced significantly in STZ alone group, and moderately, but not significantly, in STZ/NA240 group. In contrast, there was no definite decrease in those expressions of the adipose tissues in STZ alone and STZ/NA240 groups, although with the exception of decrease in IRS-2 expression of STZ alone group. Thus we assumed a possibility that the insulin resistance originating in the liver is developed with high-fat diet ingestion, although ITT did not show the development of the insulin resistance in STZ/NA240 group. As was expected, the insulin resistance induced by ingestion of high-fat diet was clearly demonstrated in STZ/NA group, and was significantly improved with a biguanide drug, buformin. This drug is known to act on the insulin resistance in the liver mainly *via* inhibition of gluconeogenesis.[22] Thus, it became clear that this mouse model developed the insulin resistance in the liver by prolonged ingestion of high-fat diet. On the other hand, glibenclamide, which is known to stimulate insulin release from β islets *via* sulfonylurea (SU) receptor,[23] did not show any hypoglycemic effect in OGTT in high-fat fed STZ/NA240 group. This fact strongly suggests that the insulin released by glibenclamide was hindered from effectively lowering PG concentration by the enhanced insulin resistance in high-fat fed STZ/NA240 group. Furthermore, high concentration of cholesterol and neutral fat in plasma of STZ/NA240 group was also detected with high-fat diet. This hyperlipidemia is strongly suggested to become the cause of insulin resistance in this fat-fed diabetic mouse model.

Pathophysiological characteristics of the present mouse model may well express the present condition of diabetic patients with lean body and poor ability of insulin secretion in the East Asia. In a majority of subjects with impaired glucose tolerance (IGT) and type 2 diabetic patients in Japan, China, and Korea, insulin reservoir is partially depleted and the ability to secrete insulin is decreased to about 20 to 40% of normal individuals.[24] Usually these patients are not obese, which is one of the most prominent differences from the appearance of the diabetic patients in Western countries. Insulin resistance does not progress much in those who suffer from IGT, so that it does not cause a serious problem. Once these non-obese IGT subjects get into habit to take high-fat diet, however, it gives rise to a serious insulin resistance.[25] Moreover, in Japan, the application of biguanides is increasing in the medical treatment of poor insulin secretion type diabetic patients who are not so obese.[26] The characters of this mouse model are also considered to follow the present condition in Japan.

These observations indicate that the present STZ/NA-induced diabetic mouse model quite well possesses the characteristics that reflect the non-obese type 2 diabetes, which is expanding in population among Far-east Asians, recently. The present diabetic mouse model is quite useful one both for investigating the molecular mechanism in the non-obese type 2 diabetes and the insulin resistance and for screening effective drugs to suppress the development of insulin resistance in the non-obese type 2 diabetes.

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