Age-Dependent Onset of Insulin Resistance in Insulin-Resistant Mice

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We have previously isolated spontaneous insulin-resistant mice (ddY-H) and non-insulin-resistant mice (ddY-L) from ddY mice. In the present study, age-dependent onset of insulin resistance in obese ddY-H mice was investigated by comparing with lean ddY-L mice. In ddY-H mice fed a standard diet, an increase in elevation of glucose-stimulated plasma insulin level, glucose intolerance in an intraperitoneal glucose tolerance test, and a reduction of hypoglycemic action of insulin were found at 9 weeks of age, but not at 6 weeks of age. When ddY-H mice were administered nateglinide, a greater elevation of plasma insulin level and a less decrease of serum glucose level were observed at 9 weeks of age. These changes developed progressively with age. These findings suggest that insulin resistance is induced at 9 weeks of age. The age-related change in insulin resistance was correlated with reductions in mRNA expression and protein content of the insulin receptor (InsR), and insulin receptor substrate (IRS)-1 and IRS-2 in the epididymal adipose tissue. On the other hand, in the liver, mRNA expression of InsR and IRS-1 did not change at any age, although that of the IRS-2 was reduced. Thus, in ddY-H mice, insulin resistance and glucose-stimulated hyper-secretion of insulin are induced at 9 weeks of age and are reciprocally affected, resulting in progression to a more severe state at an older age. Insulin resistance may be attributed, at least in part, to the decreases in the mRNA expressions and proteins of InsR, IRS-1 and IRS-2 in adipose tissue.

Key words insulin resistance; age-related onset; insulin secretion; insulin receptor

Type 2 diabetes mellitus is a complex disease characterized by hyperglycemia resulting from impaired pancreatic β-cell function and a decreased action of insulin on target tissues.1) The pathological states are mostly attributable to life styles and genetic background.2) It has been known that changes in life style may be related to an induction of hypertrophy of adipose tissue and a disturbance of adipocytokine production,3,4) The gene KCNQ1 has been reported as one of the diabetes-susceptible genes in diabetes patients.5) However the genetic background is still mostly ambiguous since multiple genes may be involved in the pathology.6) To prevent pathogenesis of Type 2 diabetes, it is important to know what phenomena are progressing in the early stage of the disease. Animal models are useful for investigating the causes and the progressive process of the disease. It is valuable for uncovering pathogenic situations in the process before symptoms become tangible. Numerous animal models of diabetes have been developed and investigated to clarify the mechanisms by which hyperglycemia is induced.7–10) We previously succeeded in isolating and breeding two strains, namely spontaneous insulin-resistant mice (ddY-H) and non-insulin-resistant mice (ddY-L) which are derived from ddY mice.11,12) ddY-H mice were found to have an age-dependent onset of mild obesity, insulin resistance and hypertriglyceridemia, which resemble a common form of Type 2 diabetes in humans. On the other hand, ddY-L mice were shown to be lean mice without diabetic symptoms in contrast to ddY-H mice.

In ddY-H mice, even when the mice maintained a standard diet ad libitum, glucose tolerance became impaired at 12 weeks of age, and serum glucose and insulin levels significantly increased after overnight fasting at 12 weeks of age. ddY-L mice indices, except that body weight was slightly low, did not differ from those of the original ddY mice, suggesting that insulin resistance is spontaneously induced in ddY-H mice at 12 weeks of age.12) Increases in urinary excretion and urinary sugar accompanied by increased body mass were observed in all ddY-H mice, but not in ddY or ddY-L mice, at 27 weeks of age, indicating the induction of diabetic symptoms.12) Also, we have reported an infiltration of macrophages into the adipose tissue13) and hepatic steatosis in relation to an increased expression of peroxisome proliferate-activated receptor-γ in ddY-H mice.14) Thus, ddY-H mice are a useful diabetic animal model with a spontaneous induction of diabetic symptoms without a need for loading, such as nutritional stress; ddY-L mice are a good contrast control animal.

In ddY-H mice, glucose intolerance was found at 12 weeks of age but not 9 weeks of age.12) However, serum glucose level and plasma insulin level were significantly higher in ddY-H mice than those in ddY-L mice when mice were re-fed for 1 h after a 12-h fasting.12,13) Also infiltration of macrophages into the adipose tissue was found in ddY-H mice at 9 weeks of age.13) These findings suggest that insulin resistance may progress at an earlier age than 12 weeks of age. Insulin exerts the hypoglycemic effect by binding to insulin receptor (InsR) in the peripheral tissues, namely adipose tissue, liver and skeletal muscle. Insulin resistance is associated with disturbances of the insulin-signaling pathway in the peripheral tissues.15) Alterations in the early events of the insulin-signaling pathways have been recognized as an important component in many insulin-resistant states.16) Insulin receptor, insulin receptor substrates (IRS)-1 and IRS-2 are involved in the early events of the pathway.17) Also it has been known that hepatic IRS-1 and IRS-2 have complementary roles in the insulin-signaling of hepatic metabolism.18) In the present study, to find out the age-dependent onset of insulin resistance and its progressive development, we investigated serum glucose levels and plasma insulin levels in ddY-H mice.

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under various conditions and expressions of InsR, IRS-1 and IRS-2 in relation to age.

MATERIALS AND METHODS

Materials Nateglinide (Starlix™) and insulin (NovoRapid™) were purchased from Astellas Pharma Inc. (Tokyo, Japan) and Novo Nordisk Pharm Ltd. (Tokyo, Japan), respectively. Phosphate-buffered saline (PBS) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) and all other chemicals used in the present study were of analytical grade.

Animal Care Male ddY-H mice and male ddY-L mice which were derived from our own colony were used.12 The mice were weaned at 4 weeks of age and were housed at 22 to 24°C with 50 to 60% humidity under artificial lighting conditions with a 12-h light/dark cycle and maintained free access to standard chow pellets (MF diet, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum until experiments were performed. For the experiments, the mice were used after a 4-h fasting (09:00–13:00) at 6, 9, 12 and 15 weeks of age. The body weights of mice (the mean±standard error of the mean (S.E.M.) of 6 mice) were 41.5±0.28 (6 weeks), 51.0±0.28 (9 weeks), 57.3±0.89 (12 weeks), 58.3±0.94 (15 weeks) in ddY-H mice and 32.3±0.79 (6 weeks), 40.8±1.30 (9 weeks), 42.6±0.78 (12 weeks), 46.4±0.37 (15 weeks) in ddY-L mice. Animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Shizuoka, and animal studies were reviewed and approved by the Animal and Ethics Review Committee of the University of Shizuoka (No. 136004).

Measurements of Serum Glucose and Plasma Insulin Serum glucose level and plasma insulin level were determined using Glucose ClII Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Mouse Insulin ELISA Kit (Morinaga Institute of Biological Sciences, Inc., Kanagawa, Japan), respectively, according to the manufacturer’s instructions.

Insulin Resistance Index Insulin resistance was assessed by the glucose and insulin levels in the serum of mice which fasted for 4 h (09:00–13:00) in the following way: glucose (mg/dL)×insulin (ng/mL) /100.12,19

Intraperitoneal Glucose Tolerance Test Mice were intraperitoneally injected glucose (3 g/kg body weight (BW)) after a 4-h fasting (09:00–13:00), and blood was collected from the caudal vein after 0, 30 and 120 min for measuring serum glucose levels. The area under the concentration–time curve (AUC) was also calculated.

Oral Administration of Glucose After a 4-h fasting (09:00–13:00), blood was taken from the caudal vein of the mice for the determination of serum glucose at 0 min and glucose (1, 2 and 3 g/kg) or water were orally administered. After 15 min, blood was taken from the caudal vein for the determination of glucose level, and also taken by decapitation for the determination of plasma insulin level.

Administration of Nateglinide Nateglinide (30 mg/kg) was orally administered after a 4-h fasting (09:00–13:00) and blood was taken from the caudal vein after 0, 30 and 60 min, and serum glucose was measured. Decreased glucose level induced by nateglinide was calculated by subtracting the glucose level at 0 min from that at the respective time. For the measurement of plasma insulin, blood was taken by decapitation at 0, 15 and 30 min after the administration of nateglinide.

Intraperitoneal Injection of Insulin Mice were intraperitoneally injected insulin (0.05 U/50 g) after a 4-h fasting (09:00–13:00). Blood was taken from the caudal vein after 0, 15, 30 and 60 min and serum glucose levels were measured.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction (q-PCR) The epididymal adipose tissue and liver were excised from mice after a 4-h fasting (09:00–13:00) and were frozen in liquid nitrogen. The total RNA of the epididymal adipose tissue and liver was isolated using a RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, U.S.A.) and Isogen® (NIPPON GENE Co., Ltd., Tokyo, Japan) in accordance with the manufacturer’s instructions, respectively. The cDNA was constructed from 0.5 μg of the total RNA by using an RNA PCR™ Kit (AMV Ver 3.0, TaKaRa Bio Inc., Shiga, Japan) as described previously.13 The quantitative q-PCR with the specific primers and SYBR® Premix Ex Taq™ GC (AMV Ver 3.0, TaKaRa Bio Inc.) was carried out using an Applied Biosystems™ 7500 Real-Time PCR System (Life Technologies, CA, U.S.A.). The following primers were used: 5'–TGG AAT CCT GTG GCA TCC ATG AAA C-3' and 5'–TAA AAC GCA GCT CAG TAA CAG TCC G-3' for β-actin20; 5’–GTA TGG TGT ATG AAG GCA ATG-3’ and 5’–CAG AGA ACG GAT GTG ACT-3’ for InsR21; 5’–TGA TGT CAC CCA GTG GTA GTT GCT-3 and 5’–TCA TGG CAT GAG AAG CAT GAG TAT-3 for IRS-121; and 5’–TGA GTG GCC TAC AAC CCT TAC CCA-3’ and 5’–TCA TCG CTC TTG CAG CTA TTG GAA GGA GGA GAT GAG TAT-3’ for IRS-2.21 The PCR condition was 1 cycle at 95°C for 30 s and 60°C for 15 s, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.11

Assays of InsR, IRS-1 and IRS-2 Protein Contents Epididymal adipose tissue was excised after a 4-h fasting (09:00–13:00) and 0.2 g of tissue was homogenized in an ice-cold PBS (10 mM/L, pH 7.2). After centrifugation for 5 min at 5000×g, the supernatant was assayed for contents of InsR, IRS-1 and IRS-2 in the epididymal adipose by an Enzyme-linked Immunosorbent Assay Kit for Insulin Receptor (ISR) (Cloud-Clone Corp., TX, U.S.A.), a Mouse Insulin Receptor substrate 1 (IRSI) Elisa kit and Mouse Insulin Receptor substrate 2 (IRS2) Elisa kit (MyBioSource com, CA, U.S.A.) in accordance to the manufacturer’s instructions, respectively. Protein concentration in homogenate was quantified by a Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, IL, U.S.A.). Contents of InsR, IRS-1 and IRS-2 were expressed as ng/mg proteins.

Statistical Analysis All data are expressed as the mean±S.E.M. Following Bartlett’s test to confirm equal variance among groups, ANOVA analysis was used to compare the means among ddY-H mice and ddY-L mice, and a Tukey’s test was used in the post hoc multiple comparisons. Statistical significance was set at p<0.05.

RESULTS

Insulin Resistance Index Serum glucose levels, plasma insulin level and insulin resistance index were examined in mice at 6, 9, 12 and 15 weeks of age after a 4-h fasting. Serum glucose levels and plasma insulin levels were constant during the experimental period in ddY-L mice (Figs. 1A–C). Those of ddY-H mice did not change from ddY-L mice at 6 weeks of age, and a tendency to elevate was found at 9 weeks of age.
Moreover, steady elevations were seen at 12 and 15 weeks of age. Insulin resistance index increased significantly from 9 weeks of age, and elevated markedly at 12 and 15 weeks of age, suggesting that insulin resistance latently developed from 9 weeks of age in ddY-H mice (Figs. 1A–C).

**Intraperitoneal Glucose Tolerance Test** In mice which fasted for 4 h, an intraperitoneal glucose tolerance test was carried out. There was no alteration of serum glucose levels and AUC in intraperitoneal glucose tolerance test in ddY-L mice at 6, 9, 12 and 15 weeks of age (Figs. 2B, C). However, an impairment of intraperitoneal glucose tolerance was found in the ddY-H mice at 9, 12 and 15 weeks of age but not at 6 weeks of age (Figs. 2A–C).

**Increased Levels of Serum Glucose and Plasma Insulin by Oral Glucose-Administration** Since an intolerance in oral glucose tolerance tests, in which glucose was administered at a dose of 3 g/kg, was not found in 9-week-old ddY-H mice in the previous study, increases in serum glucose level and plasma insulin level were examined by oral glucose-administration at lower doses such as 1, 2 and 3 g/kg. In ddY-L mice, plasma insulin levels elevated dose-dependently but serum glucose levels increased to the same degree, and a difference in regard to ages tested was not found (Figs. 3A–D). Also, no difference was observed in serum glucose levels and plasma insulin levels between ddY-H mice and ddY-L mice at 6 weeks of age in all doses (Fig. 3A). However, at 9 weeks of age, plasma insulin levels were significantly increased in ddY-H mice than in ddY-L mice at all doses, but serum glucose levels were not (Fig. 3B). Both serum glucose levels and plasma insulin levels at 12 and 15 weeks of age were found to be markedly higher in ddY-H mice than ddY-L mice at all doses (Figs. 3C, D). Together with the result of the intraperitoneal glucose tolerance tests, this result may suggest that insulin resistance appears from 9 weeks of age in ddY-H mice.

**Decrease in Serum Glucose Level and Increase in Plasma Insulin Level by Nateglinide Administration** As described above, plasma insulin levels were markedly increased in response to the glucose administration in ddY-H mice. Then, the effect of nateglinide (30 mg/kg), which increased plasma insulin levels, was examined. Figures 4A and B depict changes in plasma insulin levels and serum glucose levels of mice by administration of nateglinide, respectively. An increase in plasma insulin level and a decrease in serum glucose level by nateglinide were found to be constant at all ages of ddY-L mice (Fig. 4A). On the other hand, the efficacies of nateglinide on plasma insulin levels were markedly increased from 9 and 12 weeks of age in ddY-H mice (Fig. 4A). However, the efficacy of nateglinide to decrease the serum glucose level in ddY-H mice gradually weakened by age, but those in ddY-L mice were not changed (Fig. 4B). The results suggest that an increase of insulin release in response to stimuli and insulin resistance may be progressing in ddY-H mice from 9 weeks of age.

**Decrease in Serum Glucose Level by Insulin Injection** To confirm an appearance of insulin resistance in ddY-H mice, insulin was intraperitoneally injected and serum glucose
levels were determined. In ddY-H mice at 6 weeks of age, the serum glucose level certainly decreased in response to the injection of insulin, which action was to the same degree in ddY-L mice at all ages tested (Figs. 5A–C). In ddY-H mice, a defect in the insulin’s action to decrease the serum glucose level was found from 9 weeks of age and a marked defect was discovered at 12 and 15 weeks of age (Figs. 5A, C).

**mRNA Expression of InsR, IRS-1 and IRS-2 in Epididymal Adipose Tissue and Liver**  
As the insulin action was defective in ddY-H mice, mRNA expression of InsR, IRS-1 and IRS-2 in the epididymal adipose tissue and liver was examined.

As shown above and previously, the body weight and body mass index of ddY-H mice were significantly higher than those of ddY-L mice, suggesting the obesity of ddY-H mice. The epididymal adipose tissue weights per 100 g body weight (the mean ± S.E.M. of 6 mice) of ddY-H mice (2.40 ± 0.18 (6 weeks), 3.46 ± 0.09 (9 weeks), 3.41 ± 0.18 (12 weeks), 3.18 ± 0.21 (15 weeks)) were significantly higher than those of ddY-L mice (1.60 ± 0.15 (6 weeks), 1.99 ± 0.2 (9 weeks), 2.13 ± 0.20 (12 weeks), 2.73 ± 0.16 (15 weeks)) at the respective age ($p<0.01$). In the liver, a decreasing level of IRS-2 was also found in ddY-H mice at 9, 12 and 15 weeks of age, but the mRNA levels of InsR and IRS-1 were not different from those in ddY-L mice in all age (Figs. 6D–F).

**Contents of InsR, IRS-1 and IRS-2 Proteins in Epididymal Adipose Tissue**  
It has been known that IRS-1 and IRS-2 have complementary roles in the regulation of hepatic metabolism. Since mRNA expression InsR, IRS-1 and IRS-2 in the epididymal adipose tissue of ddY-H mice was decreased, contents of InsR, IRS-1 and IRS-2 proteins in the epididymal adipose tissue were assessed. Figure 7 depicts contents of InsR, IRS-1 and IRS-2 proteins in the epididymal adipose tissue. No difference was found between those in ddY-H mice and ddY-L mice at 6 weeks of age. In ddY-L mice, contents of InsR, IRS-1 and IRS-2 proteins were constant in all ages (Fig. 7). On the other hand, in ddY-H mice, the content of InsR protein decreased from 12 weeks of age, and those of
IRS-1 and IRS-2 proteins significantly decreased at 9 weeks of age and were at very low levels at 12 and 15 weeks of age (Fig. 7). The changes in InsR, IRS-1 and IRS-2 proteins in the adipose tissue were correlated with those of their mRNA expression. The finding suggests that the defective expression of mRNA and proteins of InsR, IRS-1 and IRS-2 in the adipose tissue is one of the causes, at least, to induce insulin resistance in ddY-H mice in the elder age.

DISCUSSION

One of the physiological functions of insulin is a hypoglycemic action which involves regulation of glucose concentration in the circulating system and metabolism in the peripheral tissues. The defect of this action contributes to the induction of diabetes in humans and rodents. Thus, it is important to know how the disturbances in the circulating concentrations of glucose and nutrients-stimulated secretion of insulin are caused in the early stage of diabetes. However, it remains poorly understood.1)

We have previously shown that glucose tolerance was spontaneously impaired at 12 weeks of age, and diabetic symptoms such as glycosuria and polyuria were found in some mice at 20 weeks of age and in all mice at 27 weeks of age.12) Thus, it was suggested that insulin resistance was induced at 12 weeks of age and diabetes developed progressively in ddY-H mice from 20 weeks of age. Although insulin resistance is a crucial factor in the development of diabetes, the precise mechanism responsible for developing insulin resistance in Type 2 diabetes is unclear.1) In the present study, the age-dependent onset of insulin resistance in ddY-H mice was investigated.

In the previous study,12) there was no difference between plasma insulin levels after a 12-h fasting in ddY-H mice and ddY-L mice at 9 weeks of age. However, plasma insulin levels were significantly high in ddY-H mice than those in ddY-L mice when mice were re-fed for 1 h after a 12-h fasting. Since, in the present study, insulin resistance index was found to be slightly high at 9 weeks of age in ddY-H mice, it was supposed that insulin resistance may be induced in ddY-H mice at 9 weeks of age and may develop progressively in relation to age. When glucose was orally administered at doses of 1–3 g/kg, the glucose-stimulated increase in plasma insulin level significantly increased, but that of the serum glucose level did not increase at 9 weeks of age in ddY-H mice. On the other hand, glucose intolerance in the intraperitoneal glucose tolerance test (ipGTT) was found at 9 weeks of age and older. The glucose tolerance test is a useful procedure to find out glucose intolerance. However, there are discrepancies in serum glucose levels and plasma insulin levels between the administration routes of glucose, such as oral-injection (OGTT), intravenous-injection (IVGTT) and ipGTT.22,23) The glucose-stimulated increase in plasma insulin is lower in ipGTT and
IVGTT compared to that in OGTT, suggesting that ipGTT and IVGTT were examined under less influence of glucose-stimulated insulin secretion. Therefore, glucose intolerance in ipGTT indicates occurrence of insulin resistance in ddY-H mice at 9 weeks of age.

To support the above proposal, age-dependent onset of insulin resistance was examined by administrations of nateglinide and insulin under circumstances where the effects of glucose-stimulated elevation of plasma insulin level were disregarded. Nateglinide, one of the sulfonylureas, is known to increase insulin secretion by acting directly on the pancreatic β cells, resulting in an increase in plasma insulin levels and a decrease of serum glucose levels. Nateglinide-induced elevation of plasma insulin level was enhanced in ddY-H mice at 9 weeks of age and older. However, the decrease in serum glucose level was significantly reduced at 9 and 12 weeks of age. The changes were not found at 6 weeks of age, and were progressive from 9-weeks of age to 12-weeks of age. In addition, when insulin was intraperitoneally injected, a defect in decreasing serum glucose level was found at 9 weeks of age and older. From these findings, together with those described above, it was strongly indicated that insulin resistance and stimulus-induced hyper-secretion of insulin occurred at 9 weeks of age. In ddY-H mice, increased insulin secretion conceals insulin resistance at 9 weeks of age, and insulin resistance appeared at 12 and 15 weeks of age when insulin resistance fully induced.

Insulin resistance does not immediately lead to onset of hyperglycemia as a consequence of a compensatory increase of insulin secretion from pancreatic islet β cells. It is well known that hyperinsulinemia is found in some diabetic patients at an early stage. Uchida et al. have shown that cyclin-dependent kinases in pancreatic islet β cells involved in compensatory hyperinsulinemia of diabetic mice. Glucose-stimulated insulin secretion in pancreatic β cells includes a sequence of events involving increased glucose metabolism, an elevation of the ATP/ADP ratio, closure of ATP-sensitive K⁺ channels, depolarization of the plasma membrane, a rise in intracellular Ca²⁺ concentration by activation of voltage-dependent Ca²⁺ channels, fusion of insulin granules to the plasma membrane, and, as a result, exocytosis of insulin. The principal target of nateglinide is the ATP-sensitive K⁺ channels. Since the nateglinide-stimulated elevation of plasma insulin level increased, it was supposed that the downstream from the ATP-sensitive K⁺ channels in the signal transduction pathway for insulin secretion accelerated in ddY-H mice. The precise mechanism by which insulin secretion is increased in ddY-H mice should be clarified by further investigation.

Insulin exerts the hypoglycemic effect by binding to InsR in the peripheral tissues and accelerating glucose transport.

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**Fig. 4. Effect of Nateglinide on Serum Glucose Levels and Plasma Insulin Levels**

Nateglinide (30 mg/kg) was orally administered after a 4-h fasting (09:00–13:00) and blood was taken by decapitation of various mice at 0, 15 and 30 min to measure the plasma insulin level (A), and was taken from the caudal vein after 0, 30 and 60 min for measuring serum glucose levels (B). Decreased glucose level induced by nateglinide was calculated by subtracting glucose level at 0 min from that at the respective time. Each column and bar represents the mean ± S.E.M. for 7 mice. *: p < 0.05, **: p < 0.01 vs ddY-L mice at the same age.
from the circulating system into tissues. InsR undergoes autophosphorylation by binding with insulin, and phosphorylates several intracellular proteins including IRS-1 and IRS-2. These proteins activate the signal transduction pathway involving various proteins such as phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (Akt). Finally, glucose transport is facilitated in the adipose tissues and muscle, primarily by translocating glucose transporter isoform 4 (GLUT4) from the intracellular pool to the plasma membrane. Insulin resistance is associated with disturbances of the insulin-signaling pathway in the peripheral tissues. Alterations in the early events of the insulin-signaling pathways have been recognized as an important component in many insulin-resistant states. Reduction in insulin receptor number in the liver membranes has been known to occur in Goto–Kakizaki rats (GK rat) with peripheral insulin resistance. Also, a decreased insulin binding was found in the livers from diabetic patients. Over expression of InsR partially improved diabetic phenotypes in db/db mice. Growth hormones (GH) antagonize insulin actions and lead to hyperglycemia, hyperinsulinemia and decreased glucose utilization. Chronic elevations of GH levels in transgenic mice over expressing growth-hormone genes are associated with the development of reduction in InsR number, resulting hyperinsulinemia and insulin resistance. Based on these findings, various disturbances in InsR of plasma membranes are considered to be associated with insulin resistance. Also, it has been shown that disruptions of IRS-1 gene and IRS-2 gene contribute to disturbance of insulin-signaling pathways and insulin resistance. IRS protein expression and phosphorylation are markedly reduced in many insulin resistant states. These evidences suggest a strong relationship between insulin resistance and disturbance in the early events for insulin-signaling pathways and lead us to investigate expressions of InsR, IRS-1 and IRS-2 in the adipose tissue and liver of ddY-H mice.

In the epididymal adipose tissue of ddY-H mice, slight reduction in mRNA expression of InsR, IRS-1 and IRS-2 were found at 9 weeks of age and the reduction progressed with age. On the other hand, in the liver, mRNA expression of InsR and IRS-1 did not changed at any age, although reduction of IRS-2 was the same as that in the adipose tissue. The knockdown of either IRS-1 or IRS-2 did not affect the insulin-signaling in liver, indicating that hepatic IRS-1 and IRS-2 have complementary roles in the regulation of hepatic metabolism. The decreased mRNA expression of IRS-1 may not be sufficient for the impairment of insulin signaling in liver. Thus, the protein contents of InsR, IRS-1 and IRS-2 in the epididymal adipose tissue were assessed. In ddY-H mice,

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

Fig. 5. Effect of Insulin Injection on Serum Glucose Levels

ddY-H mice (A) and ddY-L mice (B) at 6 (rhombuses), 9 (squares), 12 (triangles) and 15 weeks of age (circles) were intraperitoneally injected with insulin (0.05U/50g of body weight) after fasting for 4hr (09:00–13:00), and serum glucose levels were measured after 0, 15, 30 and 60 min. The decreased glucose level was calculated by subtracting the glucose level at 0 min from that at the respective time. Each symbol represents the mean of the decreased glucose levels by insulin injection for 8 mice. The decreased glucose level for 60 min was shown in (C). Each column and bar represents the mean ± S.E.M. of 8 mice (closed column: ddY-H mice, open column: ddY-L mice). **: p<0.01 vs. ddY-L mice at the same age.
reductions of protein contents of InsR, IRS-1 and IRS-2 were found to be similar to reductions of mRNA expression, except that InsR protein was not reduced at 9 weeks of age. The age-related changes of the mRNA expression and the protein contents in epididymal adipose tissue of ddY-H are correlated to changes in glucose intolerance and glucose-stimulated elevation of plasma insulin levels. These results suggest that the defective expression of mRNA and protein of InsR, IRS-1 and IRS-2 in the adipose tissue is one of the causes, at least, to induce insulin resistance and diabetes in ddY-H mice in the elder age. However, further investigation should be performed not only for expressions of proteins involving insulin-signaling pathways but also for phosphorylation of the proteins.

In conclusion, in ddY-H mice, insulin resistance and glucose-stimulated hyper-secretion of insulin spontaneously induce at 9 weeks of age, and those are reciprocally affected, resulting in progression to a more severe state at 12 and 15 weeks of age. Insulin resistance is suggested to be attributed, at least in part, to decreases in mRNA expressions and proteins of InsR, IRS-1 and IRS-2.

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Conflict of Interest The authors declare no conflict of interest.
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