**FULL PAPER**  Laboratory Animal Science

**Plekhs1 and Prdx3 are candidate genes responsible for mild hyperglycemia associated with obesity in a new animal model of F344-fa-nidd6 rat**

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**ABSTRACT.** Type 2 diabetes is a polygenic disease and characterized by hyperglycemia and insulin resistance, and it is strongly associated with obesity. However, the mechanism by which obesity contributes to onset of type 2 diabetes is not well understood. We generated rat strains with a hyperglycemic quantitative trait locus (QTL) derived from the Otsuka Long-Evans Tokushima Fatty rat and a fa/fa (Lepr

locus derived from the Zucker Fatty rat. Phenotypes for plasma glucose, and insulin levels were measured, and RNA and protein levels were determined using reverse transcription quantitative PCR and Western blot analyses, respectively. Compared with the obese control strain F344-fa (Lepr

), plasma glucose levels of the obese F344-fa-nidd6 (Lepr

and Nidd6) significantly increased, and plasma insulin levels significantly decreased. These phenotypes were not observed in the lean strains, suggesting that the Nidd6 locus harbors a diabetogenic gene associated with obesity. We measured the expression of 41 genes in the Nidd6/QTL region of each strain and found that the mRNA expression levels of the two genes significantly differed between the obese strains. The two genes, pleckstrin homology-domain-containing, family S member 1 (Plekhs1) and peroxiredoxin III (Prdx3), were differentially expressed only in the obese rats, suggesting that these two genes are involved in the mild elevation of blood glucose levels and insulin resistance in obesity.

**KEYWORDS:** diabetes, hyperglycemia, insulin

The prevalence of diabetes is predicted to increase from 171 million in 2000 to 366 million in 2030 [33]. Development of type 2 diabetes mellitus is strongly associated with obesity [13], and the prevalence of obesity is closely paralleled by the increase in the prevalence of type 2 diabetes [25]. In addition, genetic factors can contribute to the development of type 2 diabetes. However, little is known regarding how these factors interact to cause type 2 diabetes. Genome–wide association studies have identified many genes associated with type 2 diabetes or obesity [6, 8, 12, 24, 26], but only a few loci are common between obesity and type 2 diabetes [6]. Because it is difficult to identify diabetogenic genes associated with obesity and to investigate the molecular mechanisms responsible for type 2 diabetes in obese humans, inbred animal models are essential components of genetic investigations [1].

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is genetically predisposed to late-onset hyperglycemia, insulin resistance and renal complications associated with mild obesity [11]. These symptoms correspond to those observed in human with type 2 diabetes [11]. The strategic development of new diabetic-obese models using the OLETF rat is a very useful for investigations of diabetic genes associated with obesity [15, 23]. Fourteen hyperglycemic quantitative trait loci (QTLs) have been identified [19, 27, 31], and congenic lines for each QTL were constructed [14]. Since OLETF rats develop type 2 diabetes with obesity, we further generated obese congenic rats by introgressing the Zucker Fatty-derived leptin receptor mutation (Lepr

locus) into F344 rats (designated as F344-fa) [21]. These congenic strains can be used to search for QTLs that exhibit strong hyperglycemia in obese animals using homozygotes of double congenic rats generated by crossing each hyperglycemic QTL with the obese line [15, 23].

The Nidd6/QTL locus is located on rat chromosome 1 in the interval bounded by the markers, D1Rat166 and D1Rat90 (approximately 11.4 cM) [31]. A previous study demonstrated that the OLETF alleles at Nidd6 were associated with the increased 30 min glucose levels after glucose loading through acting in a dominant or additive manner [34]. In this study, we generated the double congenic obese rat strains by introgressing the Nidd6/QTL locus and Lepr

locus into F344 rats. We compared gene expression in the F344-fa and F344-fa-nidd6 strains to identify the candidate genes related to obesity-associated diabetes and identified two candidate genes, pleckstrin homology-domain-containing, family S member 1 (Plekhs1) and peroxiredoxin III (Prdx3).

**MATERIALS AND METHODS**

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Animals: All rats were maintained under specific pathogen-free conditions as described previously [23]. The Institutional Animal Care and Use Committee of Kyoto Sangyo University approved the Protocols for animal care.
Fisher-344/Sle rats, F344-nidd6 congenic rats and the F344-fa congenic rats were used as described in detail previously [23]. F344-fa rats were used as the obese control. The F344-fa-nidd6 double congenic strain with the fa/fa and Nidd6/of loci (original name: F.ZF-Lepr&Nidd6/of) was generated by crossing the F344-fa and F344-nidd6 strains and then selecting fa/fa-Nidd6/of homozygotes. The F344 and F344-nidd6 rats are lean strains, whereas the F344-fa and F344-fa-nidd6 rats are obese strains (Fig. 1A).

Oral glucose tolerance test (OGTT) and plasma insulin measurement: At 20 weeks of age, OGTT was performed by injecting glucose (2 g/kg in a 2.8 M solution) in overnight-fasted rats, and the blood samples were collected for measurement of plasma insulin levels as described previously [23]. Blood glucose levels were determined directly using the glucose oxidase method with Glutest Neo test strips (Sanwa Chemical Co., Nagoya, Japan). The area under the curve (AUC) was calculated according to the trapezoid rule from the glucose measurements at each time and is expressed as mg/dl × min. The blood samples were collected from tail veins using heparinized capillary tubes and then centrifuged to obtain plasma. Plasma insulin levels were determined using an ELISA kit that detects rat insulin (Shibayagi Co., Ltd., Shibukawa, Japan).

Insulin tolerance test (ITT): The insulin tolerance test (ITT) was performed by injecting human insulin (1 U/kg, Humulin R, Eli Lilly, Indianapolis, IN., U.S.A.) intraperitoneally into rats (16 weeks of age) after overnight fasting as described previously [23]. Blood glucose levels at 0 (fasting), 30, 60, 90 and 120 min were measured directly as described above.

Isolation of islets from rat pancreas: Animals were anesthetized with a mixture of 3% isoflurane in oxygen using a vaporizer. Then, islet isolation was performed as described by Li et al. [16] with a slight modification. The bile duct was cannulated using a 23G-needle with 10 ml of cold Hank’s balanced solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) containing 0.1 mg/ml of collagenase XI (Sigma-Aldrich). After removing the pancreas and placing it in a 50 ml tube, the tube was placed in a water bath at 37°C for 15 min. Digestion was stopped by addition of RPMI 1640 supplemented with 11 mM glucose and 1% bovine serum albumin. Islet purification was performed using a micro-capillary selecting and sucking method to isolate the islets under a microscope, as described in the embryo transfer protocol.

Metabolic assays: Body weight and abdominal fat weight were determined. The serum levels of total cholesterol (TCHO), triglycerides (TG) and non-esterified fatty acids (NEFA) were determined using T-Cho-E test kits, TG-E test kits and NEFA-C test kits, respectively, from Wako Chem. Ltd. (Osaka, Japan) as described previously [23].

Sample collection: As described in detail previously [23], at 25 weeks, sera, and liver, muscle and abdominal fat tissues were collected. The tissues were dissected and stored at −80°C until use. The adiposity index was calculated from each fat pad and body weight (percentage of fat pad weight/body weight).

Quantitative real-time PCR (RT-qPCR): mRNA was obtained from each tissue using ethanol precipitation methods, then RT-qPCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems, Tokyo, Japan), and a calibration curve method was used to analyze the data as described previously [23]. The cDNA sequences were acquired from the genome database of the United States National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Primers were designed using Primer3Plus (http://primer3plus.com) and Amplify-3 (http://engels.genetics.wisc.edu/amplify/) computer software. The relative expression levels were compared by normalization to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Takara Bio Inc. Otsu, Japan).

Western blot: Western blot analysis was performed as described by Towbin et al. [29] and Sasaki et al. [23].
Table 1. Comparison of body weight, fat weight and metabolic parameters in F344, F344-nidd6, F344-fa and F344-fa-nidd6 rats at 25 weeks of age.

| Parameter                  | F344  | F344-nidd6 | F344-fa  | F344-fa-nidd6 |
|----------------------------|-------|------------|---------|--------------|
| Body Weight (g)            | 412.03 ± 8.93 (n=8) | 406.67 ± 5.22 (n=8) | 607.30 ± 14.10 (n=10) | 595.36 ± 17.66 (n=5) |
| Fat Weight (g)             |       |            |         |              |
| Mesenteric fat             | 11.44 ± 0.88 (n=7) | 6.13 ± 0.45 (n=7)†† | 17.37 ± 1.01 (n=7) | 14.83 ± 1.15 (n=5) |
| Retropitoneal fat          | 12.6 ± 0.69 (n=7) | 11.06 ± 0.82 (n=6)† | 31.59 ± 2.51 (n=7) | 33.34 ± 1.27 (n=5) |
| Epididymal fat             | 13.23 ± 0.81 (n=7) | 10.92 ± 0.23 (n=6)† | 16.97 ± 0.31 (n=7) | 17.14 ± 1.23 (n=5) |
| Adiposity index (%)        |       |            |         |              |
| Mesenteric fat             | 2.60 ± 0.18 | 1.64 ± 0.11†† | 2.95 ± 0.17 | 2.57 ± 0.17 |
| Retropitoneal fat          | 2.87 ± 0.13 | 2.82 ± 0.18 | 5.34 ± 0.34 | 5.79 ± 0.14 |
| Epididymal fat             | 3.01 ± 0.14 | 2.79 ± 0.07 | 2.89 ± 0.06 | 2.97 ± 0.19 |
| NEFA (mEq/l)               | —      |            | —       | —            |
| TG (mg/dl)                 | —      |            | —       | —            |
| TCHO (mg/dl)               | —      |            | 2,420.60 ± 316.29 | 1,851.46 ± 150.35 |
| Fat Weight (g)             |       |            |         |              |
| Mesenteric fat             |       |            |         |              |
| Retropitoneal fat          |       |            |         |              |
| Epididymal fat             |       |            |         |              |
| Adiposity index (%)        |       |            |         |              |
| NEFA (mEq/l)               | —      |            | —       | —            |
| TG (mg/dl)                 | —      |            | —       | —            |
| TCHO (mg/dl)               | —      |            | 380.95 ± 26.30 | 339.97 ± 29.35 |

NEFA: non-esterified fatty acids; TG: triglycerides; TCHO: total cholesterol. Data are presented as the mean ± SEM. †P<0.05, ††P<0.01: F344 vs. F344-nidd6.

RESULTS

F344-fa-nidd6 rat strain characteristics: The body weight of the F344-fa-nidd6 double congenic rats was almost the same as that of the F344-fa control rats (Fig. 1B). The body weight of F344-fa single congenic rats did not differ significantly from that of F344 control rats. While the adiposity index of the mesenteric fat pad was significantly lower in F344-nidd6 rats than in F344 rats (P=0.0011), the adiposity index of the retropitoneal and epididymal fat pad did not differ significantly between the strains (Table 1). The adiposity index of the mesenteric, retropitoneal and epididymal fat pads did not differ significantly between obese F344-fa-nidd6 and F344-fa rats.

Mild glucose intolerance and insulin resistance in F344-fa-nidd6 double congenic rats: Blood glucose levels of F344-fa-nidd6 single congenic rats were significantly higher than those of F344 control rats 30 min after glucose loading (P=0.0064) (Fig. 2A). Furthermore, blood glucose levels of F344-fa single congenic rats were significantly higher than those of F344 rats at all time points after glucose loading. Moreover, F344-fa-nidd6 double congenic rats had significantly higher blood glucose levels than F344-fa single congenic rats after fasting and 60, 90 and 120 min after glucose loading.

The AUC value of F344-fa-nidd6 rats did not differ significantly from that of F344 rats (Fig. 2B). The AUC value of F344-fa-nidd6 rats was significantly higher than that of F344-fa rats (P=0.032). These results indicate that the F344-fa-nidd6 double congenic rats developed glucose intolerance. We performed ITTs to determine whether the F344-fa-nidd6 rats developed insulin resistance. As shown in Fig. 2C, the glucose-lowering effect of insulin observed in F344 rats did not differ significantly from that observed in F344-fa-nidd6 rats. However, the glucose-lowering effect was significantly lower in F344-fa rats than in F344 rats after fasting and at all time points after insulin injection. Moreover, it was significantly lower in F344-fa-nidd6 rats than in F344-fa rats 30 min after insulin injection (P=0.022). These results suggest that the F344-fa-nidd6 rats were insulin resistant.

Plasma insulin levels of F344 and F344-fa-nidd6 rats did not differ significantly (Fig. 2D). While, at fasting and 30, 60 and 120 min after glucose loading, plasma insulin levels of F344-fa-nidd6 rats were significantly lower than those in F344-fa rats. We performed an in vitro experiment using primary islets isolated from F344-fa and F344-fa-nidd6 rats. The size of isolated islets did not differ significantly between F344-fa and F344-fa-nidd6 rats (Fig. 3A). Islets were cultured using buffers containing 3 mM or 20 mM glucose, and the insulin concentration was recorded. The islets isolated from F344-fa and F344-fa-nidd6 rats both secreted low levels of insulin when incubated in 3 mM glucose buffer. However, when incubated in 20 mM glucose, islets from F344-fa-nidd6 rats secreted significantly less levels of insulin than those from F344-fa rats (P=0.016) (Fig. 3B). These results suggest that the capacity of F344-fa-nidd6 rat β-cells to secrete insulin decreased, reflecting low plasma insulin levels observed in the rat (Fig. 2D).

Metabolic assays: We measured plasma NEFA, TG and TCHO levels to investigate the metabolic components of F344-fa single congenic and F344-fa-nidd6 double congenic
Fig. 2. Phenotypes of F344, F344-nidd6, F344-fa and F344-fa-nidd6 rats. (A) Glucose levels were measured during the OGTT for overnight-fasted F344 (n=16, white triangle), F344-nidd6 (n=16, white circle), F344-fa (n=14, black triangle) and F344-fa-nidd6 (n=9, black circle) rats at 20 weeks of age. (B) The AUC of blood glucose levels was calculated from the results of the OGTT at all sampling times. (C) The insulin tolerance test (1 U/kg body weight) was performed in overnight-fasted rats (n=6–8) at 16 weeks of age. (D) Plasma insulin levels during the OGTT were determined in the rats (n=5–8) at 20 weeks of age using ELISA. Data are presented as the mean ± SEM. *P<0.05, **P<0.01 F344-fa vs. F344-fa-nidd6 rats; ††P<0.01 F344 vs. F344-nidd6 rats; ##P<0.01 F344 vs. F344-fa rats.

Fig. 3. Islet size and ability of insulin secretion in the islets isolated from the pancreas. (A) Islets freshly isolated from F344-fa (n=206) and F344-fa-nidd6 (n=138) rats in 3 mM glucose buffer were measured their diameters. (B) Islets from each strain were divided into 6 wells with 10 islets in each well. Islets were incubated in 3 mM glucose buffer for 60 min; insulin contents in the medium were then measured; the islets were further incubated in 20 mM glucose buffer for 60 min, and insulin contents in the medium were measured.
rats. However, the plasma levels of these markers did not differ significantly between F344-\textit{fa} and F344-\textit{fa-nidd6} rats (Table 1).

**Diabetogenic genes associated with obesity in the Nidd6/QTL region:** The Nidd6/QTL region on chromosome 1 peaks near D1Rat90 (281.8 Mbp) and was associated with high glucose levels 30 min after glucose challenge in OLETF rats [31, 34]. We analyzed the expression levels of genes between D1Rat166 and D1Rat90 markers (11.4 cM) in the F344-\textit{fa} and F344-\textit{fa-nidd6} strains. We found that 41 genes were expressed in the liver. Considering that high glucose levels were induced in overnight-fasted F344-\textit{fa-nidd6} rats after glucose loading, we investigated whether there were any genes within the region bounded by the markers, D1Rat166 and D1Rat90, that were differentially expressed in the livers of obese control F344-\textit{fa} and F344-\textit{fa-nidd6} rats. We detected significant differences in the mRNA levels of the following genes: \textit{Acsl5} (276.24–276.29 Mbp, \(P=0.0039\)), \textit{Vti1a} (276.31–276.49 Mbp, \(P=0.0028\)), \textit{Plekhs1} (277.26–277.29 Mbp, \(P=0.0032\)) and \textit{Prdx3} (282.24–282.25 Mbp, \(P=0.0024\)) (Fig. 4).

**Obesity-specific gene expression:** To examine whether the differential expression of four genes was specific to the obesity phenotype, we compared mRNA levels in lean F344 and F344-\textit{nidd6} rat strains under fasting and postprandial conditions. As shown in Fig. 5A and 5B, cellular levels of phosphorylated GSK3β (Ser9) did not differ significantly between F344-\textit{fa} and F344-\textit{nidd6} rats under fasting conditions, but phosphorylation decreased in obese F344-\textit{fa-nidd6} rats and were significantly lower in these rats than in F344-\textit{fa} rats after glucose loading (\(P=0.0073\)). Glycogen synthesis was repressed because of decreased phosphorylation of GSK3β, and glucose production increased in the liver of F344-\textit{fa-nidd6} rats during glucose administration.

\textit{G6pc} is a key gluconeogenic enzyme in the liver and is the final gatekeeper of glucose efflux from the cell. Under fasting conditions, \textit{G6pc} expression did not differ significantly between F344-\textit{fa} and F344-\textit{nidd6} rats (Fig. 6C). However, \textit{G6pc} gene expression was significantly higher in F344-\textit{fa-nidd6} rats than in F344-\textit{fa} rats after glucose loading (\(P=0.007\)) (Fig. 6D). These data suggest that the inhibition of glycogen synthesis and activation of gluconeogenesis contribute to hyperglycemia after glucose loading through GSK3β dephosphorylation and increased \textit{G6pc} gene expression in F344-\textit{fa-nidd6} double congenic rats.

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**Fig. 4.** Gene expression levels within the Nidd6/QTL region by RT-qPCR. Differences in gene expression levels were measured between F344-\textit{fa} (n=7) and F344-\textit{fa-nidd6} (n=8) rats at 25 weeks of age. The mRNA levels were quantified using RT-qPCR and were normalized to \textit{Gapdh} mRNA levels. Rats were sacrificed at 60 min after glucose loading, and liver RNAs were immediately isolated. Data are presented as the mean ± SEM. **\(p<0.01\)**
Fig. 5. Relative expression levels of Acsl5, Vti1a, Plekhs1 and Prdx3 mRNAs in overnight-fasting lean and obese rats (25 weeks of age) at 0 and 60 min after glucose loading. (A) Relative expression levels of Acsl5 mRNA in F344 (n=8, white column), F344-nidd6 (n=8, hatched column), F344-fa (n=8, grey column) and F344-fa-nidd6 (n=8, black column) rats. The mRNA levels were quantified using RT-qPCR and normalized to the levels of Gapdh mRNA. (B) Relative expression levels of Vti1a mRNA. (C) Relative expression levels of Plekhs1 mRNA. (D) Relative expression levels of Prdx3 mRNA. Data are presented as the mean ± SEM. *P<0.05, **P<0.01 and vs F344 in lean rats and vs. F344-fa in obese control rats, respectively.
DISCUSSION

Obesity is an important risk factor for type 2 diabetes, but not the only determinant in OLETF rats [10]. We hypothesized that some of the 14 hyperglycemic QTLs previously identified in OLETF rats [19, 27, 31] contribute to the development of type 2 diabetes in obese rats. In fact, in our previous study, we confirmed that a single QTL was sufficient to induce severe hyperglycemia after glucose loading in the double congenic line F344-\textit{fa-nidd2} [15]. We then identified coenzyme Q2 (\textit{Coq2}) and placenta specific 8 (\textit{Plac8}) as strong candidates for contributors to obesity-associated onset of type 2 diabetes in F344-\textit{fa-nidd2} rats [23]. In this study, we further generated a new double congenic rat, F344-\textit{fa-nidd6}, and analyzed the expression of genes located in the \textit{Nidd6} of QTL region in F344-\textit{fa} (\textit{Lepr}\textsuperscript{−/−}) control rats and F344-\textit{fa-nidd6} double congenic rats. The major finding of this study implicated \textit{Plekhs1} and \textit{Prdx3} in the onset of obesity-associated type 2 diabetes. In addition, we elucidated that the inhibition of glycogen synthesis and increased gluconeogenesis that cause glucose intolerance and insulin resistance are associated with GSK3\textbeta d ephosphorylation and increased \textit{G6pc} expression in the liver.

We speculate that these reactions are induced by the accumulation of reactive oxygen species (ROS) because \textit{Prdx3} is involved in the suppression of ROS. \textit{Prdx3}, also called \textit{Prx3} or \textit{MER5}, was originally isolated from murine erythroleukemia cells and implicated in erythroid cell differentiation [20, 35]. \textit{Prdx3} is expressed in various rat tissues [22]. \textit{Prdx3} protein is predominantly located in mitochondria and plays an important role in mitochondrial antioxidant defence [3, 30]. \textit{Prdx3} knockout (KO) mice exhibit increased oxidative stress in the placenta [17] and increased lung inflammation [18]. In addition, \textit{Prdx3} KO mice and \textit{Prdx3} deficient 3T3-L1 adipocytes exhibit low levels of mitochondrial biogenesis, depressed antioxidant systems and mitochondrial viability as well as increased mitochondrial oxidative stress in white adipose tissue [9]. These data demonstrate that \textit{Prdx3} acts as an important scavenger of ROS under oxidative stress in mitochondria. Therefore, reducing \textit{Prdx3} may provoke ROS in the liver of F344-\textit{fa-nidd6} rats.

ROS are produced in various tissues (liver, muscle and fat) and induce insulin resistance under diabetic conditions [2, 5, 7]. In obese lepin receptor deficient (\textit{db/db}) diabetic
mice, the level of Prdx3 protein was significantly higher than in db/m control mice [9]. This suggests that Prdx3 increases during obesity. Prdx3 overexpression transgenic (Tg) mice produced significantly less H$_2$O$_2$. Glucose homeostasis was improved by reduced blood glucose levels, and increased glucose clearance was observed in Tg mice when compared with control mice. In addition, liver GSK3 inactivation significantly increased in Tg mice compared with that in control mice [4]. These studies demonstrate that reducing mitochondrial H$_2$O$_2$ by overexpressing Prdx3 improves glucose intolerance. Because Prdx3 is a well-known antioxidant and insulin resistance is closely associated with ectopic fat accumulation in the liver [28], reducing Prdx3 expression in the liver may increase mitochondrial ROS and thus induce hyperglycemia via glucose intolerance and insulin resistance under postprandial conditions in obese F344-fa-nidd6 double congenic rats. Furthermore, higher ROS may induce less insulin secretion in islets and blood in F344-fa-nidd6.

We also identified Plekhs1 as a candidate contributor to obesity-associated type 2 diabetes onset. Plekhs1 is largely uncharacterized. In a genome-wide analysis, mutations in noncoding regions of Plekhs1 were identified in cancer patients [32]. However, Plekhs1 has not previously been associated with type 2 diabetes. We propose that Prdx3 is a stronger candidate gene than Plekhs1 in Nidd6 of QTL region for the onset of obesity-associated type 2 diabetes because the maximum logarithm of odds score of Nidd6 of QTL existed on the D1Rat90, which was the telomeric end marker at that time, and the QTL profile suggested that the real QTL peak point was closer to the telomeric end rather than the D1Rat90 [31].

In summary, our study shows that the strategic development of new animal models, such as double congenic rats combining the Nidd6 of QTL with obesity, is a useful tool for the identification of genes that contribute to the onset of obesity-associated type 2 diabetes. Here, we demonstrate that Prdx3 and Plekhs1 are such candidate genes, and we are currently performing further research to confirm this.

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