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Age-dependent changes in phenotypes and candidate gene analysis in a polygenic animal model of Type II diabetes mellitus; NSY mouse

H. Ueda[1], H. Ikegami[1], Y. Kawaguchi[1], T. Fujisawa[1], K. Nojima[1], N. Babaya[1], K. Yamada[1], M. Shibata[2], E. Yamato[3], T. Ogihara[1]

[1] Department of Geriatric Medicine, Osaka University Medical School, Osaka, Japan
[2] Department of Health, Aichi-gakuin University, College of General Education, Aichi, Japan
[3] Department of Nutrition and Physiological Chemistry, Osaka University Medical School, Osaka, Japan

Abstract

Aims/hypothesis. The Nagoya-Shibata-Yasuda (NSY) mouse closely mimics human Type II (non-insulin-dependent) diabetes mellitus in that the onset is age-dependent, the animals are not severely obese, and both insulin resistance and impaired insulin response to glucose contribute to disease development. The aim of this study was to clarify the influence of age on the pathogenesis of diabetes and to analyse a candidate gene for Type II diabetes in this strain.
Methods. Several phenotypic characteristics related to diabetes mellitus were monitored longitudinally in male NSY and control C3H/He mice. The nucleotide sequence of *Glut4*, a candidate gene for *Nidd1nsy* (a susceptibility gene for Type II diabetes) on Chromosome 11, encoding insulin-sensitive glucose transporter, was determined in NSY and C3H mice.
Results. Glucose intolerance worsened with age, and fasting blood glucose and fasting plasma insulin concentration increased with age in NSY mice. Pancreatic insulin content increased until 24 weeks of age but then decreased at 48 weeks of age in NSY mice. The hypoglycaemic response to insulin was statistically significantly smaller in NSY than in C3H/He mice. The nucleotide sequence of GLUT4 cDNA was identical in NSY and C3H/He mice, but both were different from the sequence reported previously.
Conclusion/interpretation. Insulin secretion and insulin resistance, as well as ageing possibly play an important part in the disease development in NSY mice. A decline of pancreatic insulin content in older age might cause the relative insulin deficiency in this strain. Nucleotide sequencing suggests that *Glut4* is unlikely to be a candidate gene for *Nidd1nsy*. [Diabetologia (2000) 43: 932–938]

Keywords NSY mouse, ageing, animal model, insulin resistance, glucose transporter 4.

Type II (non-insulin-dependent) diabetes mellitus is a polygenic disease characterised by insulin resistance in muscle, fat and liver, and the failure of pancreatic beta cells to adequately compensate for this resistance [1, 2]. Glucose tolerance is reported to be impaired with advancing age [3, 4]. Deterioration of glucose tolerance can be due to impaired insulin secretion or impaired insulin action or both. The relative contribution of insulin deficiency and insulin resistance to the pathogenesis of age-related glucose intolerance is still controversial [5] because of the difficulty in doing longitudinal studies in humans. Longitudinal analysis of glucose tolerance in animal models with different degrees of glucose intolerance is one of the best ways to address this question.

The Nagoya-Shibata-Yasuda (NSY) mouse strain was established as an inbred animal model with spontaneous development of Type II diabetes, by selective breeding for glucose intolerance from out-

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Corresponding author: H. Ikegami, MD, PhD, Department of Geriatric Medicine, Osaka University Medical School, 2–2 Yamadaoka, Suita, Osaka 565–0871, Japan
Abbreviations: NSY mouse, Nagoya-Shibata-Yasuda mouse; Glut4, glucose transporter 4; i.p. GTT, intraperitoneal glucose tolerance test; QTL, quantitative trait loci; MLS, maximum lod score; Chr, chromosome.

bred Jcl:ICR mice [6]. The NSY mouse closely mimics human Type II diabetes in that the onset is age-dependent, the animals are not severely obese, and both insulin resistance and impaired insulin response to glucose contribute to disease development [7]. Impaired insulin response to glucose is observed after 12 weeks of age in this strain, and this impairment progressively worsens with advancing age [7]. Although impaired insulin response to glucose seems to play an important part in the development of Type II diabetes in this strain, impaired insulin action possibly also contributes to glucose intolerance. The age-dependent development of diabetes suggests that ageing is an important factor contributing to glucose intolerance and related phenotypes in NSY mice.

The gene *Nidd1nsy* in the middle part of Chromosome (Chr) 11 shows strong evidence of linkage with a maximum lod score (MLS) of 9.50 for glucose intolerance [8]. A potential candidate gene for *Nidd1nsy* is *Glut4* [9], which encodes the insulin-sensitive glucose transporter 4 (GLUT4). Glucose transporter 4 is of particular importance in glucose homeostasis because it mediates insulin-mediated glucose uptake in skeletal muscle, the main site of glucose disposal, and in adipose tissue [10–13]. Altered GLUT4 activity is suggested to be one of the factors responsible for decreased glucose uptake in muscle and adipose tissue in patients with Type II diabetes [14]. Moreover, the development of muscle insulin resistance and diabetes is reported in heterozygous GLUT4 knockout mice [15].

The aim of this study was to examine how diabetes-related phenotypes and ageing affect the development of diabetes mellitus in humans. We used an animal model of Type II diabetes and longitudinally monitored phenotypic characteristics related to diabetes in this model. Furthermore, to investigate the possible contribution of GLUT4 to the pathogenesis of diabetes in NSY mice, we determined the nucleotide sequence of GLUT4 cDNA in NSY and control C3H/He mice.

Materials and methods

We obtained originally three pairs of NSY mice (F36) from the Branch Hospital of Nagoya University School of Medicine. The colony of NSY mice was maintained in the animal facilities of Osaka University Medical School by brother-sister mating with selective breeding for glucose intolerance. We used C3H/He mice (Charles River Laboratories, Kanagawa, Japan) as a non-diabetic control strain. Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals were followed. All mice were given a laboratory diet, MF (Oriental Yeast, Tokyo, Japan), containing 24.6 % protein, 5.6 % fat, 3.1 % fibre, 6.3 % ash and 52.8 % complex carbohydrate, and tap water ad libitum in an air-conditioned room (22–25 °C) with a 12-h light:dark cycle.

Glucose tolerance test. Intraperitoneal glucose tolerance test (i.p. GTT) was done by injecting glucose (2 g/kg as 20 % solution) intraperitoneally in overnight-fasted mice without anaesthesia at 4, 8, 12, 24, 36, 48 and 60 weeks of age. Blood samples were obtained from the tail vein. Blood glucose concentration was measured directly by glucose oxidase method using Glutest E (Kyoto Daiichi Kagaku, Kyoto, Japan) at 0, 30, 60, 90 and 120 min during i.p. GTT [8, 16]. Area under the glucose curve (AUC) was also calculated according to the trapezoid rule from the glucose measurements at baseline (0 min), 30, 60, 90 and 120 min (mmol/l · min).

Measurement of fasting blood glucose and fasting plasma insulin concentrations. Fasting blood glucose and fasting plasma insulin concentrations were monitored in overnight-fasted mice at 8, 12, 24, 36 and 48 weeks of age. Blood glucose concentration was measured directly by glucose oxidase method using Glutest E. For measurement of insulin concentration whole blood (300 μl) was collected from the orbital sinus and immediately placed on ice. Plasma was obtained by centrifugation. Plasma insulin was measured by RIA (ShionoRIA insulin, Shionogi, Osaka, Japan) using rat insulin (Novo Nordisk, Copenhagen, Denmark) as a standard [17].

Measurement of phenotypic characteristics and pancreatic insulin content. After anaesthetisation (pentobarbital 50 mg/kg, i.p.), body weight and anal-nasal length were measured. Body mass index (BMI) was calculated as body weight (g) divided by the square of the anal-nasal length (cm). The pancreas and epididymal fat pads were dissected and weighed. Insulin was extracted from the pancreas by acid ethanol method [18] and measured by RIA as described above.

Insulin tolerance test. Insulin tolerance test was done by injecting human insulin (0.5 U/kg; Humulin R, Eli Lilly, Indianapolis, Ind., USA) intraperitoneally in overnight-fasted C3H/He and NSY mice at 48 weeks of age. Blood samples were obtained from the tail vein. Blood glucose concentration was measured directly by glucose oxidase method using Glutest E at 0, 15, 30, 45 and 60 min during this test. Results are expressed as the percentage change from fasting blood glucose concentration.

Sequencing of GLUT4 cDNA. Total RNA was extracted from the muscle of NSY and C3H/He mice. The DNA sequence of the *Glut4* gene of NSY and C3H/He mice was determined by PCR amplification of muscle cDNA as four overlapping segments, using primers based on the sequence of murine GLUT4 cDNA [19]. The resulting PCR products were subcloned into pT7Blue Vector (Novagen, Madison, Wis., USA), subjected to DNA sequencing and analysed on an ABI377 sequencer (Perkin-Elmer, Foster City, Calif., USA).

Statistical analysis. All results are expressed as means ± SEM. Statistical analysis to compare NSY and C3H/He mice was done by Student's *t* test and to compare the groups of animals at different ages ANOVA was used.

Results

Longitudinal changes in blood glucose and plasma insulin concentrations. Figure 1 shows the results of the glucose tolerance test in male NSY and C3H/He mice at 48 weeks of age. Fasting blood glucose and blood

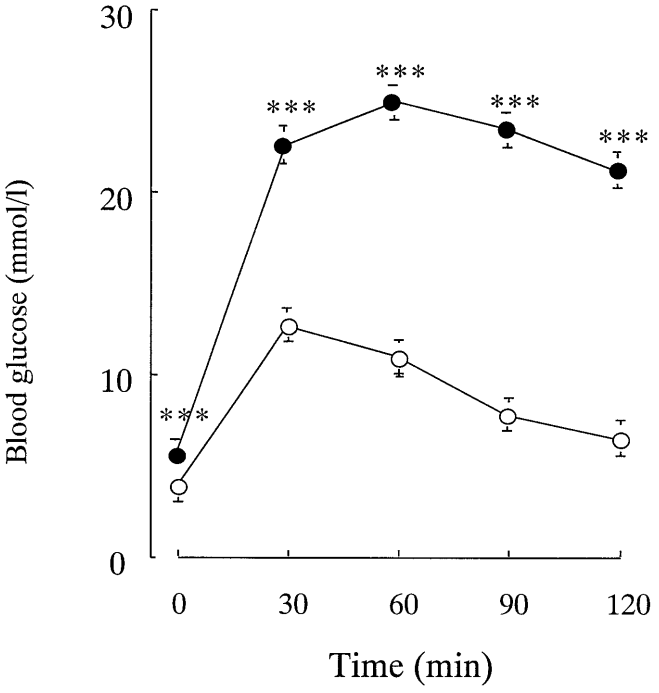

Fig. 1. Glucose tolerance test (2 g/kg body weight) in male overnight-fasted C3H/He (open circles: $n = 15$) and NSY (closed circles: $n = 15$) mice at 48 weeks of age. Values are means ± SEM. ***$p < 0.0001$ vs C3H/He mice

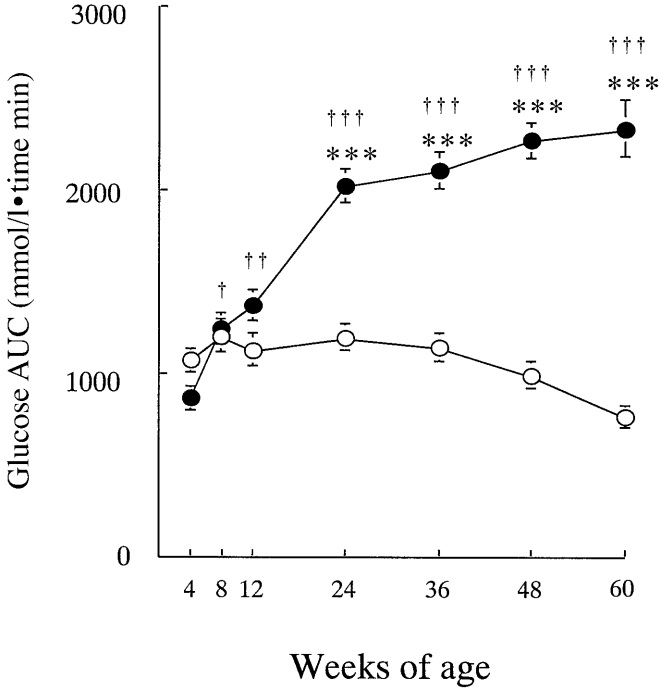

Fig. 2. Glucose area under the curve (mmol/l · min) in male, overnight-fasted C3H/He (open circles: $n = 5$–15) and NSY (closed circles: $n = 9$–15) mice from 4 to 60 weeks of age. Values are means ± SEM. ***$p < 0.0001$ vs male C3H/He mice; †$p < 0.01$, ††$p < 0.001$, †††$p < 0.0001$ vs NSY mice at 4 weeks of age

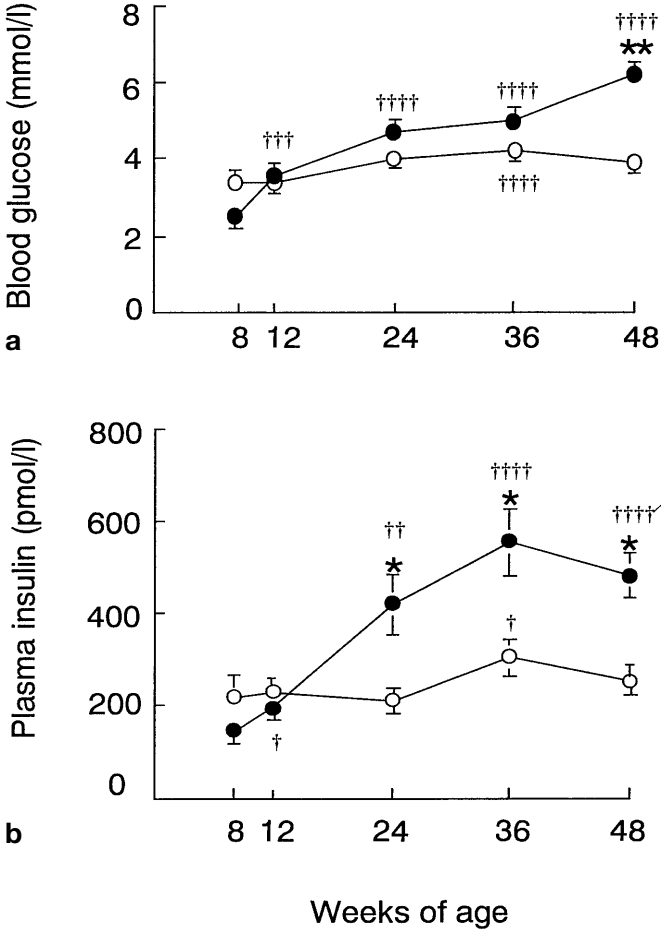

Fig. 3. Longitudinal analysis of fasting blood glucose concentration (**a**) and plasma insulin concentration (**b**) in male, overnight-fasted C3H/He (open circles: $n = 5$–7) and NSY (closed circles: $n = 5$–9) mice from 8 to 48 weeks of age. Values are means ± SEM. *$p < 0.05$ **$p < 0.001$ vs male C3H/He mice; †$p < 0.05$, ††$p < 0.01$, †††$p < 0.001$, ††††$p < 0.0001$ vs 8 weeks of age in each strain

glucose concentrations after injection of glucose were significantly higher in NSY mice than in C3H/He mice ($p < 0.0001$).

Figure 2 shows glucose area under the curve (glucose AUC) of i.p. GTT at various ages between 4 and 60 weeks of age. No significant difference was observed in glucose AUC of male NSY and C3H/He mice at 4 weeks of age (878 ± 61 and 1083 ± 53 (mmol/l · min), respectively). Glucose intolerance significantly worsened with age in NSY mice but not in C3H/He mice. Glucose AUC at 24, 36, 48 and 60 weeks of age in NSY mice was also significantly higher than at 8 weeks of age ($p < 0.0001$). Glucose AUC at 24, 36, 48 and 60 weeks of age in NSY mice was also significantly higher than at 12 weeks of age ($p < 0.0001$). Furthermore, glucose AUC at 60 weeks of age in NSY mice was also significantly higher than at 24 weeks of age ($p < 0.05$).

Figure 3 shows the longitudinal changes in fasting blood glucose and plasma insulin concentrations.

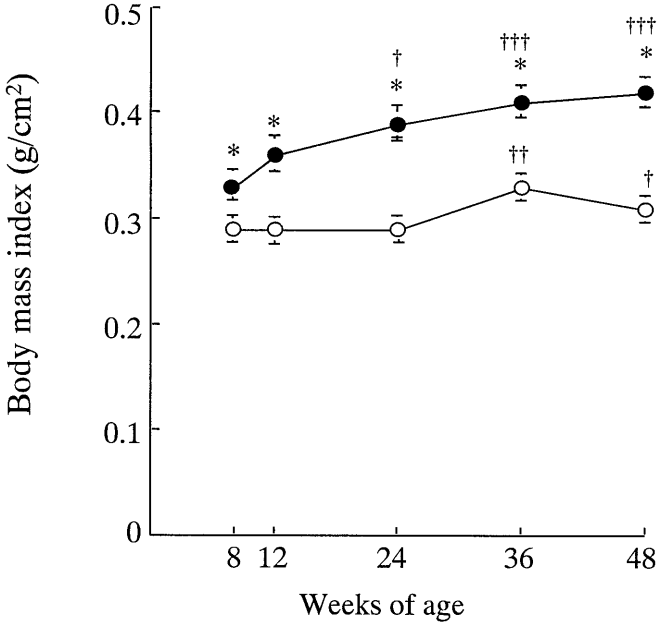

Fig. 4. Body mass index changes in C3H/He (open circles: $n = 5$–6) and NSY (closed circles: $n = 5$–8) mice from 8 to 48 weeks of age. Values are means ± SEM. *$p < 0.001$ vs C3H/He mice; †$p < 0.05$, ††$p < 0.001$, †††$p < 0.0001$ vs 8 weeks of age in each strain

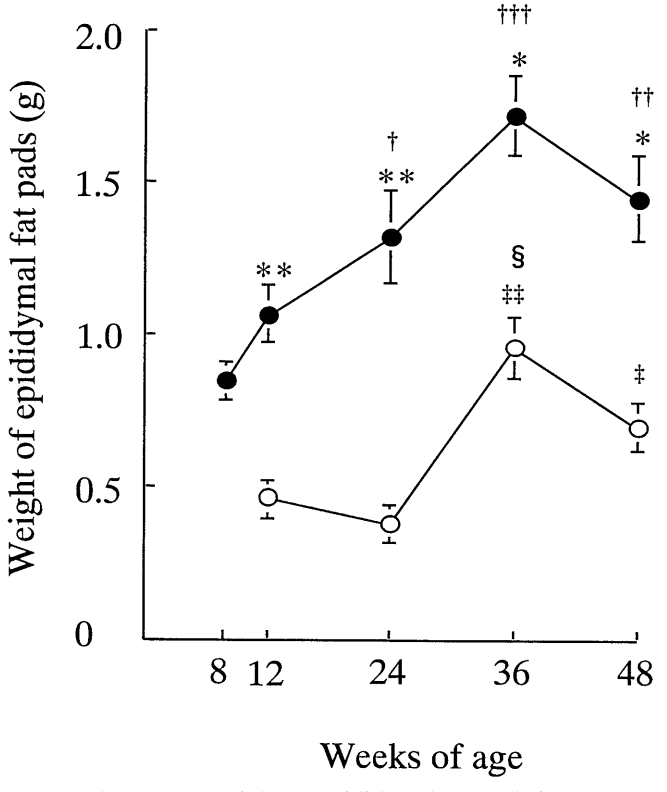

Fig. 5. Changes of weight of epididymal fat pads in C3H/He (open circles: $n = 5$–6) and NSY (closed circles: $n = 5$–8) mice from 8 to 48 weeks of age. Values are means ± SEM. *$p < 0.01$ **$p < 0.001$ vs C3H/He mice; †$p < 0.05$, ††$p < 0.01$, †††$p < 0.0001$ vs NSY mice at 8 weeks of age; ‡$p < 0.05$, ‡‡$p < 0.01$ vs C3H/He mice at 12 weeks of age; § $p < 0.05$ vs C3H/He mice at 48 weeks of age

Table 1. Phenotypic characterisation of male C3H/He and NSY mice at 8 weeks of age

	C3H/He mice $n = 5$-6	NSY mice $n = 5$-6	p
Blood glucose (mmol/l)	3.4 ± 0.1	2.5 ± 0.1	NS
Plasma insulin (pmol/l)	218 ± 21	144 ± 5.1	NS
BMI ($g \cdot cm^{-2}$)	0.29 ± 0.07	0.33 ± 0.05	< 0.001
Weight of epididymal fat pads (g)	ND	0.85 ± 0.05	–
Pancreatic insulin content ($ng \cdot mg$ wet weight $^{-1}$)	34.3 ± 6.3	33.8 ± 4.3	NS

Values are means ± SEM. NS: not significant. ND: not done

Fasting blood glucose concentration in NSY mice increased continuously with age. In contrast, fasting blood glucose concentration in C3H/He mice did not differ significantly among 8, 12, 24 and 48 weeks of age, except for a significant increase at 36 weeks of age. Fasting blood glucose was significantly higher in NSY mice than in C3H/He mice at 48 weeks of age ($p < 0.001$). Fasting plasma insulin concentration in NSY mice increased with age, whereas in C3H/He mice it was stable at all ages, except for a significant increase at 36 weeks of age. Fasting plasma insulin concentration was comparable between the two strains at 8 and 12 weeks of age. Fasting plasma insulin in NSY mice was significantly higher than in C3H/He mice at 24 ($p < 0.05$), 36 ($p < 0.05$) and 48 ($p < 0.05$) weeks of age.

Body mass index and weight of epididymal fat pads. Figure 4 shows the longitudinal changes in BMI. Up to 48 weeks of age BMI of NSY increased continuously. Until 24 weeks of age BMI of C3H/He mice did not change with age and was slightly increased at 36 and 48 weeks of age. At all ages BMI in NSY mice was significantly higher than in C3H/He mice ($p < 0.001$). Figure 5 shows the weight of epididymal fat pads. Weight of epididymal fat pads increased in NSY mice with age, whereas in C3H/He mice it was stable until 24 weeks of age with a slight increase at 36 and 48 weeks of age. Weight of epididymal fat pads did not significantly differ between 36 and 48 weeks of age in NSY mice. Weight of epididymal fat pads was significantly decreased ($p < 0.05$) in C3H/He mice at 48 weeks of age compared with 36 weeks of age. Weight of epididymal fat pads was significantly greater in NSY mice than in C3H/He mice after 12 weeks of age ($p < 0.01$). All baseline data (8-week-old animals) for fasting blood glucose, fasting plasma insulin, BMI, epididymal fat pad weight and pancreatic insulin content are in Table 1.

Pancreatic insulin content. Pancreatic insulin content in NSY mice was significantly increased at 24, 36 and 48 weeks of age from the baseline value, being

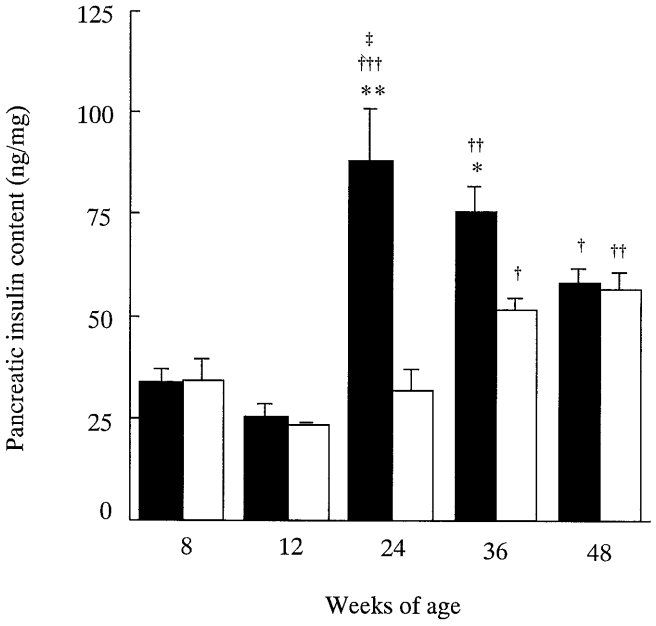

Fig. 6. Longitudinal analysis of pancreatic insulin content in male C3H/He (open bars) and NSY (closed bars) mice from 8 to 48 weeks of age. $n = 5$–6 in each group. Values are means ± SEM. $*p < 0.05$ $**p < 0.01$ vs C3H/He mice; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.0001$ vs 8 weeks of age in each strain; $\ddagger p < 0.01$ vs NSY mice at 48 weeks of age

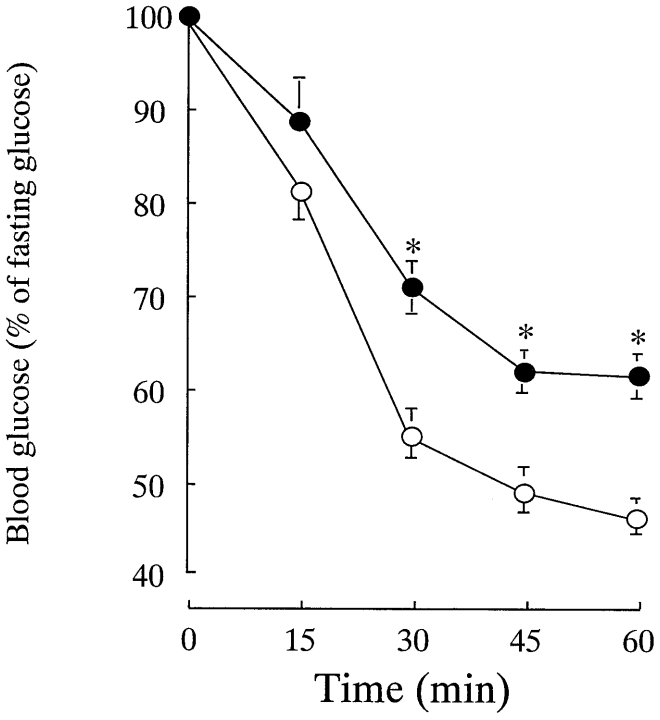

Fig. 7. Insulin tolerance test (0.5 U/kg body weight) in male overnight-fasted C3H/He (open circles: $n = 12$) and NSY (closed circles: $n = 11$) mice at 48 weeks of age. Results are expressed as the percentage change from fasting blood glucose concentration. Values are means ± SEM. $*p < 0.01$ vs C3H/He mice

the highest at 24 weeks of age and decreasing gradually thereafter (Fig. 5). Pancreatic insulin content in C3H/He mice, in contrast, did not change up to 24 weeks of age, with a significant increase at 36 and 48 weeks of age compared with 8 weeks of age. Pancreatic insulin content was comparable between the two strains at 8, 12 and 48 weeks of age but was significantly higher in NSY mice than in C3H/He mice at 24 ($p < 0.01$) and 36 ($p < 0.05$) weeks of age.

Insulin tolerance test. Insulin tolerance test was done in male NSY and C3H/He mice at 48 weeks of age (Fig. 6). The hypoglycaemic response to insulin at 30, 45 and 60 min after its injection was significantly smaller in NSY than in C3H mice. The hypoglycaemic response to insulin at 60 min after its injection was significantly smaller in NSY (61.8 ± 3.7 % of fasting glucose; $n = 11$) than in C3H mice (47.1 ± 2.3 % of fasting glucose; $n = 12$, $p < 0.01$).

Nucleotide sequence of GLUT4 cDNA. The nucleotide sequence of GLUT4 cDNA in the NSY mouse was found to be the same as in the C3H/He mouse. The nucleotide sequence in these two strains, however, was different from the previously reported sequence in 129/v strain [19]. This difference in nucleotide sequence led to the substitution of six amino acids in the predicted amino acid sequence compared with the previously reported one (Table 2). All these six amino acids were conserved in human [20] and rat [21] GLUT4, but not in murine glucose transporter 1 [19].

Discussion

Our previous report showed that NSY mice develop Type II diabetes in an age-dependent manner [7]. Ageing plays an important part in the development of diabetes in humans [3] as well as in NSY mice. Glucose intolerance worsened with age in NSY mice. In control C3H/He mice, on the other hand, glucose tolerance was stable even at older ages. Fasting plasma insulin concentration and pancreatic insulin content rose with age and then slightly decreased at an older age in NSY mice. In control C3H/He mice, in contrast, fasting plasma insulin concentration was stable at all ages, except for a significant increase at 36 weeks of age, and pancreatic insulin content was significantly increased at 36 and 48 weeks of age. These observations suggest that the islets of NSY mice retain the ability to synthesise insulin to compensate for insulin resistance up to 36 weeks of age, but the ability to synthesise insulin is decreased or exhausted at 48 weeks of age, leading to the increase of fasting blood glucose concentration. The longitudinal

Table 2. Predicted amino acid sequence of GLUT4 in NSY, C3H/He and 3T3L1 cell line (mouse 129/v)

a) MPSGFQQIGSD−−DGEPPRQRVTGTLVLAVFSAVLGSLQFGYNIGVINAP
b) ––
c) ––––––––––––VK––––––––––––––––––––––––––––––––––––
QKVIEQSYNATWLGRQGPGGPDSIPQGTLTTLWALSVAIFSVGGMISSFL
––
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IGIISQWLGRKRAMLANNVLAVLGGALMGLANAAASYEILILGRFLIGAY
––
––––––––––––––––––––––––––––––––––V––––––––––––––––
SGLTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILVAQVLGLESMLGT
––
––
ATLWPLLLALTVLPALLQLILLPFCPESPRYLYIIRNLEGPARKSLKRLT
––
––
GWADVSDALAELKDEKRKLERERPMSLLQLLGSRTHRQPLIIAVVLQLSQ
––
––
QLSGINAVFYYSTSIFESAGVGQPAYATIGAGVVNTVFTLVSVLLVERAG
––
––
RRTLHLLGLAGMCGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIG
––
––
PGPIPWFIVAELFSQGPRPAAMAVAGFSNWTCNFIVGMGFQYVADAMGPY
––
––R–––––
VFLLFAVLLLGFFIFTFLKVPETRGRTFDQISAAFRRTPSLLEQEVKPST
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ELEYLGPDEND
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(a) NSY mouse, (b) C3H/He mouse, (c) 3T3L1 cell line (mouse 129/v, from reference 19)

Dash indicates identity with NSY sequence. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession number: AB008453

characteristics of hyperinsulinaemia as observed in NSY mice were also reported in C57Bl/Ks db/db mice. In this strain, insulin concentrations rose six to ten times above normal at 3 to 6 weeks of age, and then the hyperinsulinaemia waned to below normal concentrations between 3 and 6 months of age [22]. In NSY mice, hyperinsulinaemia was much less pronounced but the hyperinsulinaemic state lasted longer. This phenomenon could reflect the delayed expression of timed genes conveying susceptibility to diabetes in NSY mice, acting through insulin deficiency, insulin resistance, or both. Indeed, longitudinal quantitative trait loci (QTL) mapping in our previous study showed that both maximum lod score (MLS) and interval of mapped loci changed and shifted with age [8]. Nagoya-Shibata-Yasuda mice might be useful to identify genes conveying susceptibility to diabetes whose expression is affected by ageing.

Insulin tolerance test showed that insulin action was impaired in NSY mice (Fig. 6). In our previous study [7], the existence of insulin resistance was only suggested by fasting hyperinsulinaemia, but this was confirmed in the present study. Thus, insulin resistance together with functional changes in pancreatic beta cells appear to play an important part in the development of diabetes in NSY mice.

Body mass index of NSY mice was significantly greater than in C3H/He mice. In addition, epididymal fat pad weight in NSY mice was significantly greater than in C3H/He mice. These characteristics together with insulin resistance as shown by insulin tolerance test are similar to those observed in human patients with insulin resistance syndrome [23, 24] in which visceral fat accumulation is a risk factor for glucose intolerance, even in patients with mild obesity [25]. Note that, an age-dependent increase in BMI and epididymal fat pad weight was similarly observed in both strains although the degree of increase was different between the two strains. The age-dependent increase in BMI and accumulation of epididymal fat pads, therefore, seem to be caused by age per se but not by a difference in genetic background between the two strains. These characteristics are similar to those observed in non-diabetic humans [26]. The greater BMI and fat pad accumulation in NSY mice, on the other hand, might reflect the different genetic background from that of C3H/He mice. The slight, but significant, decrease in weight of epididymal fat

in C3H/He mice at 48 weeks of age compared with that at 36 weeks of age could have contributed to the reduced risk for glucose intolerance in this strain.

As mentioned above, the genetic background of the NSY mouse seems to contribute in this strain to the greater BMI and fat pad accumulation which are associated with insulin resistance and glucose intolerance. A candidate gene for these characteristics is *Glut4*, encoding the insulin-sensitive glucose transporter GLUT4. A defect in GLUT4 function has been reported to result in insulin resistance and diabetes [15]. The murine *Glut4* gene is located in the central region of chromosome 11, where a strong susceptibility gene for Type II diabetes, *Nidd1nsy*, has been mapped in our whole genome scan in the NSY mouse [8]. The highest MLS which *Nidd1nsy* showed for glucose intolerance was 9.52, whereas the highest MLS which the second strongest locus, *Nidd2nsy*, showed for glucose intolerance was 4.88 [8]. Thus, we considered that *Nidd1nsy* is the most important locus in the disease development, and we focused on the analysis of a candidate gene for *Nidd1nsy*. We therefore determined the nucleotide sequence of *Glut4* in the NSY mouse and control strains. The nucleotide sequence of *Glut4* was identical in NSY and C3H/He mice, suggesting that allelic variation in *Glut4* is unlikely to be responsible for the *Nidd1nsy* effect. Although the nucleotide sequence of GLUT4 cDNA was identical in NSY and C3H/He mice, the nucleotide sequence in these strains was different from that of the previously reported cDNA sequence for murine GLUT4. The reason for the difference in *Glut4* sequence in NSY and C3H/He mice from the previously reported sequence is not clear. The previously reported sequence of cDNA was from a cell line derived from 129/v mice [19]. It is possible that the sequence of the *Glut4* gene has changed during the establishment or maintenance of the cell line. Another possibility is a strain difference. Although functional analysis of GLUT4 using two different GLUT4 is necessary to clarify how it affects the diabetic phenotype, 129/v mice were not reported as an animal model of diabetes [22]. GLUT4 of 129/v is therefore unlikely to have a strong functional defect.

In summary, insulin secretion and insulin resistance, as well as ageing, possibly play an important part in the disease development in NSY mice. A decline of pancreatic insulin content at older age could cause the relative insulin deficiency in this strain. Sequence analysis suggests that *Glut4* is unlikely to be a candidate gene for *Nidd1nsy*.

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