# Paternal-Maternal Effects on Phenotypic Characteristics in Spontaneously Diabetic Nagoya-Shibata-Yasuda Mice

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**The Nagoya-Shibata-Yasuda (NSY) mouse is an inbred strain with spontaneous development of type 2 (non–insulin-dependent) diabetes mellitus. The purpose of this study was to determine the mode of inheritance of various phenotypes related to diabetes in this strain. Two reciprocal outcrosses, female C3H/He × male NSY F1 (C3NF1) and female NSY × male C3H/He F1 (NC3F1) mice, were performed. The phenotypic characteristics in both F1 mice were investigated. The cumulative incidence of diabetes was 100% (25 of 25) in male C3NF1 mice and 97% (29 of 30) in male NC3F1 mice at 48 weeks of age, indicating that diabetes in NSY mice was transmitted to male F1 hybrids in an autosomal dominant manner. Fatty liver also showed an autosomal dominant mode of inheritance. In contrast, epididymal fat accumulation and impaired insulin secretion showed an autosomal recessive mode of inheritance. The body mass index (BMI) showed a codominant mode of inheritance. Paternal-maternal effects associated with the severity of diabetes were observed. Insulin resistance was much more severe in male F1 mice than in the parental NSY strain. These data indicate different modes of inheritance among phenotypes related to type 2 diabetes. The presence of more severe insulin resistance in F1 mice versus the parental strains suggests the interaction of both parental genomes in the development of insulin resistance. The F1 mouse is expected to be useful for studies of the pathogenesis and genetic synergism of the insulin resistance syndrome.**


TYPE 2 (non–insulin-dependent) diabetes mellitus is a heterogeneous disorder caused by both genetic and environmental factors.[1,2] The complexity of the genetic background and the influence of environmental factors make it difficult to understand the disease mechanisms in humans. Animal models are invaluable for elucidating the complex etiology of type 2 diabetes and the interaction of genetic and environmental factors. This is clearly illustrated by recent progress in the genetic analysis of type 2 diabetes using several animal models.[3-9]

The Nagoya-Shibata-Yasuda (NSY) mouse was established as an inbred strain with spontaneous development of diabetes mellitus by selective breeding for glucose intolerance from outbred Jcl:ICR mice.[10] NSY mice spontaneously develop diabetes mellitus in an age-dependent manner, a characteristic similar to human type 2 diabetes. The cumulative incidence of diabetes is 98% in male mice and 32% in female mice at 48 weeks of age. Impaired insulin secretion in response to glucose together with insulin resistance appear to contribute to the development of diabetes in this model,[11] as in the case of human type 2 diabetes.

Evidence for the involvement of mutations or deletions of mitochondrial DNA in human type 2 diabetes has been reported in previous studies.[12,13] A significant parental strain/sex effect on phenotypic variables associated with the pathophysiology of diabetes has been reported in polygenic animal models of type 2 diabetes.[5-9] The purpose of this study was to determine the mode of inheritance of various phenotypes related to diabetes in this strain. Two reciprocal crosses, female C3H/He × male NSY F1 (C3NF1) and female NSY × male C3H/He F1 (NC3F1) mice, were performed. Two reciprocal F1 mice of each sex were studied by performing a glucose tolerance test (GTT). In addition, phenotypic characteristics associated with the pathophysiology of diabetes in F1 mice were also investigated. To our surprise, insulin resistance was a much more predominant defect in F1 mice than in parental NSY mice.

## MATERIALS AND METHODS

Three pairs of NSY mice (F36) were originally obtained from the Branch Hospital of Nagoya University School of Medicine, and 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. C3H/He mice (Charles River Laboratories, Kanagawa, Japan) were used as a nondiabetic control strain. Female NSY (F41) mice were crossed with male C3H/He mice and male NSY mice were crossed with female C3H/He mice to obtain NSY × C3H/He F1 (NC3F1) and C3H/He × NSY F1 (C3NF1) mice, respectively. Two reciprocal F1 mice of each sex were used in this study. Osaka University Medical School Guidelines for the Care and Use of Laboratory Animals were followed. All mice received a laboratory diet (MF; Oriental Yeast, Tokyo, Japan) containing 24.6% protein, 5.6% fat, 3.1% fiber, 6.3% ash, and 52.8% complex carbohydrate and tap water ad libitum in an air-conditioned room (22° to 25°C) with a 12-hour light/dark cycle.

### *GTT*

An intraperitoneal (IP) GTT was performed by injecting glucose (2 g/kg as a 20% solution) IP in overnight-fasted mice without anesthesia at 12, 36, and 48 weeks of age. Blood samples were obtained from the tail vein. The blood glucose concentration was measured directly by the glucose oxidase method using Glutest E (Kyoto Daiichi Kagaku, Kyoto, Japan).[11,14] Diabetes was defined as a blood glucose concentration at 120 minutes during the IP GTT of 11.1 mmol/L or higher.

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*Body Weight, Epididymal Fat, Plasma Insulin, and Pancreatic Insulin Content*

After anesthesia (pentobarbital 50 mg/kg IP), body weight and anal-nasal length were measured. The body mass index (BMI) was calculated as body weight in grams divided by the square of the anal-nasal length in centimeters. The pancreas was dissected and weighed. Insulin was extracted from the pancreas by the acid ethanol method[15] and measured by radioimmunoassay (ShionoRIA insulin; Shionogi, Osaka, Japan) with rat insulin (Novo, Copenhagen, Denmark) as the standard.[16]

*Insulin Secretory Response to Glucose*

An IP GTT was performed by injecting glucose (2 g/kg as a 20% solution) IP in overnight-fasted mice at 48 weeks of age. Blood samples were obtained from the tail vein. The blood glucose concentration was measured at 0 and 30 minutes during the IP GTT as described before. For measurement of the insulin concentration, whole blood (300 μL) was collected from the orbital sinus at 0 and 30 minutes during the IP GTT and immediately placed on ice. Plasma was obtained by centrifugation. The plasma insulin concentration was measured by RIA. The insulinogenic index was calculated as the increment of plasma insulin (picomolars) above the basal level divided by the increment of blood glucose (millimolars) above the basal level.

*Insulin Tolerance Test*

An insulin tolerance test was performed by injecting human insulin (0.75 IU/kg Humulin R; Eli Lilly & Co, Indianapolis, IN) IP in overnight-fasted male C3H/He, NSY, and NC3F1 mice at 48 weeks of age.[17,18] Blood samples were obtained from the tail vein. The blood glucose concentration was measured as already described. Results are expressed as the percent change from fasting blood glucose.

*Fatty Liver*

Fatty liver was defined as liver tissue with a white color. The liver was dissected and then fixed in 10% Formalin neutral buffer solution, pH 7.4 (Wako Pure Chemical Industries, Osaka, Japan). Paraffin sections of the liver were stained with Sudan III. A white color of the liver tissue was correlated with fatty deposits stained red by Sudan III.

*Statistical Analysis*

All results are expressed as the mean ± SEM. Statistical analysis was performed by Student's $t$ test. For the correction of multiple comparisons, the Tukey test was performed to confirm statistical significance.

## RESULTS

*Diabetes Mellitus in F1 Mice*

Figure 1 shows the cumulative incidence of diabetes in F1 mice. Male F1 mice from both reciprocal outcrosses spontaneously developed diabetes mellitus in an age-dependent manner. The cumulative incidence of diabetes reached 100% (25 of 25) in male C3NF1 mice and 97% (29 of 30) in male NC3F1 mice at 48 weeks of age (Fig 1A). The cumulative incidence of diabetes in females was 12% (2 of 17) in C3NF1 mice and 3% (1 of 32) in NC3F1 mice at 48 weeks of age (Fig 1B). Sex differences in the incidence of diabetes were observed in both reciprocal F1 mice and NSY mice.

Figure 2 shows the results of the GTT in male C3H/He, NSY, C3NF1, and NC3F1 mice at 48 weeks of age. Fasting blood glucose was significantly elevated in NSY, C3NF1, and NC3F1 mice compared with C3H/He mice ($P < .0001$) but did not differ significantly among NSY, C3NF1, and NC3F1 mice. Blood glucose concentrations after injection of glucose were significantly higher in NSY, C3NF1, and NC3F1 mice versus C3H/He mice. Blood glucose at 120 minutes after the glucose load did not differ significantly between NSY, C3NF1, and NC3F1 mice. Blood glucose at 30 minutes after loading was slightly but significantly higher in male NSY mice versus male NC3F1 mice. Blood glucose concentrations at 60 and 90 minutes after loading did not differ significantly between NSY and NC3F1 mice. Blood glucose concentrations at 30, 60, and 90 minutes after loading were significantly higher in male C3NF1 mice than in male NC3F1 mice. Blood glucose at 30, 60, and 90 minutes after loading did not differ significantly between NSY and C3NF1 mice (Fig 2). The incremental area under the glucose curve (glucose AUC) was also calculated. The glucose AUC in male NSY, C3NF1, and NC3F1 mice (31.0 ± 1.0, 32.1 ± 1.0, and 26.9 ± 1.2 mmol/L · h, respec-

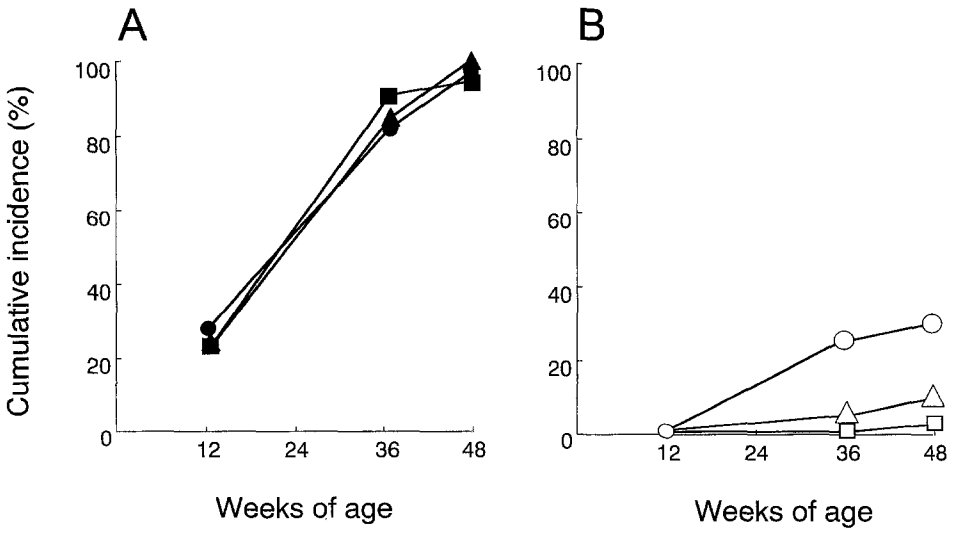


**Fig 1. Cumulative incidence of diabetes mellitus in mice at 12, 36, and 48 weeks of age. Diabetes was defined as a blood glucose value at 120 minutes during the IP GTT of 11.1 mmol/L or higher. (A) Male NSY (●), C3NF1 (▲), and NC3F1 (■) mice. (B) Female NSY (○), C3NF1 (△), and NC3F1 (□) mice.**

tively) was significantly greater than that in C3H/He mice (10.6 ± 1.0 mmol/L · h, $P < .0001$ *v* C3H/He). The glucose AUC in male NSY and C3NF1 mice was slightly but significantly greater than that in NC3F1 mice ($P < .02$ and $P < .005$, respectively). It did not differ significantly between male NSY and C3NF1 mice. In female NSY, C3NF1, NC3F1, and C3H/He mice, it was 17.3 ± 1.6 (n = 31), 11.5 ± 1.1 (n = 17), 10.4 ± 0.5 (n = 32), and 6.1 ± 1.1 (n = 8) mmol/L · h, respectively, at 48 weeks of age. The glucose AUC in female NSY mice was significantly greater than that in female C3NF1 and NC3F1 mice ($P < .02$ and $P < .001$, respectively). In female C3NF1 and NC3F1 mice, it was significantly greater than that in C3H/He mice ($P < .01$ and $P < .001$, respectively). It did not differ significantly between female C3NF1 and NC3F1 mice.

*Phenotypic Characteristics in F1 Mice at 48 Weeks of Age*

Body weight, blood glucose, plasma insulin, and pancreatic insulin content were examined in nonfasting male C3H/He, NSY, C3NF1, and NC3F1 mice at 48 weeks of age (Table 1). The body weight of NSY, C3NF1, and NC3F1 mice was significantly greater than that of C3H/He mice ($P < .0001$). The body weight of C3NF1 mice was slightly but significantly greater than that of NSY and NC3F1 mice ($P < .0001$). In female NSY, C3NF1, NC3F1, and C3H/He mice, it was 39.8 ± 1.2 g (n = 31), 44.6 ± 1.5 g (n = 17), 34.2 ± 0.8 g (n = 32), and 32.7 ± 1.5 g (n = 8), respectively, at 48 weeks of age. In female NSY and C3NF1 mice, it was significantly greater than that of female C3H/He mice ($P < .01$ and $P < .0001$, respectively). The body weight of C3NF1 mice was significantly greater than that of NSY and NC3F1 mice ($P < .05$ and $P < .0001$, respectively), but it did not differ between female NC3F1 and C3H/He mice. Although it would be useful to have the results in female mice as well, further phenotypes other than fatty liver were not investigated in female mice because of their low incidence of diabetes. Nonfasting blood glucose was slightly but significantly higher in NSY, C3NF1, and NC3F1 mice than in C3H/He mice ($P < .0001$). It was significantly higher in C3NF1 mice versus NSY and NC3F1 mice ($P < .05$). Nonfasting plasma insulin was significantly higher in NSY, C3NF1, and NC3F1 mice than in C3H/He mice ($P < .0001$). It was significantly higher in C3NF1 mice than in NC3F1 mice ($P < .05$). Pancreatic insulin content did not differ between NSY, NC3F1, and C3H/He mice.

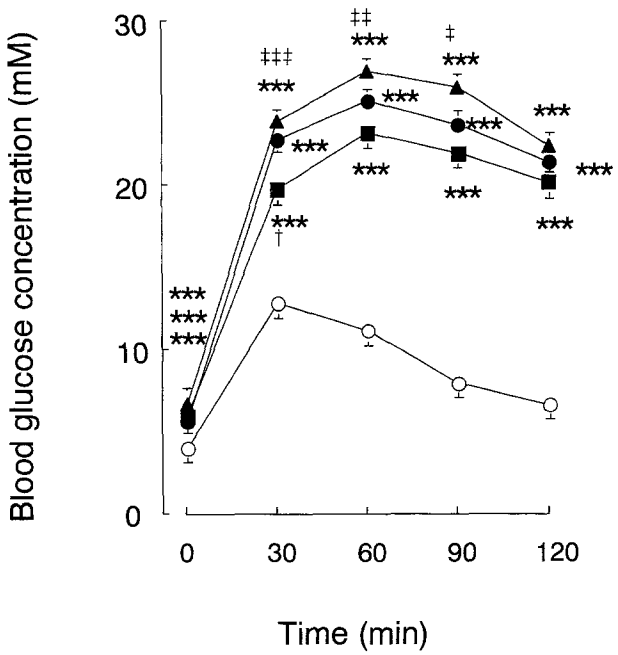


**Fig 2. GTT (2 g/kg body weight) in male overnight-fasted C3H/He (○, n = 30), NSY (●, n = 30), C3NF1 (▲, n = 25), and NC3F1 (■, n = 30) mice at 48 weeks of age. Values are means ± SEM. ***$P <$ .0001 *v* C3H/He mice, †$P < .05$ *v* NSY mice, ‡$P < .05$, ‡‡$P < .001$, ‡‡‡$P <$ .0001 *v* NC3F1 mice.**

The BMI of NSY, C3NF1, and NC3F1 mice was significantly greater than that of C3H/He mice ($P < .0001$). The BMI of NSY mice was significantly greater than that of C3NF1 ($P < .05$) and NC3F1 ($P < .0001$) mice, but it did not differ between C3NF1 and NC3F1 mice (Fig 3A). The weight of epididymal fat pads was significantly greater in NSY mice than in C3H/He, C3NF1, and NC3F1 mice ($P < .001$). It did not differ between C3H/He, C3NF1, and NC3F1 mice (Fig 3B).

*Insulin Secretion and Action in F1 Mice*

The fasting plasma insulin concentration and insulin response to glucose were examined in male C3H/He, NSY, C3NF1, and NC3F1 mice at 48 weeks of age. Fasting plasma insulin was significantly higher in NSY, C3NF1, and NC3F1 mice versus C3H/He mice ($P < .0001$), but it did not differ between NSY, C3NF1, and NC3F1 mice (Fig 4A). The insulinogenic index was significantly lower in NSY mice than in NC3F1 and C3H/He mice ($P < .05$) and tended to be lower in NSY mice versus C3NF1 mice (Fig 4B). Figure 5 shows the results of the insulin tolerance test in NSY, NC3F1, and C3H/He mice at 48 weeks of age. The hypoglycemic response to insulin at 15 minutes after its injection was significantly smaller in NSY versus C3H/He mice. The hypoglycemic response to insulin was comparable in NSY and C3H/He mice at 30, 45, and 60 minutes. The hypoglycemic response to insulin in NC3F1 mice was significantly smaller than that in NSY and C3H/He mice.

*Inheritance of Fatty Liver*

Male NSY mice spontaneously develop fatty liver in an age-dependent manner. The incidence of fatty liver was 0% (0 of 5) at 12 weeks of age, 20% (1 of 5) at 36 weeks, and 63% (17 of 27) at 48 weeks, whereas male C3H/He mice did not develop fatty liver (0 of 18) by 48 weeks of age. Both reciprocal F1 mice developed fatty liver. The incidence of fatty liver in C3NF1 mice was 92% (12 of 13) at 48 weeks of age, and in NC3F1 mice it was 80% (12 of 15) at 48 weeks of age. In contrast to the male mice, female NSY mice and reciprocal F1 females did not develop fatty liver.

Table 2 summarizes the possible modes of inheritance and phenotypes in which parental effects were observed, to contrast the characteristics that were investigated in this study.

## DISCUSSION

Two reciprocal outcrosses were performed to test the paternal-maternal effects on phenotypic characteristics in NSY mice.

**Table 1. Phenotypic Characterization of Male NSY, C3H/He, C3NF1, and NC3F1 Mice at 48 Weeks of Age**

| Parameter | C3H/He (n = 12) | NSY (n = 15) | C3NF1 (n = 12) | NC3F1 (n = 15) |
|---|---|---|---|---|
| Body weight (g) | 37.2 ± 0.5 | 49.0 ± 0.7* | 54.1 ± 0.6*§ | 45.2 ± 0.5*‡¶ |
| Blood glucose (mmol/L) | 6.1 ± 0.9 | 8.7 ± 1.1* | 10.6 ± 2.8*† | 8.0 ± 0.2*‖ |
| Plasma insulin (pmol/L) | 845 ± 78 | 2,532 ± 312* | 3,775 ± 298*† | 3,201 ± 293* |
| Insulin content (ng/mg) | 47.0 ± 6.0 | 59.7 ± 3.5 | ND | 57.2 ± 4.7 |

NOTE. Values are the mean ± SEM.
Abbreviation: ND, not determined.
*$P < .0001$ *v* C3H/He mice.
†$P < .05$, ‡$P < .001$, §$P < .0001$ *v* NSY mice.
‖$P < .05$, ¶$P < .0001$ *v* C3NF1 mice.

Both male F1 mice spontaneously developed diabetes mellitus in an age-dependent manner. The cumulative incidence of diabetes was 100% (25 of 25) in male C3NF1 mice and 98% (29 of 30) in male NC3F1 mice at 48 weeks of age. A sex difference in the incidence of diabetes was observed in F1 mice, as in the case of the parental NSY strain.[11] The cumulative incidence of diabetes did not differ between C3NF1 and NC3F1 mice. These data indicate that the susceptibility to diabetes in NSY mice was transmitted to male F1 hybrids in an autosomal dominant manner with high penetrance in these crosses. These data also suggest that X-linked genes, Y-linked genes, and mitochondrial genes are unlikely a major genetic component of diabetes in NSY mice. Among other phenotypic characteristics associated with diabetes mellitus, fatty liver also showed an autosomal dominant mode of inheritance. In contrast, epididymal fat accumulation and impaired insulin secretion in response to glucose showed an autosomal recessive mode of inheritance. The BMI showed a codominant mode of inheritance. The reason for the different inheritance of epididymal fat accumulation, impaired insulin secretion, and BMI is unclear, but it may be partly due to the polygenic nature of inheritance of each phenotype.

Although F1 mice in both reciprocal crosses showed similar characteristics in most phenotypes studied, a significant difference was observed in body weight, with C3NF1 mice showing a significantly greater body weight than NC3F1 mice. Nonfasting plasma glucose and plasma glucose concentrations during the IP GTT were also slightly but significantly higher in C3NF1 mice versus NC3F1 mice. These data indicate that a genetic factor transmitted from NSY fathers and/or C3H mothers conferred significantly higher body weight, nonfasting blood glucose, and blood glucose during the IP GTT. These results in NSY mice are consistent with previous genetic studies in rats and mice showing a significant parental strain/sex effect on phenotypic variables associated with the pathophysiology of diabetes.[5-9] Paternal effects associated with the severity of diabetes in reciprocal outcrosses between GK rats (derived by selective breeding for glucose intolerance from Wistar rats, as were NSY mice from outbred Jcl:ICR mice) and Fisher 344 rats were previously reported.[7] It is possible that a modifier gene (or genes) that contributes to glucose tolerance and body weight is located on the Y chromosome of NSY mice.

The cumulative incidence of diabetes was higher in males versus females in both NSY and F1 mice. The glucose AUC showed a codominant mode of inheritance in female F1 mice. Fatty liver was observed only in males. Sexual dimorphism in the expression of diabetic phenotypes has been well studied by Leiter et al.[19,20] The balance of estrogenic and androgenic hormones influences hepatic glucose output. Thus, an alteration of the effective concentration of estrogen or testosterone can interact with diabetes susceptibility genes to exacerbate or counteract a diabetic phenotype.

Whereas the insulin response to glucose was not impaired in F1 mice, both reciprocal F1 mice developed diabetes mellitus, as in the case of NSY mice, in which both insulin secretion and insulin action were impaired. Nonfasting plasma insulin was

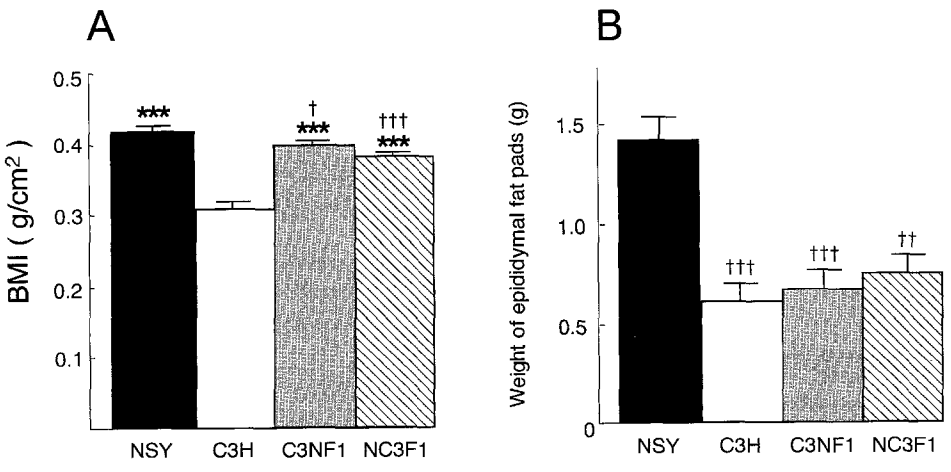


**Fig 3. (A) BMI of male NSY (n = 15), C3H/He (n = 12), C3NF1 (n = 5), and NC3F1 (n = 15) mice. (B) Weight of epididymal fat pads (g) of male NSY (n = 15), C3H/He (n = 12), C3NF1 (n = 5), and NC3F1 (n = 15) mice at 48 weeks of age. Values are means ± SEM. ***$P < .0001$ *v* C3H/He mice, †$P < .05$, ††$P < .001$, †††$P < .0001$ *v* NSY mice.**

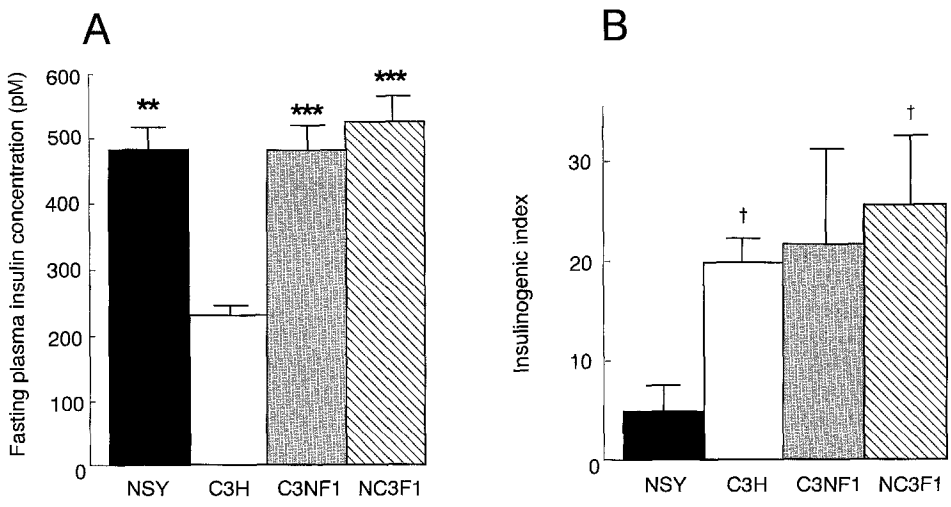


**Fig 4. (A) Fasting plasma insulin (picomolars) in male NSY (n = 15), C3H/He (n = 12), C3NF1 (n = 5), and NC3F1 (n = 15) mice. (B) Insulinogenic index (picomolars/millimolars) in male NSY (n = 15), C3H/He (n = 12), C3NF1 (n = 5), and NC3F1 (n = 15) mice at 48 weeks of age. The insulinogenic index was calculated as the increment of plasma insulin above the basal level divided by the increment of blood glucose above the basal level. Values are means ± SEM. **$P < .001$, ***$P < .0001$ *v* C3H/He mice, †$P < .05$ *v* NSY mice.**

significantly higher in C3NF1 mice versus NSY mice, and it tended to be higher in NC3F1 mice versus NSY mice. These data suggest that insulin resistance is the predominant defect in male F1 mice, and that the pathogenesis of diabetes is different between NSY and F1 mice. Indeed, the insulin tolerance test showed that insulin action was more markedly impaired in F1 mice than in NSY mice. The reason(s) that heterozygotes developed more severe insulin resistance than the parental strains is not clear, but an acceleration of the expression of phenotypes in F1 mice compared with the parental strains and genetic synergism were previously described.[9,21-24] The present study identified an example in which the offspring of two inbred mouse strains displayed a more severe phenotype than either parental strain. In addition, this study also shows the importance of gene-gene or allele-allele interactions for the expression of a polygenic phenotype. It is well known that insulin resistance plays an important role in the development of type 2 diabetes in humans and has a strong genetic background.[25,26] Male F1 mice in crosses of NSY with C3H/He mice would be a useful animal model to investigate the pathogenesis of and genetic predisposition for the development of insulin resistance.

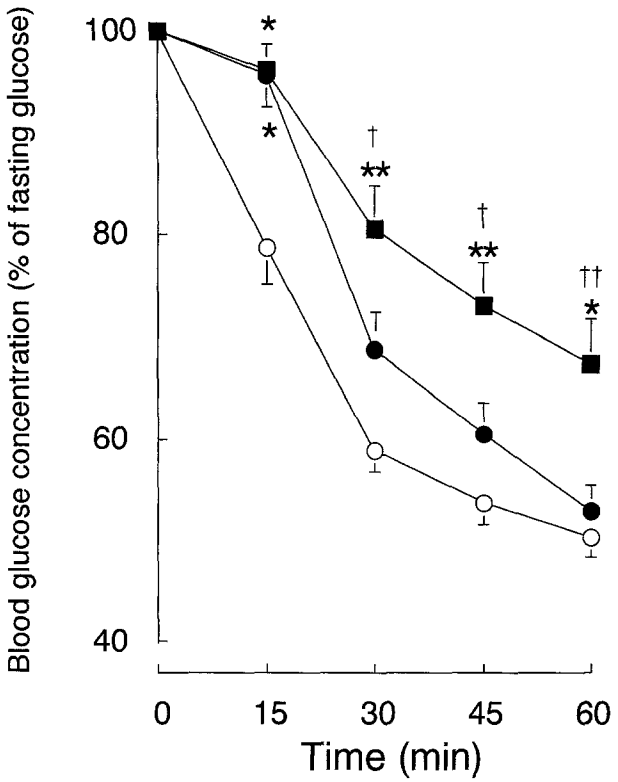


**Fig 5. Insulin tolerance test (0.75 IU/kg body weight) in male overnight-fasted C3H/He (○, n = 15), NSY (●, n = 15), and NC3F1 (■, n = 15) mice at 48 weeks of age. Results are expressed as the percent change from fasting blood glucose. Values are means ± SEM. *$P < .01$, **$P < .001$ *v* C3H/He mice, †$P < .05$, ††$P < .01$ *v* NSY mice.**

Although the precise mode of inheritance in human type 2 diabetes is unknown, recent studies suggest that most of the genetic variance of diabetes might be due to dominant genetic effects.[27-29] Our present study indicates that the major genetic components of diabetes in NSY mice seem dominant. These genetic features of diabetes in NSY mice are similar to those of human type 2 diabetes. Human type 2 diabetes is multifactorial

**Table 2. Summary of Possible Modes of Inheritance and Phenotypes in Which Parental Effects Were Observed in Male F1 Mice**

| Phenotype | Possible Mode of Inheritance | | | Parental Effect |
|---|---|---|---|---|
| | AD | AR | CD | |
| Fasting plasma glucose | X | | | No |
| Nonfasting plasma glucose | X | | | Yes |
| Glucose concentration after glucose load | X | | | Yes |
| Fasting plasma insulin | X | | | No |
| Nonfasting plasma insulin | X | | | Yes |
| Insulin secretion in response to glucose | | X | | No |
| Body mass index | | | X | No |
| Epididymal fat accumulation | | X | | No |
| Fatty liver | X | | | No |

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CD, codominant.

and is expected to be much more complex than that in inbred animal models. The NSY mouse is expected to be a useful animal model of type 2 diabetes to understand the pathogenesis and genetic complexity of type 2 diabetes. Furthermore, since insulin resistance is the predominant defect in F1 mice, F1 mice will be useful for studies of genetic synergism in complex traits like the insulin resistance syndrome.[25,26]

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