Characterization of Hyperinsulinemic Recombinant Inbred (RI) Strains (SMXA-5 and SMXA-9) Derived from Normoinsulinemic SM/J and A/J Mice

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Abstract: We discovered two mouse strains (SMXA-5 and SMXA-9) with hyperinsulinemia among the substrains and progenitor strains (SM/J and A/J) of the SMXA recombinant inbred (RI) strains, and characterized the two strains at 20 weeks of age. SMXA-5 (mean ± S.E.M: 9.6 ± 1.7 ng/ml) and SMXA-9 (7.7 ± 1.3 ng/ml) males had higher serum immunoreactive insulin levels than SM/J (1.4 ± 0.3 ng/ml) and A/J (1.1 ± 0.1 ng/ml) males in the nonfasting condition. The hypoglycemic response to insulin at 30 min after injection was significantly less in SMXA-5 males than in SM/J mice. Glucose tolerance test revealed that the incidence of impaired glucose tolerant males was 58% (11/19) in SMXA-5 and 42% (10/24) in SMXA-9 strains, but none in SM/J and A/J strains. SMXA-5 (209 ± 29 mg/dl) and SMXA-9 (235 ± 31 mg/dl) had higher serum triglyceride levels than SM/J (126 ± 14 mg/dl) and A/J (89 ± 5 mg/dl) males in the nonfasting condition. Histologic examination revealed enlarged islets in the pancreas of hyperinsulinemic SMXA-5 male mice. Moreover, SMXA-5 and SMXA-9 mice exhibited mild obesity. SMXA-5 and SMXA-9 males were therefore characterized by hyperinsulinemia, impaired glucose tolerance, hypertriglyceridemia and mild obesity which resembled some of the phenotypes of human Syndrome X, although both progenitor strains were normal so far as we examined. Since the RI strains are a powerful tool to facilitate polygenic-trait analysis, SMXA-5 and SMXA-9 mice will be useful materials to investigate the genetic basis of complex diseases, and are possible new metabolic models in relation to hyperinsulinemia.

Key words: hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance, SMXA recombinant inbred (RI) strains, Syndrome X
Introduction

Insulin resistance is regarded today as the causative factor in a number of serious civilization diseases: obesity, Type-2 diabetes, hypertension, hyperlipidemia and atherosclerosis. This cluster of metabolic abnormalities is referred to as Syndrome X and it is suggested that they are brought about by the interaction of multiple genetic and environmental factors [2, 3, 18]. Many animal models with insulin resistance have already been developed [4], but it is necessary to survey and develop other animal models in order to enrich the availability of insulin resistant animal models for analyzing this syndrome in humans. Polygenic animal models have proven their usefulness in the investigation of complex diseases such as Syndrome X. Furthermore, recent advances in genetic analysis have made it possible to identify new causative genes in these polygenic models [5–7].

Recombinant inbred (RI) strains are a powerful tool for analyzing multifactorial genetic traits [17]. The SMXA RI set consisting of 26 substrains was established from SM/J and A/J progenitor mice [16]. Detailed strain distribution patterns (SDP) of more than 400 polymorphic genetic markers in this RI set were also recently reported [15]. In order to analyze the genetic factors in hyperinsulinemia, we examined nonfasting serum immunoreactive insulin (IRI) levels in 21 SMXA RI strains as well as progenitor strains (SM/J and A/J). In the course of this study, we discovered that SMXA-5 and SMXA-9 male mice exhibited increased insulin levels in comparison with the other substrains and parental strains.

In this paper, we characterized SMXA-5 and SMXA-9 mice and provided fundamental data on these strains as new metabolic disease models in relation to hyperinsulinemia.

Materials and Methods

Animals: Twenty-one SMXA RI strains and parental (SM/J and A/J) strains were obtained from the Institute of Experimental Animals, Hamamatsu University School of Medicine, Hamamatsu, Japan. SMXA-3, SMXA-6, SMXA-19, SMXA-22 and SMXA-23 were excluded from this study due to an insufficient number of available samples. The serum sample collection of the SMXA RI strains was performed in the same facility. After the identification of strains with increased serum IRI levels, SMXA-5, SMXA-9, SM/J and A/J mice (3–5 weeks of age) were transported to Nagoya University where further characterizations were done. All mice were housed in a conventionally conditioned animal room: 23–27°C, controlled humidity (55 ± 5%) and 14L10D light. The mice were fed a commercial diet (MR Breeder, Nihon Nosan Kogyo K.K., Yokohama, Japan) from weaning until the time of sacrifice.

Sample collection: All blood sample collections for measurement of insulin and lipid levels were done in mice under unanesthetized conditions.

Serum immunoreactive insulin assay: Blood samples were obtained from the orbital sinus in nonfasted mice at 10 and 20 weeks of age. Serum samples were collected after centrifugation and stored at ~80°C until assay. Serum IRI levels were measured by radioimmunoassay (ShionoRIA, Shionogi, Osaka, Japan) with the rat insulin standard. Dilution of mouse serum resulted in a curve parallel to that of rat insulin.

Insulin tolerance test: For insulin tolerance test (ITT), human insulin (Humulin R U-40®, Shionogi, Osaka, Japan) was injected (0.5 IU/kg BW) intraperitoneally in 12–14 hr fasted mice at 20 weeks of age. Blood samples were collected from the tail vein and blood glucose levels were measured by the glucose oxidase method with a Glucose-B Test kit (Wako, Osaka, Japan). Serum glucose levels were measured prior to the injection (0), and 15 and 30 min afterwards.

Intraperitoneal glucose tolerance test: For intraperitoneal glucose tolerance test (IPGTT), a 20% glucose solution was administered intraperitoneally (2 g/kg BW) in 12–14 hr fasted mice at 10, 15 and 20 weeks of age. Blood samples were collected from the tail vein. Blood glucose levels were measured prior to the injection (0), and 30 and 120 min afterwards by the glucose oxidase method with a Glutest E analyzer (Kyoto Daiichi Chemicals Co. Ltd., Kyoto, Japan). Impaired glucose tolerance classification was based on the guidelines provided by the WHO Study Group on Diabetes Mellitus [21]. Impaired glucose tolerant mice are defined as those which had glucose levels in excess of 160 mg/dl at 120 min after glucose loading in IPGTT.

Serum lipid assay: Serum samples were separated from whole blood obtained from the orbital sinus in nonfasted 20 week old mice. Serum triglyceride (TG) and total cholesterol (TC) concentrations were mea-
sured enzymatically with an Autoanalyzer (Cobas Mira Plus, Nippon Roche, Japan).

**Body mass index:** Body weight and anal-nasal length were measured in 10 and 20 week old mice in the anesthetized condition. The body mass index (BMI) was calculated as body weight (g) divided by the square of the anal-nasal length (cm).

**Histological examination:** The pancreas was dissected and collected from 20-week old mice. Tissue samples were fixed in 10% neutral buffered formalin. Paraffin sections of the pancreas were stained with haematoxylin-eosin by the standard method.

**Statistical analysis:** Values were expressed as the mean ± S.E.M. Statistical analysis was performed by ANOVA with a Statview 4.5 program for the Macintosh Computer (Abacus Concepts Inc., Berkeley, CA). Means of differences between groups were compared by one-way ANOVA. Statistically significant differences between means were evaluated by Scheffe’s test, which was applied when the one-way ANOVA indicated significant differences (P<0.05).

### Results

We examined serum IRI levels in males of the 21 SMXA RI strains with their parental strains (SM/J and A/J) at 8–24 weeks of age in nonfasting conditions (Fig. 1). All strains were arranged in increasing order of serum IRI levels. SMXA-5 and SMXA-9 males had higher serum IRI levels than the other substrains and parental strains. We selected these two strains (SMXA-5 and SMXA-9) and compared them with their parental strains by using different metabolic parameters.

At 10 weeks of age, there were no significant differences in the serum IRI levels among SM/J (1.45 ± 0.30 ng/ml), A/J (0.90 ± 0.10), SMXA-5 (1.66 ± 0.25) and SMXA-9 (3.70 ± 1.02) males. At 20 weeks of age, SMXA-5 and SMXA-9 males had relatively higher serum IRI levels, but only SMXA-5 males were significantly different from SM/J and A/J males (Table 1). SMXA-9 males were not significantly different from SM/J (p=0.0927) and A/J (p=0.0707) males. In females of the four strains, there were no significant

**Fig. 1.** Distribution of serum immunoreactive insulin (IRI) levels in males of the 21 SMXA RI and the two parental strains (SM/J and A/J) at 8–24 weeks of age in the nonfasting condition. Each vertical bar represents the mean ± S.E.M. of 10–20 animals/strain.
differences in serum IRI levels at 10 weeks (SM/J, 0.61 ± 0.26 ng/ml; A/J, 0.59 ± 0.07; SMXA-5, 0.92 ± 0.16; SMXA-9, 1.25 ± 0.20) and 20 weeks of age (SM/J, 1.10 ± 0.17 ng/ml; A/J, 0.58 ± 0.07; SMXA-5, 1.81 ± 0.94; SMXA-9, 1.35 ± 0.20).

Serum TG levels in SMXA-5 and SMXA-9 males were significantly higher than in SM/J and A/J males at 20 weeks (Table 1). SMXA-5 females (205 ± 9 mg/dl) also showed a similar tendency toward hypertriglyceridemia compared with SMXA-9 (110 ± 7), SM/J (109 ± 9) and A/J (105 ± 7) females. Blood glucose and serum TC levels in males of the four strains were not significantly different (Table 1). Both parameters in females also had a similar tendency to the males (data not shown).

The BMI of males of SMXA-5 (0.289 ± 0.014 g/cm²), SMXA-9 (0.296 ± 0.004) and A/J (0.272 ± 0.007) at 10 weeks of age was significantly greater than that of SM/J (0.248 ± 0.006 g/cm²) males. Likewise, SMXA-5 (0.251 ± 0.012 g/cm²), SMXA-9 (0.248 ± 0.008) and A/J (0.243 ± 0.006) females had a significantly greater BMI than SM/J (0.173 ± 0.002 g/cm²) females. At 20 weeks of age, SMXA-5 males had a significantly greater BMI than SMXA-9, SM/J and A/J males. SMXA-9 males also had a significantly greater BMI than SM/J and A/J males (Table 1). Among females, SMXA-5 (0.326 ± 0.028 g/cm²) also had a significantly greater BMI than SM/J (0.238 ± 0.003) but SMXA-9 (0.255 ± 0.005) was not significantly different from SMXA-5, SM/J and A/J (0.259 ± 0.009).

Blood glucose levels during IPGTT in males of the four strains are shown in Fig. 2. SMXA-5 (107 ± 6 mg/dl) males had significantly higher fasting blood glucose levels than SMXA-9 (78 ± 3) and SM/J (61 ± 3). There was no significant difference between SMXA-5 and A/J (90 ± 7 mg/dl) males. Blood glucose levels reached peak values at 30 min after glucose loading in the four strains, and returned to near fasting values after 120 min in SM/J (110 ± 7 mg/dl) and A/J males (132 ± 15) but blood glucose levels failed to return to fasting values in SMXA-5 (181 ± 16 mg/dl) and SMXA-9 (174 ± 9) males. In females, there were no significant differences between blood glucose levels at fasting (SM/J, 70 ± 8 mg/dl; A/J, 94 ± 9; SMXA-5, 97 ± 10; SMXA-9, 52 ± 1) and at 120 min after glucose loading (SM/J, 93 ± 2 mg/dl; A/J, 89 ± 6; SMXA-5, 114 ± 20; SMXA-9, 74 ± 6).

The incidence of impaired glucose tolerant males is shown in Table 2. During the course of the experiment, the incidence of impaired glucose tolerant males in SMXA-5 was higher than in SMXA-9, and tended to increase gradually until 20 weeks of age (58% in SMXA-5 and 42% in SMXA-9). Eleven out of fourteen SMXA-5 and ten of fourteen SMXA-9 males, all of which had their serum IRI levels measured, showed signs of impaired glucose tolerance and were hyperinsulinemic. Impaired glucose tolerant mice were not observed in SM/J and A/J mice or in the females of SMXA-5 and SMXA-9.

The hypoglycemic response to exogenous insulin in SMXA-5 and SMXA-9 males was compared with that of SM/J and A/J males (Fig. 3). At 30 min after the insulin injection, the rate of decrease in blood glucose levels in SMXA-5 males (31% of the initial level) was

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**Table 1.** Comparison of metabolic features of male mice from four strains at 20 weeks of age in the nonfasting condition

<table>
<thead>
<tr>
<th>Traits</th>
<th>SM/J</th>
<th>A/J</th>
<th>SMXA-5</th>
<th>SMXA-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (g/cm²)</td>
<td>0.267 ± 0.001a (12)</td>
<td>0.297 ± 0.006a (12)</td>
<td>0.371 ± 0.010a (14)</td>
<td>0.339 ± 0.007b (19)</td>
</tr>
<tr>
<td>Blood glucose level (mg/dl)</td>
<td>132 ± 9 (9)</td>
<td>135 ± 5 (9)</td>
<td>137 ± 7 (14)</td>
<td>140 ± 5 (14)</td>
</tr>
<tr>
<td>Serum IRI level (ng/ml)</td>
<td>1.4 ± 0.3a (9)</td>
<td>1.1 ± 0.1a (9)</td>
<td>9.6 ± 1.7b (14)</td>
<td>7.7 ± 1.3b (14)</td>
</tr>
<tr>
<td>Serum TG level (mg/dl)</td>
<td>126 ± 14a (8)</td>
<td>89 ± 5a (8)</td>
<td>209 ± 29b (8)</td>
<td>235 ± 31b (8)</td>
</tr>
<tr>
<td>Serum TC level (mg/dl)</td>
<td>77 ± 4 (8)</td>
<td>80 ± 3 (8)</td>
<td>82 ± 7 (8)</td>
<td>78 ± 10 (8)</td>
</tr>
</tbody>
</table>

Values are the mean ± S.E.M. Number of mice is indicated in parenthesis. a,b,c Means in one row without common superscript letter are significantly different at P<0.05 (ANOVA, Scheffe’s Test).
Fig. 2. Blood glucose levels during intraperitoneal glucose tolerance test (IPGTT) in male mice of four strains (SM/J, A/J, SMXA-5 and SMXA-9) at 20 weeks of age. Values are shown as the mean ± S.E.M. *: P<0.05 (ANOVA, Scheffe test), SMXA-9 vs SM/J. **: P<0.01 SMXA-5 vs SM/J. There was significant difference observed between parental strains SM/J and A/J (P<0.01) and between SMXA-5 and SMXA-9 (P<0.01) at 60 min. Significant difference between SMXA-5 and SMXA-9 was observed at 30 min (P<0.05). Number of mice: SM/J, 10; A/J, 11; SMXA-5, 19; SMXA-9, 24.

Fig. 3. Blood glucose changes during insulin tolerance test (ITT) in male mice of four strains (SM/J, A/J, SMXA-5 and SMXA-9) at 20 weeks of age. Mice were intraperitoneally administered human insulin (0.5 IU/kg body weight) after 12-hr fasting. Results are expressed as the percentage change from the fasting glucose level. Values are shown as the mean ± S.E.M. **: P<0.01 (ANOVA, Scheffe test), SMXA-5 vs SM/J. There was significant difference observed between SMXA-5 and SMXA-9 at 30 min (P<0.05). Number of mice: SM/J, 8; A/J, 9; SMXA-5, 12; SMXA-9, 12.

Table 2. Incidence of impaired glucose tolerant male mice from four strains at 10–20 weeks of age

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of Impaired glucose tolerant mice&lt;sup&gt;a)&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>10 wks</td>
</tr>
<tr>
<td>SM/J</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>A/J</td>
<td>0 (0/12)</td>
</tr>
<tr>
<td>SMXA-5</td>
<td>38 (5/13)</td>
</tr>
<tr>
<td>SMXA-9</td>
<td>21 (4/19)</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Impaired glucose tolerant mice are those which had blood glucose levels higher than 160 mg/dl after an intraperitoneal dose of glucose (2.0 g/kg BW).

significantly lower than in SM/J males (59% of the initial level) but there were no significant differences observed in the decrease in blood glucose levels among SMXA-9, A/J and SM/J males. There were no significant differences in the rate of decrease in blood glucose levels among the four strains (SM/J, 33%; A/J, 43%; SMXA-5, 58%; SMXA-9, 43% of the initial level) at 60 min after insulin injection.

We were not able to find any noticeable histologic changes in the pancreatic islets of SM/J, A/J and SMXA-9 males at 20 weeks of age so far as we examined. Enlarged islets were observed in the pancreas of hyperinsulinemic SMXA-5 males (Fig. 4).
**Discussion**

We measured serum IRI levels in the 21 SMXA RI strains and the progenitor strains in nonfasting conditions (Fig. 1). We identified two strains (SMXA-5 and SMXA-9) to show increased insulin levels and further examined the metabolic features of these strains and the parental strains SM/J and A/J. SMXA-5 and SMXA-9 males showed signs of hyperinsulinemia in the nonfasting condition (Table 1). Hyperinsulinemia in the two strains was more clearly recognized at 20 weeks of age than at 10 weeks of it, but the changes in the serum IRI levels in the mice as they aged further remain to be investigated. The existence of insulin...
resistance was evidenced in SMXA-5 males by the significantly suppressed hypoglycemic response to insulin at 30 min after injection in comparison with that in SM/J and SMXA-9 males (Fig. 3). SMXA-5 males also had impaired glucose tolerance and the incidence of impaired glucose tolerant mice was 58% in SMXA-5 and 42% in SMXA-9 males at 20 weeks of age (Table 2). Most of the impaired glucose tolerant males in both strains had corresponding increased serum IRI levels. Hypertriglyceridemia and mild obesity were also observed in the males of SMXA-5 and SMXA-9 (Table 1). Histological examination of the pancreas revealed the presence of enlarged islets in hyperinsulinemic SMXA-5 males (Fig. 4). The existence of hyperinsulinemia and insulin resistance correlated with the observation of enlarged pancreatic islets in SMXA-5 males. SMXA-5 and SMXA-9 males had various degrees of severity in the traits examined. SMXA-9 males had milder metabolic changes compared to SMXA-5 males (Table 1; Figs. 2 and 3).

Most monogenic and polygenic rodent models of obesity and NIDDM exhibit a Syndrome X spectrum of altered metabolic parameters commonly associated with human obesity. The fundamental phenotype(s) for these models include obesity of various degrees and onset, hyperinsulinemia, some demonstration of hyperglycemia (either transient or sustained), and more variable hyperlipidemia [4]. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is a representative example of polygenic models with hyperinsulinemia in rodents. OLETF rats had three to fourfold higher fasting insulin concentrations than control Long-Evans Tokushima Otsuka (LETO) rats [8]. The OLETF rats also had sustained hyperglycemia, glycosuria and mild obesity [9]. SMXA-5 and SMXA-9 males had hyperinsulinemia, with serum IRI levels six to sevenfold higher than the parental strains SM/J and A/J males in the nonfasting condition (Table 1), but SMXA-5 and SMXA-9 males had normoglycemia and mild obesity (Table 1). Hypertriglyceridemia in SMXA-5 and SMXA-9 males reached 1.5-fold the levels of the parental strains (Table 1) and is milder than in OLETF rats which had a fivefold rise in TG levels compared to the control strains [10]. Fat droplet deposition in the islets associated with the degree of hypertriglyceridemia in OLETF rats has been reported [13] but SMXA-5 and SMXA-9 males did not have any such changes in the islets (Fig. 4). In addition, OLETF rats had increased systolic blood pressure compared to control LETO rats [20]. Metabolic changes in the cardiovascular system of SMXA-5 and SMXA-9 mice remain to be investigated. These results indicated that SMXA-5 and SMXA-9 males exhibited milder symptoms than OLETF rats. The results also suggested that SMXA-5 and SMXA-9 mice could be new models for impaired glucose tolerance with hyperinsulinemia, hypertriglyceridemia and mild obesity in the absence of glycosuria and hyperglycemia. These findings indicate that SMXA-5 and SMXA-9 possess some of the phenotypes resembling Syndrome X.

Hyperinsulinemic and impaired glucose tolerant mice occurred among males of SMXA-5 and SMXA-9 but not in females. Greater sensitivity to insulin of female mice has generally been recognized in various genetic obese models [see 11]. Genes controlling sex-steroid balance such as estrogen sulfotransferase in insulin-sensitive tissue may have a significant impact on glucose homeostasis [12]. The gender-related differences in glucose metabolism of SMXA-5 and SMXA-9 mice may also be related to sex-steroid balance.

Among 21 SMXA RI strains, we characterized two strains (SMXA-5 and SMXA-9) as having mild obesity, hyperinsulinemia, hypertriglyceridemia and impaired glucose tolerance. It is very interesting to note that the parental strains (SM/J and A/J) of SMXA-5 and SMXA-9 have never had these symptoms. The occurrence of these phenotypic traits in SMXA-5 and SMXA-9 strains may reflect an effect of novel combinations of genes due to random assortment of parental alleles [14]. It is also possible to consider that accumulation of complementary alleles at multiple loci inherited from the two parents may have led to the appearance of some strains characterized by phenotypes that are unlike the progenitor phenotypes [19]. Despite a common genetic origin, the recombination of the parental alleles may have led to the emergence of distinct strains such as SMXA-5 and SMXA-9. As the detailed genetic profile of the SMXA RI set has been recently reported [15], the results of this study will be of primary advantage in the analysis of the complex basis of hyperinsulinemia in these mice. The identification of genetic loci for cellular defects in glucose and fatty acid metabolism was recently reported in the HXB rat strains derived from SHR/Ola and BN. Lx, which manifest many of the defining features of Syndrome X [1]. Thus RI strains have been of
value in investigating the genetic basis of Syndrome X. It is expected that the SMXA RI strains will contribute to the analysis of genetic factors in Syndrome X.

In conclusion, our results provided the fundamental characteristics of the new hyperinsulinemic strains (SMXA-5 and SMXA-9) discovered among the SMXA RI strains. The interesting traits exhibited by the two strains can be subjected to genetic analysis in future studies. With the well-established genetic profile of the SMXA RI set, SMXA-5 and SMXA-9 mice will be useful animal models for the genetic analysis of such complex phenotypic traits.

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