Genetic susceptibility to diabetes in inbred strains of mice: measurements of proinsulin mRNA and response to dexamethasone

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Summary. The insulin resistance produced by the recessive db mutation has led to more severe diabetes in C57BL/KsJ mice relative to that in C57BL/6J mice, suggesting genetic differences between the two strains affecting insulin production or insulin action. To assess these parameters blood glucose, serum insulin, pancreatic insulin, and proinsulin mRNA were measured in both normal and diabetic (db/db) KsJ and 6J strains. The mice were compared at 5 weeks of age, prior to the development of insulin lack known to occur with age in KsJ db/db mice. As a further provocation to insulin production, another group of the normal and db/db mice were given dexamethasone for 4 days. In normal mice there were no strain differences in blood glucose, serum insulin, pancreatic insulin, or proinsulin mRNA. Dexamethasone, presumably by augmenting insulin resistance, induced increases in serum insulin and proinsulin mRNA to the same extent in KsJ and 6J mice. In db/db mice, while blood glucose, serum insulin, and proinsulin mRNA were considerably higher than in normal mice, there were no strain differences observed. After dexamethasone the db/db mice exhib-

ited strain differences which included higher blood glucose and higher serum insulin levels in KsJ mice. These findings were compatible with greater insulin resistance in KsJ than in 6J db/db mice. While dexamethasone treatment increased serum insulin in KsJ db/db mice, there was no augmentation of proinsulin mRNA in either strain, suggesting a limit to the insulin synthesis. Analysis of serum insulin/glucose and proinsulin mRNA/glucose ratios demonstrated a dexamethasone-induced increase in serum insulin/glucose in normal and diabetic mice of both strains. An increase in dexamethasone induced proinsulin mRNA/glucose ratio was observed in all but the KsJ db/db mice. This analysis suggested that although insulin secretion in KsJ db/db mice was augmented, the capacity for insulin synthesis had been exceeded. A limitation of insulin production at the level of insulin synthesis might explain the enhanced diabetes susceptibility of this strain.

Key words: Genetic susceptibility to diabetes, db mutation, dexamethasone, inbred mouse strains, proinsulin mRNA.

In man, the occurrence of Type 2 (non-insulin-dependent) diabetes mellitus has been related to strong genetic influences [1]. In mice, the autosomal recessive "diabetes" (db) mutation [2–4] results in metabolic changes similar to those observed in Type 2 diabetes in man. The relative diabetes susceptibility observed among certain inbred strains carrying either the db mutation on chromosome 4, or the "obesity" (ob) mutation on chromosome 6, provides evidence of genetic differences [4, 5]. While the nature of this genetic influence is unknown, both db/db and ob/ob mice exhibit profound resistance to insulin [6–8].

Augmented diabetes susceptibility has led to studies of immune response genes [9] and of retroviral genes expressed in the pancreatic islets of KsJ mice [10, 11], but a convincing relationship of these factors to the development of diabetes has not been demon-

strated. The more profound expression of diabetes in male than in female KsJ mice has suggested sex-related genetic factors [11, 12], but diabetes susceptibility related to sex does not account for the strain differences. It is likely that the action of genes conferring relative diabetes susceptibility in KsJ mice might be manifested by: 1) resistance to insulin action [6–8], or 2) a limitation of ability to augment insulin production [13–17].

Diabetes susceptible male db/db C57BL/KsJ (KsJ) mice were previously observed [18] with longitudinal measurements of fasting and fed blood glucose, serum insulin, pancreatic insulin, and pancreatic proinsulin messenger RNA (mRNA). At 5 weeks of age, the occurrence of hyperglycaemia in db/db mice was associated with a 4-fold increase in serum insulin and in pancreatic proinsulin mRNA above that in control

mice, apparently quantifying the ability of the KsJ mice to respond to the insulin resistance imposed by the db/db genotype. Persistent hyperglycaemia, however, suggested that their augmented insulin synthesis was inadequate to meet metabolic demands. At 10 and 13 weeks of age a marked loss of insulin production, manifested by parallel decreases in serum insulin and proinsulin mRNA and a modest decrement in pancreatic insulin content, was seen in conjunction with more severe hyperglycaemia; these losses were less marked and hyperglycaemia was less severe when caloric intake was restricted, suggesting a potential to preserve but not to further augment insulin production.

In the current studies, insulin biosynthetic capacity has been assessed by quantifying proinsulin mRNA levels before and after administration of dexamethasone, which has been shown to augment insulin synthesis in previous *in vivo* studies [19]. The validity of using proinsulin mRNA in this assessment followed correlations established between proinsulin mRNA levels and the incorporation of ^3H -leucine into extractable proinsulin in mouse islets *in vitro* [20] and in rat islets both *in vitro* [20] and *in vivo* [19, 22], suggesting comparable ability of these methods to assess insulin synthesis. The ability of diabetes-susceptible KsJ mice and of diabetes-resistant C57BL/6J (6J) mice to adapt to the db mutation, to dexamethasone administration, and to the combination of these perturbations, has been assessed by measuring serum insulin, pancreatic insulin content, and proinsulin mRNA levels. Diabetic (db/db) mice of both strains did not increase proinsulin mRNA in response to dexamethasone. A striking strain difference was found in a disparate response of serum insulin and of proinsulin mRNA in the db/db mice provoked by dexamethasone treatment. This finding suggests a primary defect in KsJ mice limiting their ability to synthesize insulin.

Materials and methods

C57BL/6J and C57BL/KsJ mice were obtained from the Jackson Laboratories (Bar Harbor, Me, USA) after weaning at roughly 28 days of age. These mice were maintained briefly on an Emery Moss 911a diet during shipment to St. Louis, Missouri, then were fed *ad libitum* with Purina Rodent Chow (Ralston Purina, Inc., St. Louis, Mo, USA). Mice were shipped in groups based on genetic background (6J or KsJ) and presence of diabetes (db) genes. Among the 6J mice normal (+/+) and diabetic (db/db) mice were the products of separate breeding stocks which had been previously identified. Among the KsJ mice identification of the mice and segregation into normal (+/+), and homozygous diabetic (db/db) groups were accomplished at the Jackson Laboratory prior to shipment by methods previously reported [2–4].

Animals were caged in groups of five with two groups established randomly for each combination of strain and db gene status. Beginning at 5 weeks of age, each group was assigned to receive either isotonic saline or isotonic saline containing dexamethasone, $0.125 \text{ mg} \cdot \text{kg body weight}^{-1} \cdot \text{day}^{-1}$, for 4 days. This supraphysiologic dosage of dexamethasone had been previously defined for stimulation of insulin synthesis in rats [19]. For 3 days prior to initiation of

the injections, then for 4 days during administration of either the saline alone or the dexamethasone solution, the food intake of each group was measured each day by subtracting the weight of a food remnant obtained each morning from the aliquot given the previous day. A small decrement in food intake was noted on the first day following the peritoneal injection of $0.025 \text{ ml/g body weight}$ (using a 27-gauge needle); subsequently food intake returned to levels noted prior to starting the injections in both the saline and dexamethasone-treated groups. Body weight of each mouse was recorded on the morning of the first day of injection, and again at the time it was killed 4 days later.

Blood glucose was measured in each mouse prior to killing by clipping 0.5 cm from the end of the tail and milking a drop of blood to a Dextrostix glucoseoxidase reagent strip, then developing the strip after 1 min and measuring the result with an Ames Glucometer II (Miles Laboratories, Elkhart, Ind, USA). Care was taken to maintain the animals in an unstressed state during blood letting.

Killing was accomplished in the fed state in all cases by administering sodium pentobarbital, $10 \text{ mg/kg body weight}$, by peritoneal injection. Dissection, cardiac puncture, and removal of the pancreas were accomplished prior to cardiorespiratory arrest, then dissection and division of the pancreas were accomplished as previously described [23].

Serum insulin was measured from serum samples frozen shortly after killing. A double antibody radioimmunoassay with a rat insulin standard was used; all samples were assayed concurrently.

Pancreatic insulin was extracted using acid alcohol as previously described [23] from aliquots from pancreas frozen in liquid nitrogen and then stored at -80°C . All assays were performed concurrently.

Pancreatic RNA was extracted from aliquots of pancreas immediately after dissection by homogenisation in a guanidine thiocyanate solution. RNA was purified from the homogenate by repeated precipitation from guanidine thiocyanate, guanidine hydrochloride, and water by the method of Chirgwin et al. [24] as previously described [19, 23]. Assessment of purity and degradation was accomplished by measurement of absorbance at 260 and 280 nm and by measurement of 28S/18S ribosomal RNA ratios conforming to previous standards [19, 23].

Proinsulin mRNA was measured employing filter hybridisation of duplicate $5 \mu\text{g}$ aliquots of total pancreatic RNA from each mouse, and of internal pooled RNA standards to assess the linear response of the hybridisation and autoradiography, as previously described [18, 23]. Filters were prepared separately for normal (+/+) mice and diabetic (db/db) mice, so that strain-related differences could be assessed on the same filter. Hybridisation to the radiolabelled pCRI 354 cDNA probe, washing, and auto-radiography were performed concurrently, then densitometric tracings obtained from the autoradiographs were subsequently compared using the internal standards.

Statistical analysis

Standard statistical methods were employed. The food intake measurements (Tables 1, 2) are presented as the mean $\pm$ standard error of the mean (SEM) of the four daily food intake measurements for each cage of mice, divided by the five mice in each cage. All other data (Tables 1, 2, Fig. 1) are presented as the mean $\pm$ SEM for the five mice in each group. Values in the tables and figures are compared using a non-paired Student's *t*-test.

Results

The data delineating body weight, food intake, fed blood glucose and serum insulin, pancreatic weight, pancreatic insulin and RNA content, proinsulin mRNA concentration (in total RNA), and total proinsulin mRNA content are listed in Tables 1 and 2. Comments concerning the observed differences related to

Table 1. Differences in normal (+/+) mice of the C57BL/KsJ and C57BL/6J strains before and after dexamethasone administration

Strain	Body weight (g)	Food intake (g/day)	Blood glucose (mmol/l)	Serum IRI (ng/ml)	Pancreatic			Proinsulin	mRNA
					Weight (g)	IRI (mg)	RNA (mg)	Concentration (ng/mg RNA)	Content (ng)
<i>Before dexamethasone</i>									
6J	19.4±0.3	3.4±0.2	6.6±0.2	1.4±0.4	0.21±0.01	20.1±2.0	3.9±0.3	0.44±0.03	1.71±0.17
KsJ	13.4±0.7 ^a	2.4±0.4 ^b	6.5±0.5	1.2±0.1	0.17±0.01 ^b	13.8±1.8 ^b	3.0±0.3 ^b	0.53±0.04	1.62±0.23
<i>After dexamethasone</i>									
6J	19.4±0.6	3.2±0.2	6.3±0.4	2.0±0.2 ^d	0.22±0.01	15.8±1.4	3.2±0.3	0.68±0.13 ^d	2.16±0.40
KsJ	15.2±0.5 ^b	2.8±0.2	5.9±0.1	2.2±0.2 ^c	0.21±0.01	11.1±2.0 ^b	3.1±0.4	0.77±0.04 ^c	2.41±0.35 ^d

Data are presented as mean ± SEM, of four measurements of the daily food intake, and of five mice in each of the other groups. Footnotes designate differences between groups as follows: ^{a, b} difference of the KsJ from the 6J strain, ^{c, d} change following dexamethasone administration. ^{a, c} $p < 0.01$; ^{b, d} $p < 0.05$

Table 2. Differences in homozygous diabetic (db/db) mice of the C57BL/KsJ and C57BL/6J strains before and after dexamethasone administration

Strain	Body weight (g)	Food intake (g/day)	Blood glucose (mmol/l)	Serum IRI (ng/ml)	Pancreatic			Proinsulin	mRNA
					Weight (g)	IRI (mg)	RNA (mg)	Concentration (ng/mg RNA)	Content (ng)
Before dexamethasone									
6J	34.7±1.9 ^e	6.0±0.2 ^e	17.9±1.4 ^c	7.7±0.7 ^e	0.27±0.02 ^c	18.6±1.5	4.4±0.3	3.69±0.34 ^e	16.38±2.49 ^e
KsJ	29.7±1.3 ^{a, e}	3.8±0.2 ^{a, f}	17.1±1.8 ^e	7.0±1.8 ^e	0.27±0.01 ^f	15.2±2.8	5.0±0.6 ^e	2.68±0.24 ^{b, e}	13.21±1.55 ^e
After dexamethasone									
6J	36.3±2.5 ^c	5.7±0.2 ^c	11.3±2.3 ^{d, e}	7.9±0.5 ^e	0.26±0.01 ^c	15.9±2.1	4.0±0.2 ^f	4.16±0.44 ^c	16.93±2.42 ^e
KsJ	28.5±0.8 ^e	3.5±0.4 ^b	18.6±1.3 ^{b^e}	12.4±1.5 ^{b, d, e}	0.25±0.01 ^c	12.8±2.1	4.4±0.1 ^c	3.18±0.60 ^e	13.76±2.19 ^e

Data are presented as mean ± SEM of four measurements of the daily food intake, and of five mice in each group. Foot notes designate differences between groups as follows: ^{a, b} difference of the KsJ from the 6J strain; ^{c, d} change following dexamethasone administration; ^{e, f} difference from normal (+/+) mice. ^{a, c, e} $p < 0.01$; ^{b, d, f} $p < 0.05$

strain under the control condition and following dexamethasone administration among normal (+/+) and diabetic (db/db) mice are given below.

Normal (+/+) mice

Body weight and daily food intake were greater in 6J mice. There were no differences in fed blood glucose or serum insulin. Pancreatic weight, insulin content, and RNA were significantly greater in 6J mice, while neither proinsulin mRNA concentration nor proinsulin mRNA content differed. Following 4 days of dexamethasone administration, there was no change in blood glucose in either strain, but comparable increases in serum insulin, pancreatic proinsulin mRNA concentration, and proinsulin mRNA content were noted in the two strains.

Diabetic (db/db) mice

Comparison to normal (+/+) mice. In general, mice homozygous for the diabetes mutation were more obese, demonstrated a greater daily food intake, and had higher fed blood glucose concentration than the normal (+/+) mice. In addition, there was significant hyperinsulinaemia ($p < 0.01$), as well as marked increases in proinsulin mRNA concentration in db/db

mice relative to that in normal mice of both strains. Pancreatic proinsulin mRNA content was augmented almost 7-fold in db/db mice in the presence of an approximately 2-fold increase in body weight relative to that in +/+ mice. Pancreatic insulin content, however, was not different between normal and diabetic mice. A close relationship between the levels of serum insulin and proinsulin mRNA was also maintained in db/db mice as previously demonstrated [18].

Strain differences in db/db mice. Both body weight and food intake were greater in 6J db/db mice than in KsJ db/db mice. Blood glucose, serum insulin, pancreatic insulin content, and proinsulin mRNA concentration and content did not differ between the two strains.

Following 4 days of dexamethasone treatment there was a significant decline in blood glucose concentration in 6J mice (17.9 ± 1.4 mmol/l to 11.3 ± 2.3 mmol/l, $p < 0.05$), but not in KsJ mice (17.1 ± 1.8 mmol/l vs 18.6 ± 1.3 mmol/l, NS), so that in the presence of dexamethasone the blood glucose was higher in the KsJ mouse ($p < 0.05$). The difference in blood glucose between the two strains was accompanied by no change in serum insulin in 6J mice, but a marked rise in KsJ mice (7.9 ± 0.5 ng/ml vs 12.4 ± 1.5 ng/ml, $p < 0.05$). There were no differences observed in pancreatic insulin or proinsulin mRNA pro-

Fig. 1. **a** Serum insulin/fed blood glucose ratios (ng·ml⁻¹·mmol⁻¹) and **b** proinsulin mRNA content/fed blood glucose ratios (ng·mmol⁻¹·l) compared in 6J and KsJ mice in the absence (-) or presence (+) of dexamethasone (Dex). There were significant increases in serum insulin/fed glucose ratios induced by dexamethasone in both normal and db/db mice, * $p < 0.01$, ** $p < 0.05$, while there were no strain-related differences. A significant strain related difference in the proinsulin mRNA/fed blood glucose ratio following dexamethasone was noted, *** $p < 0.01$. Open bars = 6J mice, hatched bars = KsJ mice

duced by dexamethasone in either strain, and no differences in these parameters between the strains.

Discussion

These studies were designed to assess differences in diabetes susceptibility between C57BL/KsJ and C57BL/6J mice, with particular attention to insulin production. The protocol allowed observations of the two strains with graded increases of insulin resistance, imposed by exogenous dexamethasone administration and by the genetically acquired db mutation. In normal (+/+) mice, no significant strain differences in insulin synthesis, secretion, or action were apparent. Dexamethasone treatment, presumably by augmenting insulin resistance and gluconeogenesis, induced increases in serum insulin and proinsulin mRNA in both strains, but the changes were comparable between

strains. The response to dexamethasone in these mice were comparable to those noted in normal rats [19, 25]. In the diabetic db/db mice, even higher levels of blood glucose, serum insulin, and proinsulin mRNA were observed, suggesting that the db mutation results in even greater insulin resistance than that resulting from dexamethasone treatment. Under these conditions strain differences were observed (Table 2), including a higher blood glucose and serum insulin in KsJ mice compared to 6J mice after dexamethasone. The levels of proinsulin mRNA and minimal changes in proinsulin mRNA content induced by dexamethasone were not different between strains.

Previous experiments assessing blood glucose, serum insulin, and pancreatic proinsulin mRNA have documented their interdependence and have shown that glucose is a positive modulator of both serum insulin and proinsulin mRNA levels [18–23, 26–29]. Thus, in this study the insulin and proinsulin mRNA data have been evaluated with respect to blood glucose levels, as illustrated by serum insulin/blood glucose and proinsulin mRNA/blood glucose ratios (Fig. 1). Dexamethasone administration to normal mice resulted in a significant but comparable 2-fold to 3-fold increase in the serum insulin/blood glucose ratio in both strains (Fig. 1a). The obesity in the db/db mice of both strains imposed comparable 2-fold to 3-fold increase in the serum insulin/blood glucose, but again there were no significant strain differences. Even in these obese, insulin resistant db/db animals, dexamethasone administration produced an even greater increase in the serum insulin/blood glucose ratio, yet the response did not differ between 6J and KsJ mice. Taken together, these data might suggest that neither differences in the steady state level of insulin secretion nor differences in insulin sensitivity account for the difference in diabetes susceptibility between the two strains. Yet a striking finding separating KsJ from 6J mice was the higher blood glucose and serum insulin in db/db mice following dexamethasone administration (Table 2). This could be interpreted to reflect impaired glucose-stimulated insulin release and/or increased insulin resistance in the KsJ mice; to evaluate these interpretations further study is warranted.

While serum insulin/blood glucose ratios (Fig. 1a) showed no strain differences, the proinsulin mRNA/blood glucose data (Fig. 1b) showed an important difference between 6J and KsJ diabetic (db/db) mice. Following the administration of dexamethasone to 6J mice, there was a greater than 2-fold increase in the proinsulin mRNA/blood glucose ratio compared to that in the KsJ strain ($p < 0.01$). Furthermore, the db/db mice of the KsJ strain failed to show an increase of the proinsulin mRNA/blood glucose ratio after dexamethasone, while a significant increase was observed in all other groups.

The observation that male KsJ diabetic (db/db) mice were capable of generating a substantial increase

in serum insulin but did not augment their total proinsulin mRNA in response to dexamethasone administration may be a clue to the mechanism of dexamethasone effects, and to the nature of diabetes susceptibility in these mice. Except for this group all of the data are compatible with a comparable effect either of the dexamethasone itself or of a single mediator which promotes both insulin synthesis and insulin secretion. But in the male KsJ diabetic mice the data for insulin secretion and synthesis are uniquely uncoupled, implying either a defect specifically in the dexamethasone-induced modulation of proinsulin mRNA or a lack of an ability to further increase proinsulin mRNA levels regardless of the stimulus. The observation that normal (+/+) male KsJ mice had significant dexamethasone-induced increases in both serum insulin and proinsulin mRNA speaks against a genetically determined restriction of the effects of dexamethasone, and favours an overall limitation of insulin synthetic capacity in male KsJ mice. In normal Sprague-Dawley rats, the administration of pharmacologic doses of dexamethasone for 4 days, a period sufficient to induce only modest changes in B-cell number [25], resulted in a coordinate 2-fold increase in ³H-leucine incorporation into proinsulin and in pancreatic proinsulin mRNA concentration [19]. No change in either parameter was observed, however, after rats made hyperglycaemic according to a neonatal streptozotocin injection protocol were given dexamethasone, leading to the conclusion that their capacity for insulin synthesis already had been exceeded.

It should be noted that the blood glucose levels in the male 6J db/db mice are higher than previously reported [2-5, 10, 11]. This was confirmed by E. Leiter of the Jackson Laboratory (personal communication), thus eliminating potential differences in diet or animal handling in this laboratory. There is no explanation for this increased blood glucose in the 6J db/db male mice relative to levels previously reported, but it clearly indicates that the strain differences observed in this report with dexamethasone may represent minimal estimates of limited capacity to synthesize insulin between the two strains. Genetic experiments between KsJ mice and a strain even more resistant to diabetes than the 6J strain can test this hypothesis.

It has been observed that female mice are more resistant than male mice to diabetes induced by obesity mutations [3, 30], viruses [31], and B-cell toxins [32]. Female 6J and KsJ, both normal and diabetic, were evaluated in the current studies exactly as male mice (data not shown). In general, the hyperglycaemia was less marked and strain differences were less prevalent. It is interesting to note that dexamethasone administration to +/+ and db/db genotypes was associated with a significant increase in proinsulin mRNA concentration and total proinsulin mRNA content only in female mice of both strains. These data further suggest a sex difference in the capacity to augment insulin biosyn-

thesis in mice which might play a role in the noted sex difference in diabetes susceptibility.

Current hypotheses addressing the pathogenesis of diabetes mellitus in C57BL mice and of Type 2 diabetes in man [33] are related to (1) the development of insulin resistance, and/or (2) the limitation of the capacity for insulin secretion. Observations have amply confirmed the presence of insulin resistance accompanying obesity both in these mice and in man. The question which remains unanswered in either case is whether insulin resistance alone is sufficient to produce hyperglycaemia, or whether capacity to produce insulin must also be pathologically limited before significant hyperglycaemia occurs. These observations of the KsJ and 6J mice are interpreted to demonstrate that there is indeed limitation of the capacity of male KsJ mice to produce insulin demonstrable in the face of extreme insulin resistance, and that this limitation may be at the level of insulin synthesis. While it is well-known that pharmacologic steroids can provoke diabetes in man, similar observations can be expected only when the means to measure insulin synthesis in man are defined.

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