The Possible Role of Age-Related Increase in the Plasma Glucagon/Insulin Ratio in the Enhanced Hepatic Gluconeogenesis and Hyperglycemia in Genetically Diabetic (C57BL/KsJ-db/db) Mice

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ABSTRACT—Genetically diabetic db/db mice and their normoglycemic littermates (+/+ mice) were studied to determine plasma levels of glucose, glucagon and insulin and hepatic gluconeogenic enzyme activities. Plasma glucose levels did not differ significantly between the 5-week-old db/db and +/+ mice, but increased with age in the former until the animals were 16-week-old. Similar age-associated changes were observed in the activities of the gluconeogenic enzymes, glucose-6-phosphatase (G-6-Pase) and fructose-1,6-diphosphatase (F-1,6-DPase). While the plasma levels of insulin and glucagon that peaked at 7 weeks of age did not parallel the hyperglycemia, the plasma glucagon/insulin (G/I) ratio roughly paralleled the hyperglycemia. Analysis of individual values for the db/db mice revealed statistically significant (P<0.001) correlations between plasma glucose levels and hepatic G-6-Pase (r=0.78) or F-1,6-DPase (r=0.74) activity. There were also significant correlations between the G/I ratio and plasma glucose levels (P<0.001, r=0.66), hepatic G-6-Pase (P<0.01, r=0.48) or F-1,6-DPase (P<0.01, r=0.57) activity. It is thus concluded that the relative predominance of glucagon over insulin action plays an important role in the age-associated development of hyperglycemia in db/db mice. Glucagon presumably activates the hepatic gluconeogenic enzymes to enhance hepatic glucose output.

Keywords: Diabetic (db/db) mouse, Glucagon/insulin ratio, Gluconeogenesis, Hyperglycemia

Genetically diabetic (db/db) mouse was first described by Hummel et al. (1) as an animal model of NIDDM (non-insulin-dependent diabetes mellitus). Detailed studies from the same laboratory have later showed that hyperinsulinemia was the first detectable abnormality in the mice (2–4). Interestingly, the blood insulin levels which decrease with age appear to be inversely correlated with the blood glucose levels. On the other hand, we have recently demonstrated that insulin resistance occurred in db/db mice before the manifestation of hyperglycemia and remained constant during the course of developing hyperglycemia (5). We thus speculate that insulin resistance causes compensatory insulin release that causes age-associated insulin depletion and leads to development of hyperglycemia. Decreased glucose utilization due to insufficient insulin release was considered to be an explanation for the hyperglycemia in db/db mice.

It has also been postulated that accelerated glucose production contributes to the hyperglycemia of db/db mice (6). The speculation was extended by Chan et al. (7), who showed that hepatic glycogenolytic and gluconeogenic enzyme activities increased in db/db mice. While glucagon enhances hepatic glucose production by stimulating gluconeogenesis and glycogenolysis, db/db mice are hyperglucagonemic (4, 8) presumably due to enhanced pancreatic glucagon release (9). These results suggest that hyperglucagonemia which enhances hepatic glucose production plays an important role in the development of hyperglycemia in db/db mice.

As described above, there are many papers reporting hyperglycemia, hyperglucagonemia, hyperinsulinemia and increased hepatic gluconeogenesis in db/db mice; however, few papers refer to the correlations between them during the course of developing hyperglycemia. On the other hand, there is no doubt that insulin and glucagon have reciprocal roles in regulating blood glucose levels and that the plasma glucagon/insulin (G/I) ratio is an essential unit in regulating hepatic carbohydrate metabolism in vitro and in vivo (10–12). We thus determined the plasma glucagon and insulin levels simultaneously with hepatic gluconeogenic enzyme activities in db/db mice aged 5–16 weeks when the hyperglycemia
dramatically developed and examined the relationships between these parameters and the G/I ratio.

MATERIALS AND METHODS

Animals
Female C57BL/KsJ (db/db) and their normoglycemic littermates (+/+ ) mice aged 4 weeks were purchased from Jackson Laboratory (Bar Harbor, ME, USA). They were kept in our laboratories at least one week in a temperature-controlled environment (22±1°C) and used in the studies at 5, 7, 10 and 16 weeks of age. Throughout the experiment, they were allowed free access to water and standard diet under a 12:12 hr light/dark cycle with lights on at 08:00 a.m.

Determination of plasma glucose, insulin and glucagon levels
At around 10:00 a.m., approximately 1 ml of blood was taken from the heart under ether anesthesia (for about 30 sec) and then mixed with 200 units/ml of aprotinin (Boehringer Mannheim, Mannheim, Germany) and 2.4 mg/ml of EDTA·2Na (Nacalai Tesque, Kyoto). The mixture was centrifuged at 8,000 × g for 10 min, and the resulting supernatant (plasma) was stored at -80°C until used.

Glucose concentration in the plasma was determined by the glucose-oxidase method with a commercial kit (Wako Pure Chemical, Osaka). Plasma insulin and glucagon levels were determined with commercial RIA kits (insulin RIA kit; Kabi Pharmacia Diagnostics, Uppsala, Sweden; glucagon RIA kit; Daiichi Radioisotope Labs., Tokyo).

Enzyme assay
The liver was isolated immediately after blood sampling and frozen in liquid nitrogen. The frozen liver was homogenized in 5 volumes of 50 mmol/l Tris/HCl buffer solution (pH 7.5) containing 0.25 mol/l sucrose and 500 units/ml of aprotinin under ice/water cooling. The homogenate was centrifuged at 100,000 × g for 40 min. The resulting supernatant (plasma) was stored at -80°C until used.

G-6-Pase in the liver homogenate was assayed by essentially the same method as described by Swanson (13). The whole homogenate was preliminary solubilized with 2 mg/ml deoxycholic acid (Nacalai). The standard reaction mixture containing 60 mmol/l cacodylic acid (Nacalai) and 18 mmol/l glucose-6-phosphate (Sigma, St. Louis, MO, USA) was adjusted to pH 6.5 with NaOH, and incubated with 20 µl/ml (containing about 30 mg protein/ml sample) of the sample for 30 min at 37°C. The reaction was terminated by the addition of 30 mg/ml trichloroacetic acid (TCA, Nacalai). The concentration of inorganic phosphate in the TCA extract was determined by the method of Fiske and Subbarow (14) using a commercial kit (Daiichi Pure Chemical, Tokyo). One unit of the enzyme was defined as the amount that liberated 1 µmol of inorganic phosphate with linear kinetics in 1 hr under the above conditions.

F-1,6-DPase activity was determined in the liver supernatant by the rate of inorganic phosphate release from fructose-1,6-diphosphate according to the method described by McGilvery (15). The standard reaction mixture contained 20 mmol/l triethanolamine/HCl buffer solution, pH 7.5, containing 1 mmol/l MgCl₂ and 10 mmol/l fructose-1,6-diphosphate (Sigma), and it was incubated with 10 µl/ml of the sample (containing about 15 mg protein/ml sample) for 30 min at 37°C. The subsequent assay procedures were the same as described for G-6-Pase. One unit of the enzyme was defined as the amount that liberated 1 µmol inorganic phosphate with linear kinetics in 1 hr under the above conditions.

To examine the effect of ether-anesthesia on these gluconeogenic enzyme activities, the enzyme activities were determined in the liver obtained from +/+ and db/db mice (7-week-old) by decapitation and under ether-anesthesia (for about 30 sec). There were no significant changes in the activities between these treatments in either of the mice (data not shown).

Protein concentration was determined by the method of Lowry et al. (16) with bovine serum albumin (fraction V powder, Sigma) as a standard.

Statistical analyses
The data are presented as the mean±S.E. and were statistically analyzed by Student’s t-test. A difference was considered significant at P<0.05.

RESULTS

Body weight and food intake
Table 1 shows the body weight and daily food intake of +/+ and db/db mice at 5, 7, 10 and 16 weeks of age. At 5 weeks of age, the db/db mice weighed more than the +/+ mice, and the difference increased with age. The food intake of the db/db mice was rather constant throughout the experimental period and ranged from 4.7 to 5.2 g/animal/day. The food intake of the +/+ mice was significantly less than that of the db/db mice at 5 and 7 weeks of age, and increased to almost the same values as the db/db mice from week 10 onward.

Plasma glucose
Plasma glucose levels were not significantly different between the 5-week-old db/db and +/+ mice, but in-
creased age-dependently in the former to reach 47.5 mmol/l at 16 weeks of age (Fig. 1A). The levels were almost constant in the +/+ mice.

Hepatic gluconeogenic enzymes activities
The activity of G-6-Pase did not differ between the 5-week-old db/db and +/+ mice, and it gradually increased with age in the former but remained almost constant in the latter (Fig. 1B). There were statistically significant differences in the activity between the mice at 7, 10 and 16 weeks of age.

In +/+ mice, the F-1,6-DPase activity at 7 weeks of age was slightly but significantly ($P<0.001$) decreased compared with that at 5 weeks of age (Fig. 1C). The activity gradually increased with age in the db/db mice, and there were statistically significant differences in the activity between the +/+ and db/db mice at 7, 10 and 16 weeks of age.

Individual values of G-6-Pase activity significantly correlated with those of plasma glucose levels in the db/db mice at ages of 5–16 weeks (Fig. 2A), and there also was a good correlation between F-1,6-DPase activities and plasma glucose levels (Fig. 2B).

Plasma insulin and glucagon
Plasma insulin levels were significantly higher in the db/db mice than in the +/+ mice at 5 weeks of age, and the levels in the former peaked at 7 weeks and decreased to the levels of the +/+ mice at 10 and 16 weeks (Fig. 3A). The plasma insulin levels of the +/+ mice were almost constant throughout the experimental period. There was no significant correlation ($r=-0.354$) between the individual values of plasma insulin and glucose levels in the db/db mice, while the insulin levels significantly correlated with G-6-Pase ($r=-0.400$, $P<0.05$) or F-1,6-DPase ($r=-0.438$, $P<0.05$) activity.

Plasma glucagon levels were always higher in the db/db

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Type</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Food intake (g/body/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+/+</td>
<td>7</td>
<td>21.0±0.4</td>
<td>2.8±0.5</td>
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<tr>
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<td>db/db</td>
<td>8</td>
<td>24.6±0.6***</td>
<td>4.7±0.5*</td>
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<tr>
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<td>+/+</td>
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<td>23.3±0.4</td>
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<td>5.1±0.4**</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>db/db</td>
<td>7</td>
<td>50.5±1.8***</td>
<td>4.7±0.4</td>
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</tbody>
</table>

Values are presented as the mean±S.E. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs +/+ mice at the same age.

Fig. 1. Age-associated changes in plasma glucose levels (A), hepatic G-6-Pase (B) and F-1,6-DPase (C) activities in +/+ and db/db mice. [Z], +/+ mice; [●], db/db mice. Values are presented as means±S.E. of 7–8 mice. **$P<0.01$, ***$P<0.001$ vs +/+ mice at the same age. *$P<0.01$, **$P<0.001$ vs db/db mice at 5 weeks of age.

Table 1. Body weight and food intake in +/+ and db/db mice
Fig. 2. Relationship between plasma glucose levels and hepatic G-6-Pase (A) or F-1,6-DPase (B) activity in db/db mice aged 5–16 weeks (n=29). Plasma glucose levels and G-6-Pase activity were significantly related: y=0.71x+5.1, r=0.779 (P<0.001). Plasma glucose levels and F-1,6-DPase activity were significantly related: y=0.29x−0.19, r=0.738 (P<0.001).

Fig. 3. Age-associated changes in plasma insulin (A), glucagon (B) levels and glucagon/insulin (G/I) ratio (C) in +/+ and db/db mice. □, +/+ mice; ■, db/db mice. Values are presented as means±S.E. of 7–8 mice. *P<0.05, **P<0.01, ***P<0.001 vs +/+ mice at the same age. aP<0.05, bP<0.01, cP<0.001 vs db/db mice at 5 weeks of age.
mice than in the +/+ mice, although the difference was not statistically significant at 5 weeks of age (Fig. 3B). The difference of plasma glucagon levels between the two groups was maximal at 7 weeks and then decreased with age. There was no significant correlation \((r=0.340)\) between the individual values of plasma glucagon and glucose levels in the db/db mice. Plasma glucagon levels also did not significantly correlate with G-6-Pase \((r=0.071)\) or F-1,6-DPase \((r=0.118)\) activity.

It has been reported that the insulin and glucagon levels of the peripheral blood are different from those of the portal blood (17). We therefore measured the hormone levels in plasma obtained from the portal vein and compared the values with those obtained from the heart in the +/+ and db/db mice. Plasma insulin levels were 2-3 times higher in the portal vein than in the cardiac pouch, and plasma glucagon levels were 2-4 times higher in the former than in the latter (data not shown). However, age-associated changes in the portal vein blood G/I ratio were almost the same as those of the heart blood G/I ratio in both +/+ and db/db mice (data not shown).

**DISCUSSION**

Extending the observations by previous authors (3, 6, 7), the present study demonstrated that the hepatic activities of gluconeogenic enzymes, G-6-Pase and F-1,6-DPase, increase in db/db mice and coincide with the manifestation of hyperglycemia. In addition, it is a novel finding that there was a statistically significant correlation \((P<0.001)\) between plasma glucose levels and G-6-Pase or F-1,6-DPase activity in 5-16 week old db/db mice. Although we did not determine other gluconeogenic enzyme activities or gluconeogenic precursors levels, previous studies have demonstrated that phosphoenolpyruvate carboxykinase and pyruvate carboxylase activities (3, 6) as well as circulating free fatty acids and lactate (7, 18, 19) increase in db/db mice after the onset of hyperglycemia. Chang and Schneider demonstrated in vivo that incorporation of radioactivity from \([14C]\)-pyruvate into glucose was enhanced in db/db mice (6). On the other hand, administration of 2,5-anhydro-D-mannitol, which inhibits glucose production from several gluconeogenic substrates in rat hepatocytes, potently improved the hyperglycemia in db/db mice (20). We thus speculate that elevated glucose production, mediated by increased gluconeogenesis flux, contributes to the age-associated development of
hyperglycemia in db/db mice. Although glycogen phosphorylase A activity has been reported to be enhanced in db/db mice (4, 7, 21–23), the change does not seem to parallel the degree of hyperglycemia (4, 22). In addition, there have been evidence suggesting that glycolytic enzyme activity, e.g., glucokinase and pyruvatekinase, were rather accelerated in the liver of db/db mice at 2 months of age (6, 7). Changes in the hepatic glycogenolysis and glycolysis may not play an important role, if any, in the manifestation of hyperglycemia in the mice.

The present study clearly demonstrated that hyperinsulinemia occurred in db/db mice before the manifestation of hyperglycemia and that neither a negative nor positive correlation was observed between the plasma insulin and glucose levels in the db/db mice. Likewise, the plasma glucagon levels, though enhanced in the db/db mice as compared with the +/+ mice, were not correlated with the plasma glucose levels. These results coincide with those in the earlier reports (2–4), suggesting that either hyperinsulinemia or hyperglucagonemia per se may not play an important role, if any, in the development of the hyperglycemia in db/db mice.

The most important finding of the present study is that there was a statistically significant correlation between the G/I ratio in the peripheral blood and hepatic gluconeogenic enzymes activity or plasma glucose levels in db/db mice. Furthermore, the age-associated changes in the G/I ratio determined for portal blood were almost the same as those determined for peripheral blood. While glucagon is known to increase G-6-Pase and F-1,6-DPase activities and accelerate the rate of gluconeogenesis and glucose output in the liver, insulin suppresses the effects of glucagon (10, 11, 24–26). In addition, it has been well established that the G/I ratio but not the absolute concentration of either hormone determines hepatic carbohydrate metabolism in vitro and in vivo (10–12). These results coincide with the above discussion suggest that the relative predominance of glucagon over insulin activities may induce an elevation of hepatic gluconeogenic enzymes activity and contribute to the age-dependent development of hyperglycemia in db/db mice. The assumption is also based on the finding that insulin action, though being depressed in the db/db mice compared with +/+ mice, remained constant during the course of developing hyperglycemia in the former (5).

Another important finding of the present study is that the G/I ratio was significantly less in db/db than +/+ mice at 5 weeks of age (before the manifestation of hyperglycemia) (5). These taken together indicate that higher insulin levels relative to glucagon are required to maintain normoglycemia in the prediabetic db/db than in the +/+ mice.

In conclusion, this study demonstrated that there is a good correlation between plasma glucose levels and G/I ratio or hepatic gluconeogenic enzymes activities in db/db mice. These results lead us to speculate that enhanced hepatic gluconeogenesis due to increased G/I ratio and gluconeogenic enzymes activities are responsible for the age-dependent development of hyperglycemia in the mice.

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