Differential Hypoglycemic Effect of 2,5-Anhydro-D-Mannitol, a Putative Gluconeogenesis Inhibitor, in Genetically Diabetic (db/db) and Streptozotocin-Induced Diabetic Mice

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ABSTRACT—2,5-Anhydro-D-mannitol (AM), a putative gluconeogenesis inhibitor, completely reversed the hyperglycemia in genetically diabetic (db/db) mice that exhibited hyperinsulinemia and enhanced hepatic gluconeogenic enzyme (glucose-6-phosphatase (G-6-Pase) and fructose-1,6-diphosphatase (F-1,6-DPase)) activities compared with the control +/+ mice. In contrast, AM only partially reversed the hyperglycemia of streptozotocin (STZ)-treated +/+ mice in which the hepatic gluconeogenic enzyme activities were enhanced to the same degree as in the db/db mice, whereas the blood insulin level was depressed. In the db/db mice, the STZ-treatment attenuated the hyperinsulinemia and exaggerated the hyperglycemia as well as the hepatic gluconeogenic enzyme activities, and it greatly reduced the hypoglycemic action of AM. Not only the dose-response curve of AM but also the time-course of the blood glucose level (expressed as % of pre-treatment value) following 320 mg/kg of AM were almost identical between +/+ , STZ-treated +/+ and STZ-treated db/db mice. In the STZ-treated +/+ mice, a combination treatment of insulin (320 µg/kg) with AM (320 mg/kg) caused hypoglycemia that was greater than that induced by AM or insulin alone. On the other hand, in vitro studies with purified F-1,6-DPase revealed that phosphorylated AM (AM-1,6-diphosphate) but not AM itself inhibited the gluconeogenic enzyme activities. These results suggest that inhibition of gluconeogenesis is responsible, at least in part, for the hypoglycemic activity of AM. AM appears to inhibit hepatic gluconeogenic enzyme activities after being phosphorylated by an insulin-dependent mechanism.

Keywords: 2,5-Anhydro-D-mannitol, Insulin, Gluconeogenesis, db/db Mouse, Streptozotocin

Genetically diabetic (C57BL/KsJ-db/db) mice, first described by Hummel et al. as an animal model of NIDDM (non-insulin-dependent diabetes mellitus) (1), are characterized by several abnormalities in hepatic carbohydrate metabolism (2–5) and insulin action (insulin resistance) (6). Extending the previous finding that the rate of gluconeogenesis increased in the liver of db/db mice (3, 4), we have recently demonstrated that there was a strong correlation between individual plasma glucose levels and hepatic gluconeogenic enzyme activities during the development of hyperglycemia in the mice (7). In addition, we have recently reported that insulin resistance occurred in the db/db mice before the manifestation of hyperglycemia and remained constant during the development of hyperglycemia (6). We thus speculated that enhanced glucose release due to increased hepatic gluconeogenesis rather than depressed glucose utilization due to insulin resistance was directly linked to the development of hyperglycemia in the mice.

2,5-Anhydro-D-mannitol (AM), an analog of β-D-fructose, inhibits glucose release from isolated rat hepatocytes and has a hypoglycemic action in fasting normal and diabetic rodents (8–10). In vitro studies have demonstrated that AM is converted by fructokinase and phosphofructokinase to AM-1,6-diphosphate which potently inhibits fructose-1,6-diphosphatase (F-1,6-DPase) activity (11–14). Since insulin is a major stimulator of these two kinase activities, the hormone could be essential for the phosphorylation of AM. It is thus possible that the hypoglycemic action of AM differs between NIDDM and IDDM (insulin-dependent diabetes mellitus), which represent hyperinsulinemia and hypoinsulinemia, respectively.

The present study was designed to examine the role of insulin in the hypoglycemic action of AM. We determined
the acute effect of AM on plasma glucose levels in hyperinsulinemic db/db mice, insulin-deficient diabetic mice (streptozotocin (STZ)-treated +/+ and db/db) and STZ-treated +/+ mice supplemented with insulin. Our results are discussed in relation to the in vitro effects of AM and AM-1,6-diphosphate on the gluconeogenic enzyme activities.

MATERIALS AND METHODS

Reagents
Bovine pancreas insulin, 2,5-anhydro-D-mannitol, streptozotocin, fructose-1,6-diphosphate, glucose-6-phosphate, rabbit liver fructose-1,6-diphosphatase, yeast glucose-6-phosphate dehydrogenase and yeast phosphoglucone isomerase were from Sigma Co. (St. Louis, MO, USA); aprotinin, from Boehringer Mannheim (Mannheim, Germany); Glucose assay kit (glucose-oxidase method), from Wako Pure Chemical (Osaka); insulin RIA kit, from Kabi Pharmacia (Uppsala, Sweden). 2,5-Anhydro-D-mannitol-1,6-diphosphate was synthesized by New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd. (Osaka).

STZ treatment
Female C57BL/KsJ-db/db (db/db) mice aged 5 weeks and their normoglycemic littermates (+/+) were purchased from Jackson Labs. (Bar Harbor, ME, USA). The +/+ and db/db mice were injected intraperitoneally with 150 mg/kg and 100 mg/kg of STZ, respectively, designated in this article as +/+-STZ and db/db-STZ mice. The control mice were injected with only vehicle (2 mmol/l-citric acid/NaOH buffered saline, pH 4.5). The mice were provided free access to standard mouse chow (Clea Japan, Tokyo) and tap water. Three weeks after the injection, the mice (8-week-old) were used for the present study.

AM administration
Time-course study: Non-fasted +/+, +/+ -STZ, db/db and db/db-STZ mice were given an oral dose (320 mg/kg) of AM or only its vehicle (saline, control). Chow was removed immediately after dosing. About 30 μl of blood was taken from the orbital sinus using heparinized-capillary tubes at 0, 1, 2 and 4 hr thereafter and was used for plasma glucose determinations. The plasma glucose levels of the AM dosing group was expressed as a percent of those of the respective control groups (+/+, +/+ -STZ, db/db, db/db-STZ mice).

Dose-response study: Zero, 32, 100, 320 and 1000 mg/kg of AM were orally administered to the non-fasted +/+, +/+ -STZ, db/db and db/db-STZ mice. Feed was removed immediately after the administration. Four hours later, about 30 μl of blood was taken from the orbital sinus and used for measurement of plasma glucose levels. For determining plasma insulin levels, blood samples were taken from the heart under ether anesthesia and mixed with 500 units/ml of aprotinin and 1.2 mg/ml of EDTA·2Na. The liver was immediately removed after the blood sampling and stored at −80°C until used. The frozen liver was homogenized in five volumes (v/w) of 50 mmol/l Tris/HCl buffer solution, pH 7.4, containing 0.25 mmol/l sucrose and 500 units/ml of aprotinin under ice/water cooling. The homogenate was centrifuged at 12,000xg for 30 min at 4°C. The resulting supernatant was collected as the source of F-1,6-DPase for assay. The whole homogenate was also collected and solubilized with 2 mg/ml deoxycholic acid, which was used for determining glucose-6-phosphatase (G-6-Pase) activity. These enzyme activities were determined by the rate of inorganic phosphate release from the respective substrates, fructose-1,6-diphosphate and glucose-6-phosphate, as reported previously (15, 16). Protein concentration was measured according to Lowry et al. (17) using bovine serum albumin (BSA, fraction V powder) as a standard.

Insulin administration
+/+-STZ mice were given simultaneously with insulin (0 or 320 μg/kg dissolved in saline with 0.01 mol/l HCl, s.c.) and AM (0 or 320 mg/kg, orally). Blood sampling and analytical methods were the same as described above (AM administration, Time-course study).

Effect of AM and AM-1,6-diphosphate on F-1,6-DPase activity in vitro
F-1,6-DPase activity was measured by the coupled spectrophotometric assay as described previously (18). The standard assay mixture contained 0.025 mol/l-Tris/histidine, pH 6.5, 0.1 mmol/l EDTA, 5 mmol/l MgSO4, 0.19 mmol/l NADP⁺, 3.2-32 μmol/l fructose-1,6-diphosphate, 10 units/l F-1,6-DPase, 100 units/l glucose-6-phosphate dehydrogenase, and 100 units/l phosphoglucone isomerase in a final volume of 2 ml at 35°C. The reaction was initiated by the addition of F-1,6-DPase. The F-1,6-DPase activity was determined in the absence or presence of AM-1,6-diphosphate (1 and 10 μmol/l) or AM (10 μmol/l).

Statistical analysis
Data are expressed as means±S.E. Group differences were analyzed by analysis of variance, and differences between different doses of AM were assessed by Dunnett’s test. Differences between paired groups were assessed by Student’s t-test.
Table 1. Parameters in +/+ and db/db mice with or without STZ treatment

| Type          | Body weight (g) | Plasma glucose (mmol/l) | Plasma insulin (pmol/l) | F-1,6-DPase (units/mg protein) | G-6-Pase (units/mg protein) |
|---------------|-----------------|-------------------------|-------------------------|-------------------------------|-----------------------------|
| +/+           | 22.7±0.3        | 13.4±0.3                | 34±6                    | 5.2±0.3                       | 16.3±0.5                    |
| +/+-STZ       | 12.8±0.6***     | 31.2±0.9***             | 14±6*                   | 8.1±0.5***                    | 25.6±2.1**                  |
| db/db         | 37.9±0.8***     | 23.6±1.6***             | 186±24***               | 8.9±1.1*                      | 27.8±1.8***                 |
| db/db-STZ     | 22.0±1.3g#      | 40.6±1.6###             | 47±28g#                 | 10.3±0.3                      | 29.1±0.8                    |

Values are presented as the means±S.E. of 8 mice. *P<0.05, **P<0.01, ***P<0.001 vs +/+ mice. g#P<0.01, g#P<0.001 vs db/db mice (Student's t-test).

RESULTS

Characteristics of diabetic mice

Body weight, basal plasma parameters and hepatic gluconeogenic enzyme activities of the four groups of normal and diabetic mice are given in Table 1. A 150-mg/kg dose of STZ injected in +/+ mice significantly decreased basal plasma insulin levels by 59%, reduced body weight and caused hyperglycemia. Db/db mice, aged 8 weeks, showed obesity and significant increases in basal plasma glucose and insulin levels compared with the +/+ mice. After STZ injection at 100 mg/kg, the plasma insulin of db/db mice was reduced by 75% and reached a value similar to that observed in the +/+ mice, whereas plasma glucose levels were considerably elevated.

Significant increases in F-1,6-DPase activity were found in the liver supernatants obtained from the +/+-STZ and db/db mice when compared with the +/+ mice. These diabetic mice also showed a significant increase in G-6-Pase activity in their liver homogenates.

Effect of AM in normal and diabetic mice

Plasma glucose levels were reduced time-dependently after an oral dose (320 mg/kg) of AM in +/+, +/+-STZ and db/db mice (Fig. 1). The dose of AM exerted a greater hypoglycemic effect in the db/db mice, although the dynamics were similar among the three types. On the other hand, the plasma glucose levels in db/db-STZ mice were almost constant during the experimental period.

Plasma glucose levels also were dose-dependently decreased in the +/+, +/+-STZ and db/db mice at 4 hr after the administration of AM (Fig. 2). Compared by the dose-response curves, AM was most effective in the db/db mice, where 320 mg/kg of AM significantly decreased plasma glucose levels by 48%, from 23.6±1.6 to 12.2±0.6 mmol/l, which was almost the same value as that in the normal mice (13.4±0.3 mmol/l). In the +/+ and +/++STZ mice, respectively, the same dose of AM reduced plasma glucose levels by only 22 and 24%. STZ treatment diminished the hypoglycemic activity of AM in the db/db mice. The dose of AM decreased plasma glucose levels in the db/db-STZ mice by only 16% from 40.6±1.6 to 34.0±3.2 mmol/l, and the change was not statistically significant.

AM dose-dependently decreased plasma insulin levels in the db/db mice, but hardly changed those in the +/+, +/+-STZ and db/db-STZ mice (Fig. 3). AM at 320 mg/kg significantly decreased plasma insulin levels in the db/db mice from 186±24 to 120±18 pmol/l, although this was still 3.5 times higher than the level (34.0±6 pmol/l) in the +/- mice.

Effect of insulin on the AM action in +/+-STZ mice

AM at 320 mg/kg slightly reduced plasma glucose levels in the +/+-STZ mice by 16%, from 28.6±0.7 to 24.0±0.7 mmol/l, at 4 hr after administration; and the reduction was enhanced by a simultaneous injection of insulin (320 μg/kg) (Fig. 4). The same dose of AM reduced
plasma glucose levels in the insulin-treated +/+-STZ mice by 75%, from 18.3±2.1 to 4.6±1.1 mmol/l.

Effect of AM and AM-1,6-diphosphate on F-1,6-DPase activity in vitro

AM-1,6-diphosphate competitively inhibited the F-1,6-DPase activity, and the \( K_i \) (inhibition constant) value was calculated to be \( 1.3 \times 10^{-6} \) mol/l (Fig. 5). On the other hand, 10 \( \mu \)mol/l of AM hardly affected the F-1,6-DPase activity.

DISCUSSION

The most important finding of the present study is that the hypoglycemic activity of AM was greater in genetically diabetic db/db compared with STZ-induced diabetic +/+ mice. Among the parameters determined, body weight, plasma glucose and insulin levels differed significantly between the mice, which could be involved in the differential activity of AM. Although various factors that were not determined in the present study may also contribute to the difference, the present study clearly
Fig. 4. Effect of insulin on the hypoglycemic action of AM in +/+ -STZ mice. A, control; B, AM alone; C, insulin alone; D, insulin+AM. Values are presented as the means±S.E. of 8 mice. Significant differences between groups: **P<0.01, ***P<0.001 by Student's t-test.

demonstrated that the strong hypoglycemic activity of AM in db/db mice was diminished by STZ which attenuated the hyperinsulinemia. In contrast, the activity of AM in STZ-treated +/+ mice was potentiated by the simultaneous administration of insulin. These results indicate that hyperinsulinemia in db/db mice is important for the AM-induced attenuation of blood glucose levels. The mechanism of the hypoglycemic action of AM may involve, at least in part, an insulin-dependent pathway. It has been demonstrated that AM is phosphorylated by insulin-dependent kinases such as fructokinase and phosphofructokinase to AM-6-phosphate and AM-1,6-diphosphate, which potently inhibit glycogen phosphorylase and F-1,6-DPase activity (10-12). These findings taken together lead us to the speculation that insulin could potentiate the hypoglycemic action of AM through accelerating the conversion of AM to its phosphorylated metabolites. This assumption is supported in part by the present in vitro experiment demonstrating that AM-1,6-diphosphate but not AM itself inhibited the activity of F-1,6-DPase. Although we did not determine the hepatic levels of the phosphorylated AM metabolite, it is noteworthy that the hepatic insulin-dependent kinase activities of db/db mice are above normal at 8 weeks of age (4) when the hyperinsulinemia was clearly observed.

The body weight significantly differed between db/db (37.9 g) and +/+ (22.7 g) mice, which were reduced by the STZ-treatment to 22.0 and 12.8 g, respectively. The difference in the body weight could also contribute to the difference in the hypoglycemic activity of AM between the mice; however, the following results obtained in the present study may argue against this possibility. Increasing the dose of AM did not overcome the difference in the response; AM at the dose of 320 mg/kg in +/+ or STZ-treated db/db mice induced less response than 100 mg/kg in db/db mice, while the body weight difference between the former two and the latter mice was less than twofold. Furthermore, the time-course after AM treatment was almost identical between +/+ , STZ-treated +/+ and STZ-treated db/db mice in spite of the body weight difference.

AM significantly decreased plasma insulin levels in db/db mice. Although the hypoinsulinemic effect of AM in db/db mice looks incompatible with its hypoglycemic effect, this could be explained as follows: The degree of the change in insulin levels following AM treatment (about 25% at its maximum) is too small to cause hyperglycemia. In fact, STZ caused more than a 50% decrease in the basal insulin levels of +/+ or db/db mice. Another explanation is that the hyperglycemia due to the reduced insulin levels may be antagonized by the above-mentioned inhibitory effect of AM on gluconeogenesis. On the other hand, it is noteworthy that AM hardly changed the plasma insulin levels in +/+ mice. This raises the possibility that the drastic hypoglycemia caused by AM rather than the direct effect of the drug on insulin release contributes to the depression of plasma insulin levels in db/db mice. We have previously reported that db/db mice reveal severe hyperinsulinemia at 5 weeks of age when their plasma glucose levels are still nearly normal (6, 7). In db/db mice, there appears to be a compensatory insulin release to restore the hyperglycemia in mice.
The present observation that STZ-treatment exaggerated the well-developed hyperglycemia in db/db mice is in line with the assumption.

The above discussions not only clarify the mechanism of action of AM but also strongly substantiate our previous speculation about the etiological role of gluconeogenesis in the development of hyperglycemia in db/db mice. Db/db mice develop hyperglycemia and show increases in the activities of hepatic gluconeogenic enzymes such as F-1,6-DPase and G-6-Pase with age (3, 4). Extending the studies, we recently found that there is a good correlation between individual plasma glucose levels and these enzyme activities in 5- to 16-week-old db/db mice (7). In addition, Chang and Schneider found increased incorporation of radioactivity from (14C)-pyruvate into serum glucose in db/db mice (3). We thus speculated that enhanced hepatic glucose output possibly due to increased gluconeogenesis plays a crucial role in the development of hyperglycemia in the mice.

In conclusion, AM improves not only hyperglycemia but also hyperinsulinemia in db/db mice. Insulin-dependent pathways may be involved in the hypoglycemic action of AM. A clinical trial of AM in NIDDM patients is of interest, because a similar mechanism may be involved in the pathogenesis of hyperglycemia in NIDDM patients and db/db mice. Elevated rates of basal glucose output have been documented by numerous investigators in patients with NIDDM, and a strong correlation exists between the degree of fasting hyperglycemia and the rate of hepatic glucose output (19-23).

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