Potential Benefit of Inhibitors of Advanced Glycation End Products in the Progression of Type II Diabetes: A Study With Aminoguanidine in C57/BLKsJ Diabetic Mice

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Prolonged hyperglycemia in type II diabetic patients is linked both with diabetic complications and with further impairment of glucose homeostasis, possibly due to glucose toxicity of the β cell. While the connection between the accumulation of extracellular advanced glycation end products (AGEs) and the development of complications is well established, it has only recently been suggested that intracellular glycation may be equally adverse and could be involved in the pathogenesis of glucose toxicity in vitro. Aminoguanidine is a recognized inhibitor of the formation of both extracellular and intracellular AGEs. In this study, we show that the development of diabetes, measured by increased water intake and concomitant midday blood glucose levels in type II genetically diabetic mice, is reduced by treatment with aminoguanidine at a dosage of 500 mg/kg/d for 12 weeks in the diet. In addition, at the end of the study, aminoguanidine reduced the decline in serum and pancreatic insulin levels and the degree of pancreatic islet morphological degeneration, all of which are associated with pancreatic insufficiency following prolonged hyperglycemia in this animal model. These results suggest that AGEs may be involved in the aggravation of type II diabetes in vivo and aminoguanidine may be beneficial in its treatment.

THE RESULTS of the Diabetes Control and Complications Trial have established a causal relationship between chronic hyperglycemia and diabetic complications.[1] Increasing evidence points to a major role for advanced glycation end products [AGEs] the outcome of nonenzymatic modification of tissue proteins by physiological sugars in vivo as the link between these features of the disease.[2-4]

The glucose transporter in the β-cell membrane, GLUT 2, ensures that rapid fluctuations in extracellular glucose are matched by similar changes in glucose concentrations within the β cell. Consequently, circulating hyperglycemia will be paralleled by an equivalent elevation of glucose within the β cell. It has been suggested that chronic hyperglycemia has adverse effects on β-cell function in vivo leading to an impairment in insulin biosynthesis and a worsening of diabetes.[5] Indeed, insulin secretion in patients with type II diabetes often deteriorates with the duration of the disease, which may be, in part, due to the effects of glucose toxicity.

Recent studies have shown that glycation within the β cell can occur in vitro under hyperglycemic conditions, leading to a loss of glucose responsiveness that can be improved by inhibitors of glycation.[6-8] If AGE formation does play a role in glucose toxicity, then treatment with agents like the AGE inhibitor aminoguanidine (a compound currently undergoing clinical trials for the treatment of diabetic complications) may be of benefit in retarding the deterioration of β-cell function in type II diabetic patients.

The purpose of this study was to determine if aminoguanidine has any protective effect on the development of diabetes in vivo in the C57/BLKsJ *db/db* mouse, an animal model of type II diabetes that eventually develops pancreatic insufficiency.[9,10]

MATERIALS AND METHODS

Animals and Drug Treatment

Female C57/BLKsJ *db/db* mice aged 4 to 5 weeks and their +/? nondiabetic controls were purchased from Jackson Laboratories (Bar Harbor, ME) and housed seven per cage under quarantine conditions at 26° ± 2°C. They were allowed free access to powdered RM3 diet and tap water. All experimental procedures were performed according to the UK Animals (Scientific Procedures) Act of 1986, and were approved by the SmithKline Beecham UK procedures review panel.

On day 0, mice were randomized into three groups of diabetic and two groups of nondiabetic mice (n = 7) according to the midday blood glucose level (vide infra). One group of diabetic and one group of nondiabetic mice were killed to act as baseline controls. Another group of diabetic mice was treated with 500 mg/kg/d aminoguanidine in the diet for 12 weeks. The remaining groups of mice acted as time-matched untreated diabetic and nondiabetic controls.

Food Intake and Body Weight Gain

Collective food intake was measured daily, and individual body weight was measured weekly.

Disease progression was determined by monitoring the development of hyperglycemia throughout the study and comparing pancreatic and serum insulin levels and pancreatic morphology in mice killed at the end of the study versus mice killed at the beginning of the study.

The development of hyperglycemia was determined by measuring collective daily water intake for each group of mice and individual midday blood glucose levels on day 0, day 63, and at the end of the study. A 10-μL blood sample was taken from the tail by tail-snip and hemolyzed with 1 mL hemolysis reagent. Glucose levels were determined enzymatically using a Ciba Corning 550 Express Analyser (Halstead, Essex, UK).

Terminal Samples

Mice were killed by cervical dislocation either on day 0 or at the end of the treatment period and neck-bled for measurement of serum insulin levels by radioimmunoassay. Part of the pancreas was removed and weighed, the insulin was extracted by homogenization in ice-cold acid ethanol (150 mL ethanol, 30 mL water, and 30 mL concentrated hydrochloric acid), and the content per milligram of tissue was determined as already described.

The rest of the pancreas was fixed in 4% buffered Formol saline, and 4-μm paraffin sections were cut and stained with hematoxylin and eosin for morphological examination. Insulin and glucagon in serial sections

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were detected by immunocytochemistry using specific antibodies and peroxidase-conjugated secondary antisera using diaminobenzidine to visualize the enzyme.

Microscopic examination was performed blind by a pathologist. The pancreatic islets were scored for the degree of morphological degeneration and for insulin and glucagon content.

Scoring System for Pancreatic Islet Morphology

No degeneration (o). Islets consist of uniform light-staining cords of cells in compact rounded or ellipsoid groups. Centrally located β cells containing moderate amounts of insulin comprise 80% to 90% of the islets. The α cells are peripherally located.

Minimal degeneration (±). Approximately 10% of the β cells are hypertrophied, with minimal insulin content. The α cells are peripherally located.

Mild degeneration (+). Approximately 25% of the β cells are hypertrophied, with minimal insulin content. The α cells are scattered throughout the islet.

Moderate degeneration (++). Approximately 50% of the surviving β cells are hypertrophied. The islets are clearly smaller, with minimal insulin content. The α cells are scattered throughout the islet.

Marked degeneration (+++). This involves anything more severe than as described for moderate degeneration.

Reagents

RM3 801 187W powdered diet was supplied by Special Diet Services (Witham, Essex, UK). Aminoguanidine (Sigma, Poole, Dorset, UK) was incorporated into the diet by mixing in a T2C Turbula Shaker (Glen Creston, Middlesex, UK). Hemolysis reagent was made from 50 mg/mL digitonin and 100 mg/mL maleimide, both from Sigma. Linco rat insulin [^{125}I]insulin radioimmunoassay kits, which exhibit 100% specificity for mouse insulin, were supplied by Biogenesis (Poole, UK). For immunocytochemistry measurements, guinea pig antiporcine insulin, rabbit antiporcine glucagon, and their respective peroxidase-conjugated secondary antisera were obtained from Dako (High Wycombe, UK). Diaminobenzidine was obtained from Sigma.

Statistical Analysis

Results are presented as the mean ± SEM. The level of significance was determined by unpaired *t* test or by ANOVA with post hoc comparison using Tukey's honest significance test. The level of significance was set at P less than .05.

Collective water intake was analyzed by the Statistical Sciences Department of SmithKline Beecham UK. Curves were fitted to the data for each group using the GLM procedure in the SAS 6.11 software (SAS Institute, Cary, NC, *SAS/STAT User's Guide,* Version 6, vol 2, ed 4). The fitted parameters for the slope and curvature for the three groups were compared using contrast analysis. This splits the variation in the slope and curvature between groups into pairwise comparisons of groups.

RESULTS

Baseline Measurements

At the beginning of the experiment, body weight, circulating blood glucose, and serum insulin were markedly elevated in *db/db* mice compared with +/? mice, while pancreatic insulin levels in both phenotypes were similar (Table 1). The pancreas from +/? mice showed normal architecture, with the islets consisting of uniform light-staining cords of cells in compact rounded or ellipsoid groups. Eighty percent to 90% of the cells in the islets were centrally located, insulin-containing β cells. Located around the periphery of the islets were glucagon-containing α cells. The pancreas from *db/db* mice was beginning to show morphological changes associated with the disease such as islet disorganization and β-cell degeneration.

Table 1. Body Weight, Midday Blood Glucose, Serum Insulin, and Pancreatic Insulin in *db/db* Mice, *db/db* Mice Treated With Aminoguanidine, and +/? Mice on Days 0 and 85

Group	Body Weight (g)	Midday Blood Glucose (mmol/L)	Serum Insulin (ng/mL)	Pancreatic Insulin (ng/mg)
Day 0				
db/db control	26.7 ± 1.0*	10.4 ± 0.5*	127 ± 37.3*	84.5 ± 15.9
+/?	15.1 ± 0.7	7.8 ± 0.3	3.0 ± 0.9	70.0 ± 9.5
Day 85				
db/db control	49.9 ± 0.9*	28.2 ± 1.2*	11.7 ± 1.8*	30.0 ± 2.6
db/db AG-treated	49.4 ± 1.14	21.2 ± 1.7†	23.3 ± 4.1†	64.3 ± 17.9
+/?	21.8 ± 0.58	6.7 ± 0.3	2.7 ± 0.4	53.3 ± 8.8

Abbreviation: AG, aminoguanidine.
**db/db* control *v* +/?.
†*db/db* AG-treated *v db/db* control.

Development of Diabetes in db/db Mice

The *db/db* mice gained weight throughout the study, while the weight of +/? mice plateaued after about 4 weeks into the study, despite the same average daily food intake between the two phenotypes (data not shown). By the end of the study, the body weight of *db/db* mice was 56% greater than for the lean phenotype (Table 1).

Cumulative water intake in +/? mice was constant throughout the study, while water intake in *db/db* mice increased with time, with a significant difference in the slope of the water intake curve (Fig 1a). This was accompanied by a 152% increase in the midday blood glucose level by day 63 (data not shown; Cawthorne MA, Chapman HC, Lister CA, April 1980) and a 171% increase by the end of the study (Table 1 and Fig 1b), supporting previous unpublished data that the development of hyperglycemia is paralleled by an increase in water intake.

Despite the persistent hyperglycemia, the elevated serum and pancreatic insulin in *db/db* mice on day 0 declined by the end of the study to a level similar to that found in +/? mice (Table 1). At this time, the histology of the pancreas from diabetic mice showed marked pancreatic islet disorganization involving a loss of cord architecture and β-cell hypertrophy/degeneration (especially toward the center of the islets) characteristically involving cytoplasmic compartmentalization with a perinuclear acidophilic cytoplasm and peripheral basophilia. The remaining β cells were hypertrophied, with a slightly basophilic finely vacuolated cytoplasm. β-Cell changes were associated with a reduction in insulin content (data not shown). The loss of β cells was demonstrated by the appearance of α cells scattered throughout the islets, considered to indicate inward collapse (Table 2). In contrast, the pancreas from +/? mice showed a normal islet architecture and insulin and glucagon content as described for the day 0 phenotype (Table 2). This supports previous findings that progression of diabetes in *db/db* mice leads to a loss of normal islet morphology and islet insulin secretion capacity.[9]

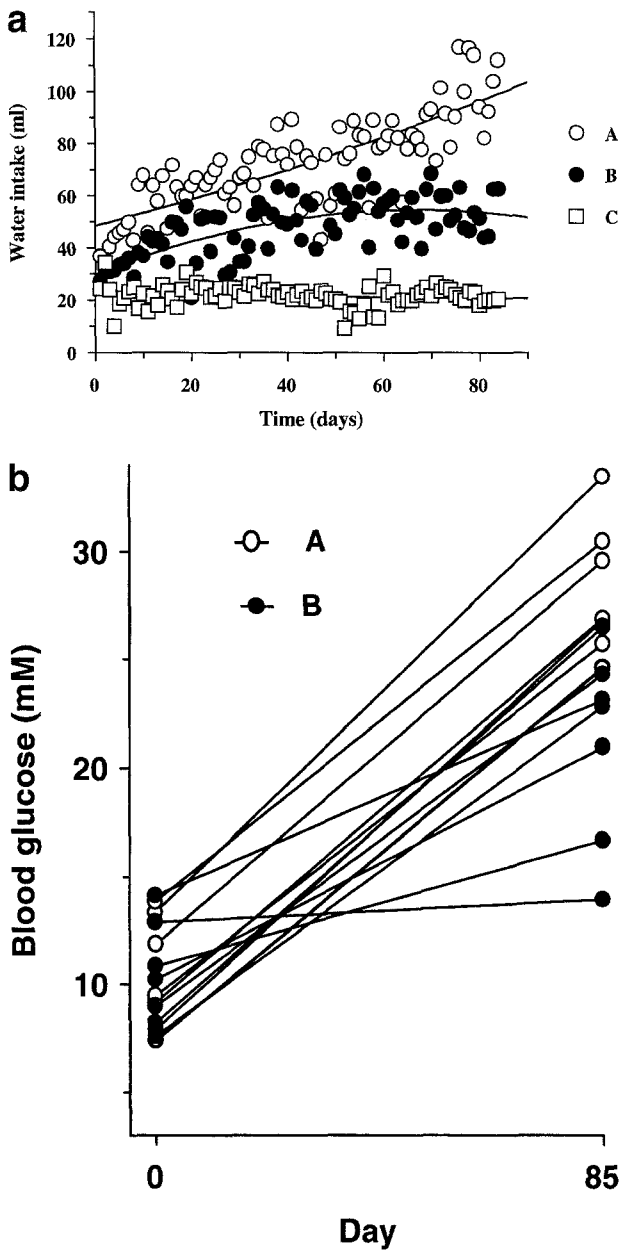

Fig 1. (a) Daily water intake over 12 weeks in *db/db* mice (A), *db/db* mice treated with aminoguanidine (B), and +/? mice (C). The differences in intercept, slope, and curvature for pairwise tests are as follows: A *v* C, 25.6071 ($P = .0001$), 0.5201 ($P = .0156$), 0.0013 ($P = .6038$); B *v* C, 7.0363 ($P = .0756$), 0.7770 ($P = .0004$), −0.0057 ($P = .0213$); A *v* B, 18.5708 ($P = .0001$), −0.2569 ($P = .2338$), 0.0069 ($P = .0050$). (b) Individual blood glucose levels on day 0 and day 85 for *db/db* mice (A) and *db/db* mice treated with aminoguanidine (B).

Effects of Aminoguanidine on the Development of Diabetes in db/db Mice

There was no difference in the day 0 body weight (data not shown) or midday blood glucose level in drug-treated diabetic mice compared with controls (Fig 1b). Food intake was the same for both groups throughout the study (data not shown), and there was no difference in body weight gain (Table 1).

The shape of the cumulative water intake curve was different for aminoguanidine-treated *db/db* mice versus untreated diabetic mice, and the difference was significant (Fig 1a). In addition, there was a significant difference in the terminal midday blood glucose level between the two groups (Table 1 and Fig 1b). These data suggest that aminoguanidine reduced the development of hyperglycemia in *db/db* mice.

Terminal circulating insulin levels were significantly greater in aminoguanidine-treated mice compared with the diabetic controls (Table 1). Terminal insulin levels in the pancreas were also higher in the drug-treated diabetic mice when visualized by immunocytochemistry (data not shown) and quantified by radioimmunoassay (Table 1), although this did not reach significance. Remarkably, islets from aminoguanidine-treated mice showed normal morphology despite a perceived loss of β cells as demonstrated by the scattered distribution of α cells (Table 2).

Taken together, these data suggest that aminoguanidine had a protective effect on the progression of diabetes in *db/db* mice.

DISCUSSION

The primary causal factor for the development of most diabetic complications such as nephropathy, neuropathy, and cardiovascular disease is now considered to be prolonged exposure to hyperglycemia.[1] There is substantial evidence linking hyperglycemia with the nonenzymatic reaction of sugars with proteins and the accelerated formation of highly reactive intermediate and terminal adducts (AGEs) that may contribute to these complications.[2-4]

Although glucose is the major physiological stimulator of insulin secretion and biosynthesis, extensive exposure of pancreatic β cells to high levels of glucose in vitro can cause β-cell dysfunction that is associated with impaired insulin secretion and biosynthesis,[11] possibly through suppression of insulin gene promoter activity[6] or impairment of proinsulin processing.[12] Thus, in addition to the diabetic complications already described, prolonged poor glycemic control in type II diabetic patients often leads to a decline in insulin secretion from pancreatic β cells and a worsening of the diabetic state.[5]

In this 12-week in vivo study using *db/db* mice, which are paradigms for human type II diabetes, there was a progressive increase in polydipsia associated with a threefold increase in terminal circulating blood glucose levels. The worsening of glycemic control was accompanied by a dramatic decrease of

Table 2. Summary of Major Histopathological Findings in Pancreatic Islets From *db/db* Mice (A) Compared With Age-Matched *db/db* Mice Treated With AG for 12 Weeks (B) and +/? Mice (C)

	Animal No.						
Group	1	2	3	4	5	6	7
Islet degeneration							
A	++	++	++	+	+	+	++
B	0	0	0	0	0	0	0
C	0	0	0	0	0	0	0
Glucagon cell distribution within the islet							
A	S	S	S	S	S	S	S
B	S	S	S	S	S	S	S
C	P	P	P	P	P	P	P

Abbreviations: 0, no degeneration; +, mild degeneration/content; ++, moderate degeneration; P, peripheral; S, scattered throughout.

terminal serum and pancreatic insulin levels and a loss of islet architecture, all of which are consistent with a loss of pancreatic insulin content and insulin secretory capacity.

Treatment with 500 mg/kg/d of the AGE inhibitor aminoguanidine[13] reduced the increase in polydipsia and the terminal hyperglycemia in diabetic mice. Moreover, aminoguanidine reduced the decrease in serum and pancreatic insulin levels and maintained pancreatic islet organization. These data therefore indicate that aminoguanidine treatment had a protective effect against the progression of diabetes in these mice and attenuated the loss of normal islet morphology and secretory capacity.

It was originally thought that glycation of proteins only occurred in the extracellular matrix, but there is now evidence to suggest that rapid intracellular AGE formation can occur in cells in which glucose is not regulated by insulin.[2] Pancreatic cells are highly efficient at glucose uptake when exposed to high glucose due to the abundance of the equilibrium glucose transporter GLUT 2, such that intracellular glucose concentrations mirror the extracellular environment in diabetes.

Recent studies have shown that glycation of proteins within the β cell can occur in vitro under hyperglycemic conditions, leading to a loss of glucose responsiveness that can be improved by inhibitors of glycation.[7,8]

Long-term treatment with 25 or 20 mg/kg/d aminoguanidine has been shown to be efficacious for inhibiting AGE formation in diabetic rats.[13-16] Using the consideration scaling phenomenon, which necessitates an increase in the dose required for mice compared with rats,[17,18] treatment of *db/db* mice with 500 mg/kg/d aminoguanidine for 12 weeks in this study protected against the progression of diabetes. While we did not measure any markers of glycation in the *db/db* mice, pancreatic extracts from hyperglycemic *ob/ob* mice, which are from the same genetic background as *db/db* mice,[19] have been shown to contain significantly elevated amounts of glycated immunoreactive insulin compared with nondiabetic controls.[12] It is therefore possible that the partial amelioration of pancreatic insufficiency observed in *db/db* mice by aminoguanidine in the present study could be the result of inhibition of AGE formation.

Aminoguanidine has also been shown to be a selective inhibitor of inducible nitric oxide synthase,[20] and nitric oxide has been implicated in the pathogenesis of diabetes in the Zucker diabetic fatty rat.[21] We believe this is unlikely to be the mechanism of action in this study with *db/db* mice, since there was no evidence of inflammation in the pancreas from diabetic mice at any time point, nor have we been able to show any beneficial effects of treatment with nicotinamide in this animal model (manuscript in preparation).

Although further studies are needed to confirm this, we suggest that the beneficial effects of aminoguanidine in this study occurred via inhibition of the formation of AGEs.

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