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Improvement by Aminoguanidine of Insulin Secretion from Pancreatic Islets Grafted to Syngeneic Diabetic Rats

Shinsuke Hiramatsu, Kaoru Inoue,* Yuji Tajirl* and Valdemar Grill†‡*

*Department of Molecular Medicine, Endocrine and Diabetes Unit, Karolinska Hospital, Karolinska Institute, S-17176, Stockholm, Sweden; and †Department of Internal Medicine, Section of Endocrinology, Norwegian University of Science and Technology, Trondheim, N-7006, Norway

ABSTRACT. Prolonged hyperglycemia inhibits B-cell function by mechanisms that are largely unclarified. We investigated the involvement of advanced glycation end products (AGEs), using aminoguanidine as well as the AGE-breaking compound ALT-711 in a transplantation model. Islets from Wistar–Furth rats were transplanted under the kidney capsule of syngeneic streptozocin-diabetic recipients. Aminoguanidine was administered as 1 g/L in the drinking water. Graft-bearing kidneys were isolated and perfused to investigate insulin secretion, and grafts were excised to measure preproinsulin mRNA contents. In all transplants to diabetic rats, insulin responses to 27.8 mM glucose were abolished and aminoguanidine failed to correct this abnormality. However, aminoguanidine treatment for 8 weeks following transplantation increased preproinsulin mRNA contents of the grafts ($P < 0.05$). In addition, treatment with aminoguanidine enhanced the insulin secretory response to arginine ($P < 0.05$). Arginine-induced insulin secretion was also enhanced when aminoguanidine treatment was started after an initial 2-week implantation period rather than immediately after transplantation. On the other hand, treatment with ALT-711 (0.1 mg/kg by gavage) for 8 weeks completely failed to affect B-cell function of grafts, and ALT-711 was also ineffective under *in vitro* conditions. Our findings indicate that aminoguanidine effects *in vivo* are to a major extent not coupled to AGEs or nitric oxide synthetase inhibition, but possibly to oxidative modifications accomplished by the guanidine compound. BIOCHEM PHARMACOL **60**;2: 263–268, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. aminoguanidine; islet transplantation; insulin secretion; insulin biosynthesis; oxidation; glycation

Prolonged hyperglycemia gives rise to complications of diabetes, such as retinopathy, neuropathy, and nephropathy. It has been proposed that these adverse effects of glucose are linked to the formation of AGEs§ [1–3]. This hypothesis is largely based on results with aminoguanidine, which is known to inhibit the formation of AGEs [4]. This guanidine compound has been shown to retard the evolution of diabetic complications in several models of animal diabetes. More recently, however, it was realized that aminoguanidine also had effects independent from those on AGE formation [5], such as inhibition of both nitric oxide synthases [6, 7] and glycooxidation processes [8].

In addition to causing the well-known diabetic complications of diabetes, hyperglycemia also time dependently decreases B-cell function both *in vitro* [9] and *in vivo* [10]. We therefore wondered whether prolonged hyperglycemia might damage B-cells by mechanisms akin to those operative in "classic" complications of diabetes. This notion was supported by a previous study *in vitro* [9]. There, we demonstrated that a six-week culture period of rat pancreatic islets at a high glucose concentration led to a deterioration of B-cell function that could be partly protected against by the inclusion of aminoguanidine into culture media. The effects of aminoguanidine were accompanied by a reduction of elevated concentrations of AGEs in the islets. However, the previous study did not answer the question of the extent to which these findings reflected *in vivo* conditions. We have presently addressed this question by testing effects of aminoguanidine *in vivo* in a rat transplantation model using a standard dosage of the compound that is known to affect diabetic complications. Upon finding beneficial effects, we compared them with those of ALT-711. ALT-711 is a stable 4,5-dimethyl thiazolium derivative of the prototype compound *N*-phenacylthiazolium bromide that has been documented to specifically break AGEs both *in vitro* and *in vivo* [11, 12].

‡ Corresponding author: Prof. Valdemar Grill, Department of Internal Medicine, Section of Endocrinology, Norwegian University of Science and Technology, N-7006, Trondheim, Norway. Tel. +47-73868042; FAX +47-73867546; E-mail: valdemar.grill@medisin.ntnu.no

§ *Abbreviations:* AGEs, advanced glycation end products.

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TABLE 1. Body weights and blood glucose levels of rats before and after treatment

Animals	N	Body weight (g)		Blood glucose level (mmol/L)	
		Before treatment	End of treatmemt	Before treatment	End of treatment
Experiments with aminoguanidine protocols					
A 0–2 weeks, without washout					
Normal rats	5	205 ± 10	239 ± 8	4.8 ± 0.2	6.3 ± 1.1
Control diabetic rats	7	187 ± 15	215 ± 14	26.0 ± 2.0	21.0 ± 2.5
Aminoguanidine-treated diabetic rats	6	175 ± 7	205 ± 6	21.7 ± 1.4	18.4 ± 2.3
B 0–8 weeks, without washout					
Normal rats	11	350 ± 9	381 ± 9	5.0 ± 0.2	4.6 ± 0.1
Control diabetic rats	11	252 ± 7	281 ± 7	22.2 ± 0.6	21.0 ± 1.0
Aminoguanidine-treated diabetic rats	12	233 ± 4	265 ± 6	23.7 ± 0.6	20.1 ± 0.6
C 0–2 weeks, after washout					
Control diabetic rats	6	225 ± 4	230 ± 4	25.8 ± 2.1	25.8 ± 1.7
Aminoguanidine-treated diabetic rats	6	230 ± 6	238 ± 6	25.6 ± 0.9	25.2 ± 0.7
D 2–10 weeks, after washout					
Control diabetic rats	5	223 ± 16	233 ± 15	21.7 ± 0.6	26.0 ± 2.4
Aminoguanidine-treated diabetic rats	5	224 ± 17	238 ± 10	20.8 ± 0.3	24.9 ± 1.6
Experiments with ALT-711					
Control diabetic rats	7	202 ± 7	230 ± 7	25.5 ± 1.1	25.6 ± 0.5
ALT-711-treated diabetic rats	7	206 ± 7	229 ± 5	25.9 ± 0.9	25.2 ± 1.0

MATERIALS AND METHODS

Animals

The experimental protocols were approved by the Stockholm Ethics Committee for Animal Experiments. Male Wistar–Furth rats (B & K Universal) were made diabetic by intravenous injection with 60 mg/kg of streptozocin (Sigma). Diabetes was confirmed by stable levels of blood glucose > 20 mmol/L. These diabetic rats then served as transplant recipients. In addition, male non-diabetic rats of the same strain were used as recipients in some experiments.

Isolation and Transplantation of Islets

Islets of Langerhans were isolated by collagenase treatment from 12–15-week-old female Wistar–Furth rats. Islets were cultured overnight in RPMI-1640 (Life Technologies), after which 200 islets were brought into a capillary and transplanted under the left kidney capsule using a stereo microscope for guidance [13]. Islets from a single donor were always divided to transplant both control and treated rats in each experiment. This procedure served to minimize the influence of individual variations in B-cell function of donors, including a varying impact of the collagenase digestion procedure on islet functions.

Experimental Protocols

EXPERIMENTS WITH AMINOGUANIDINE. Rats were treated with aminoguanidine (Sigma) in the drinking water at the concentration of 1 g/L according to the protocols specified below. Protocol A: Following transplantation, aminoguanidine was administered for a period of 2 weeks. Transplant-bearing kidneys were then perfused sequentially to the treatment with aminoguanidine. Protocol B: Following transplantation, aminoguanidine was administered for 8 weeks. Kidneys were then perfused sequentially to the treatment with aminoguanidine. Protocol C: Following transplantation, aminoguanidine was administered for 2 weeks. Treatment was then stopped for 48 hr after which the kidneys were perfused. Protocol D: Aminoguanidine was administered during weeks 2–10 after transplantation. Treatment was then stopped for 48 hr after which the kidneys were perfused.

IN VIVO EXPERIMENTS WITH ALT-711. ALT-711 was dissolved in water. An amount of 0.1 mg/kg was given by gavage tube at 9 a.m. once a day for 8 weeks. Control rats received tap water by gavage. Perfusion experiments were performed 48 hr after the last administration. Body weights and non-fasting blood glucose levels were measured weekly.

Kidney Perfusion

Graft-bearing kidneys were isolated and initially perfused with Krebs–Ringer bicarbonate buffer supplemented with 20 g/L of bovine serum albumin (Sigma), 20 g/L of dextran T-70 (Pharmacia), and 3.3 mmol/L of glucose. After an equilibration period of 10 min, stimulation was performed with 27.8 mmol/L of glucose, followed by 10 mmol/L of arginine on a background of 3.3 mmol/L of glucose [13]. Effluents were collected every minute and stored at −20° until insulin assay. Grafts were retrieved after perfusion study and stored at −70° until total RNA extraction.

In Vitro *Experiments with ALT-711*

Islets of female Wistar rats were isolated as described above [9]. They were then cultured in RPMI together with 11 or

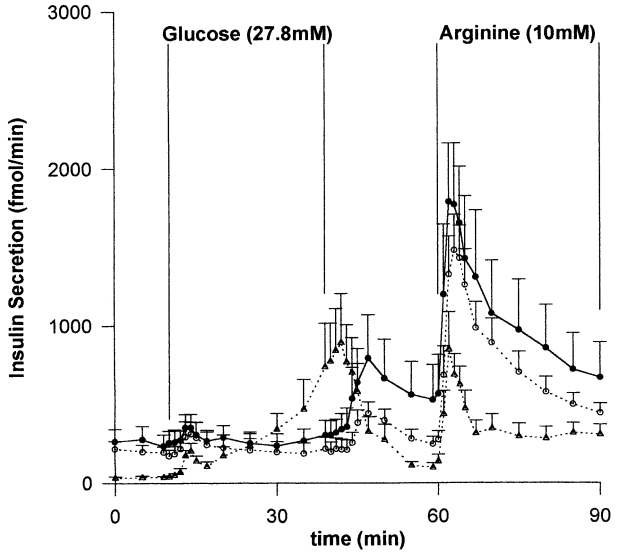

FIG. 1. Insulin secretion from graft-bearing kidneys 2 weeks after transplantation (protocol A). Triangles denote results from normal rats, open circles from control diabetic rats and closed circles from aminoguanidine-treated rats. Aminoguanidine treatment was started immediately after transplantation. Values are means ± SEM of 5–7 experiments.

38 mmol/L of glucose for 6 weeks with or without the further addition of 0.1 mmol/L of ALT-711. Culture medium was exchanged every second day. Six weeks of culture was followed by 24 hr of additional culture in RPMI with 11 mmol/L of glucose in the absence of ALT-711. This procedure served to provide a washout period for any ambient effect of the compound. Insulin release was evaluated by batch-type incubation in Krebs–Ringer bicarbonate buffer containing 3.3 or 28 mmol/L of glucose. Insulin content of islets was measured as previously reported [9] from islets retrieved at the end of incubations.

Assays

Immunoreactive insulin was measured by radioimmunoassay [14]. Blood glucose determinations were carried out using a glucose oxidase method (Accutrend Sensor Glucose, Boehringer Mannheim). Total amounts of RNA in islet grafts were extracted by an acid guanidinum–phenol–chloroform method [15], and preproinsulin mRNA levels were determined by a non-saturated solution hybridization assay as previously reported [9].

Presentation of Data and Statistical Analysis

The total secretion of insulin was calculated from the area under the curve by trapezoidal rule and converted to moles per minute. Results in tables and in figures are expressed as means ± SEM. Because of the sizable variations between the different series (which were performed over several years), the results were normalized in relation to results of the control rats included in each particular series of experiments. Tests of significance were done using Student's *t*-test (two-tailed). A probability value < 0.05 was considered to be significant.

RESULTS

Characteristics of Rats

The marked hyperglycemia of the diabetic recipient rats remained stable after transplantation. These levels were unaffected by aminoguanidine or ALT-711 treatment. Body weights of the recipient rats were also unaffected by the two compounds (Table 1).

Effect of Aminoguanidine Treatment on Insulin Secretion from Islet Grafts

A 2-week transplantation period to the diabetic recipients totally abolished an insulin response to glucose from the transplanted grafts, whereas the insulin response to arginine was upheld compared to non-diabetic recipients. Treatment with aminoguanidine for these 2 weeks (protocol A) failed to affect glucose- or arginine-induced insulin secretion from the grafts (Fig. 1, Table 2). Prolonging the transplantation period in the diabetic recipients from 2 to 8 weeks led to a further

TABLE 2. Effect of aminoguanidine on B-cell function of islet grafts

Protocols	Animals	N	Insulin secretion (% of control diabetic rats)			Preproinsulin mRNA content (% of control diabetic rats)
			3.3 mM glucose	27.8 mM glucose	10 mM arginine	
A	0–2 weeks without washout					
	Normal rats	5	15 ± 4	131 ± 50	47 ± 12	86 ± 10
	Control diabetic rats	7	100 ± 22	100 ± 36	100 ± 18*	100 ± 10
	Aminoguanidine-treated diabetic rats	6	126 ± 44	126 ± 37	135 ± 45	82 ± 19
B	0–8 weeks without washout					
	Normal rats	11	63 ± 11	411 ± 62	233 ± 61	369 ± 25
	Control diabetic rats	11	100 ± 15	100 ± 16†	100 ± 18	100 ± 22†
	Aminoguanidine-treated diabetic rats	12	164 ± 21‡	135 ± 12	202 ± 40*	183 ± 20‡

*$P < 0.05$ versus normal rats.
†$P < 0.01$ versus normal rats.
‡$P < 0.05$ versus control diabetic rats.

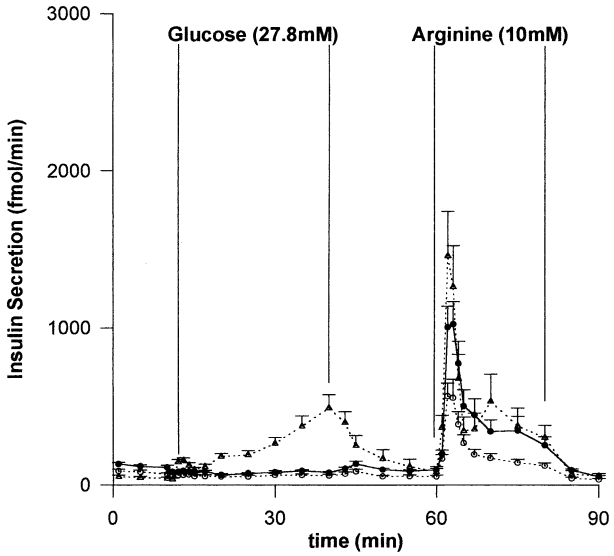

FIG. 2. Insulin secretion from graft-bearing kidneys 8 weeks after transplantation (protocol B). Symbols are as in Fig. 1. Aminoguanidine treatment was started after transplantation and continued for 8 weeks. Values are means ± SEM of 11–12 experiments.

reduction of B-cell function compared to non-diabetic recipients (Fig. 2, Table 2). The secretory response to 10 mmol/L of arginine was thus reduced to 43%. Treatment of diabetic recipients with aminoguanidine during the 8 weeks following transplantation (protocol B) significantly improved arginine-induced insulin release (Fig. 2, Table 2). However, this treatment failed to restore to any degree glucose-induced insulin release from the grafts.

Our previous studies *in vitro* indicated that a washout period could enhance the beneficial effect of aminoguanidine [9]. As this washout effect was presumably due to a documented negative effect of ambient aminoguanidine on insulin secretion [16], we concluded that this negative effect could partly reduce a beneficial effect of long-term aminoguanidine treatment. We therefore re-tested the influence of aminoguanidine treatment for 2 weeks, adding to the experimental protocol a 48-hr period without drug administration before kidney isolation and perfusion (protocol C). Under these conditions, treatment with aminoguanidine was followed by a definite improvement in arginine-induced insulin secretion (512 ± 129%, N = 6, $P < 0.05$).

The importance of early versus late effects of aminoguanidine during transplantation was further investigated by delaying aminoguanidine treatment until two weeks after the date of transplantation. Treatment with aminoguanidine was then continued for the following 8 weeks (protocol D). Also under these conditions aminoguanidine treatment did increase arginine-induced insulin secretion (564 ± 116% increase, N = 5, $P < 0.05$).

Effect of Aminoguanidine Treatment on Preproinsulin mRNA

An 8-week treatment period with aminoguanidine following transplantation was associated with a significant increase in preproinsulin mRNA content (Table 2). In protocol D (aminoguanidine treatment during weeks 2–10), treatment with aminoguanidine affected, but not significantly, preproinsulin mRNA contents of islet grafts (192 ± 50%, N = 5, $P = 0.16$). No effect on preproinsulin mRNA was observed in experiments with aminoguanidine treatment during weeks 0–2 without washout (Table 2, protocol A) or with washout (82.3 ± 18.3% of control, protocol C).

Effect of ALT-711 Treatment on B-cell Function of Islet Grafts

An 8-week treatment with ALT-711 failed to improve any parameters of B-cell function of islet grafts. There were no significant differences in insulin secretion or preproinsulin mRNA content (Table 3).

Effect of ALT-711 on B-cell Function of Cultured Rat Islets

A 6-week period of culture at 38 mmol/L of glucose impaired insulin secretion (Table 4). The inclusion of 0.1 mmol/L of ALT-711 did not affect this B-cell dysfunction. ALT-711 did not increase, but rather reduced the insulin content of high-glucose-cultured islets.

DISCUSSION

Previous studies have demonstrated that aminoguanidine prolongs the survival of islet xenografts in diabetic recipients [17], these effects likely being secondary to effects on immunological and/or inflammatory parameters. The present study has demonstrated, for the first time, that aminoguanidine *in vivo* improves B-cell function in islet syngeneic grafts, an experimental situation in which rejection processes are not an obvious problem. The present data

TABLE 3. Effect of ALT-711 on B-cell function of transplanted islets

Animals	*n*	Insulin secretion (% of control diabetic rats)			Preproinsulin mRNA content (% of control diabetic rats)
		3.3 mM glucose	27.8 mM glucose	10 mM glucose	
Control diabetic rats	7	100 ± 0	100 ± 98	100 ± 31	100 ± 11
ALT-711-treated diabetic rats	7	100 ± 0	14 ± 10	84 ± 26	98 ± 12

TABLE 4. Effect of ALT-711 on B-cell function of cultured islets

Culture conditions	N	Insulin secretion (pmol/islet hr)		Insulin content (pmol/islet)
		3.3 mM glucose	28 mM glucose	
11 mM glucose	11	0.0 ± 0.0	2.6 ± 0.4	13.4 ± 3.1
38 mM glucose	11	0.2 ± 0.1*	0.6 ± 0.1*	9.1 ± 0.9
38 mM glucose with ALT-711	11	0.2 ± 0.1	0.7 ± 0.1	3.8 ± 0.7†

*$P < 0.01$ versus 11 mM glucose.
†$P < 0.01$ versus 38 mM glucose.

corroborate our previous findings of beneficial effects with aminoguanidine *in vitro* in 6-week-cultured islets. In further correspondence with the *in vitro* results, we found evidence that a "washout" period without drug treatment can, under some conditions, enhance the beneficial effects of aminoguanidine. The importance of a "washout" period was evident from the two protocols in which 2 weeks of aminoguanidine were administered: only the protocol that included a "washout" period showed a beneficial effect on insulin secretion. The beneficial effects of aminoguanidine were selective in so far that arginine-induced, but not glucose-induced, insulin secretion was improved. This selectivity was anticipated, since our previous studies indicated that overstimulation rather than any effect by glucose *per se* leads to selective inhibition of glucose-induced insulin secretion [18]. The enhancing effect of aminoguanidine on preproinsulin mRNA could reflect an effect on insulin biosynthesis. Alternatively, or additionally, the effect may reflect an increase in the B-cell mass of the transplants. B-cell morphometry would be necessary to establish this point.

The question remains as to the mechanisms by which aminoguanidine exerts its beneficial effects. We previously hypothesized that the formation of AGEs in B-cells impairs B-cell function. In an earlier *in vitro* study, improvement of B-cell function by aminoguanidine was seen in conjunction with reduced islet AGE-associated fluorescence [9]. These data supported the notion of a relationship between the formation of AGEs and the deterioration of B-cell function. However, it was realized that the *in vitro* study did not prove a cause and effect relationship and furthermore, that other effects of aminoguanidine than those on AGE formation could be important for B-cell function in the *in vivo* situation.

Aminoguanidine inhibits the ubiquitous enzyme nitric oxide synthase in different tissues [6, 7] including pancreatic islets [19], and inhibition of nitric oxide production is reported to retard allograft rejection [20]. Although the present transplantation procedure involved syngeneic animals, it seems possible that surgical trauma and hypoxia increased the production of nitric oxide after transplantation and that this production could play a role in the engraftment of islets following operation. The process of engraftment is known to be almost completed during the first 2 weeks following transplantation [21], and nitric oxide production was reported to be highest 2 days after islet portal transplantation and to decline thereafter [22]. We therefore tested whether aminoguanidine would also exert a beneficial effect when treatment with the drug was delayed to start 2 weeks after the date of transplantation. Our results show this indeed to be the case. Hence, the effect of aminoguanidine on nitric oxide synthase does not appear at least to be the major one behind the beneficial effects observed herein.

To gain further insight into the mechanism(s) by which aminoguanidine influences B-cell function, we evaluated the ability of another AGE reducing compound, the newly developed AGE-breaking compound ALT-711 [11, 12]. This drug was tested in concentrations documented to be biologically effective, i.e. those normalizing large artery properties affected by diabetes [11]. In contrast to experiments with aminoguanidine, ALT-711 failed to affect B-cell function *in vivo*. It was also ineffective *in vitro* under the same high-glucose conditions where aminoguanidine was previously shown to be effective [9]. The failure of ALT-711 to reproduce *in vivo* and *in vitro* the effects of aminoguanidine thus indicates that other effects of aminoguanidine than those reported on AGEs are equally or more important for the beneficial effects observed in the present study.

Antioxidant agents have been reported to protect against hyperglycemia-induced reduction of insulin gene promoter activity [23], and hyperglycemia may also trigger apoptosis by oxidative modifications [24]. Being a reactive hydrazine, aminoguanidine has the potential to interact with carbonyl groups of different biological constituents. It was recently reported that aminoguanidine potently inhibits semicarbazide-sensitive amine oxidase, which could lead to a lesser production of cytotoxic formaldehyde and methylglyoxal [8]. The importance of an antioxidant effect is supported by the finding that beneficial effects of aminoguanidine in a B-cell line were shared by the antioxidative *N*-acetyl-L-cysteine [25]. Antioxidant, non-AGE-linked effects could thus be important for the effects of aminoguanidine we observed. However, it is recognized that the mechanisms whereby aminoguanidine exerts its beneficial effects in the present study have not been fully elucidated and may involve more than one mechanism.

In conclusion, this study has shown that aminoguanidine *in vivo* improves B-cell function of rat islets transplanted to diabetic recipients. The beneficial effects of aminoguanidine are exerted beyond the implantation period, making it

unlikely that effects on nitric oxide synthase are crucially important. The effects exerted by aminoguanidine are not reproduced by the AGE-breaking compound ALT-711, making it unlikely that inhibition of AGE accumulation can explain the beneficial effects. We hypothesize that the effects of aminoguanidine on B-cells *in vivo* are, to a major extent, secondary to the reduction of glycooxidation processes.

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