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Dimethylarginine dimethylaminohydrolase inhibition and asymmetric dimethylarginine accumulation contribute to endothelial dysfunction in rats exposed to glycosylated protein: Effects of aminoguanidine

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Abstract

Objectives: To determine whether alterations of endogenous asymmetric dimethylarginine (ADMA) concentration and dimethylarginine dimethylaminohydrolase (DDAH) activity are involved in endothelial dysfunction induced by glycosylated bovine serum albumin (GBSA) in rats and effects of aminoguanidine on them.

Methods: Endothelium-dependent relaxation of aortic rings from Sprague–Dawley rats after treatment with GBSA in vitro and in vivo was tested. Serum concentrations of ADMA, nitrite/nitrate, and activities of aortic DDAH, nitric oxide synthase (NOS) and superoxide dismutase were measured in GBSA-treated rats. Moreover, serum contents of glycosylated serum protein, and malondialdehyde were also assayed.

Results: Endothelium-dependent relaxation was significantly impaired either by incubation of aortic rings with GBSA (1.70 mmol/l) in vitro for 60 min or by injection of GBSA (35 mg/kg/d, i.v.) to normal rats for 4 weeks, and serum ADMA levels were remarkably elevated in GBSA-treated rats, which was accompanied by decreases of nitrite/nitrate concentrations, NOS and DDAH activities. Furthermore, elevated glycosylated serum protein, malondialdehyde levels, and reduced superoxide dismutase activity were also observed in GBSA-treated rats. Treatment with aminoguanidine not only improved impairment of endothelium-dependent relaxation but also prevented elevation of endogenous ADMA, which were concomitant with increases of nitrite/nitrate concentration, NOS and DDAH activity. Serum levels of glycosylated serum protein, malondialdehyde, and vascular superoxide dismutase activity were also normalized after aminoguanidine treatment.

Conclusions: Decreased DDAH activity and elevated endogenous ADMA is implicated in endothelial dysfunction of rats exposed to GBSA. Aminoguanidine can protect endothelium of rat aorta against injury induced by GBSA both in vitro and in vivo.

Keywords: Dimethylarginine dimethylaminohydrolase; Asymmetric dimethylarginine; Endothelium-dependent relaxation; Glycosylated bovine serum albumin; Aminoguanidine

1. Introduction

Diabetes mellitus is associated with accelerated atherosclerosis and an increased prevalence of cardiovascular diseases, which have become the principal cause of morbidity and mortality in individuals with diabetes mellitus [1,2]. Endothelial dysfunction, characterized by impaired nitric oxide (NO)-mediated endothelium-dependent vasodilatation, is a common pathogenesis in the development of diabetic vascular diseases [3,4]. It is well known that advanced glycosylated end products (AGEs), which are produced by a nonenzymatic reaction between glucose and proteins, play an important role in endothelial dysfunction associated with diabetes mellitus [5–7]. Many studies have demonstrated that the extent of impairment of endothelium-dependent vasodilatation was

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positively correlated with serum concentrations of AGEs in patients [5] and animals [6] with diabetes mellitus. Exposure of isolated vessels to exogenous AGEs might mimic the impairment of endothelium-dependent relaxation observed in diabetes mellitus [7]. Although AGEs have been reported to accelerate the inactivation of NO via oxidative stress [6,8], the precise mechanisms underlying endothelial dysfunction induced by AGEs remain to be elucidated.

Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase (NOS), has been considered to be an important and novel risk factor for endothelial dysfunction. Our previous studies demonstrated that serum concentrations of endogenous ADMA were significantly elevated in animals and patients with hypercholesterolemia [9,10], diabetes mellitus [11–13], and ageing [14]. ADMA is thought to derive from the catabolism of proteins containing methylated arginine residues and release as the proteins are hydrolyzed [15], and its major catabolism is via the enzyme dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and L-citrulline [16]. It has been reported that pharmacological inhibition of DDAH leads to local accumulation of ADMA [17]. Therefore, DDAH activity is a key determinant of endogenous ADMA concentrations. Many studies have demonstrated that endogenous ADMA were significantly elevated in experimental animals and patients with diabetes mellitus [11–13,18,19], and DDAH activity was inhibited in aorta of diabetic rats and in endothelial cells exposed to high glucose, resulting in the accumulation of ADMA [20]. Serum levels of glycosylated protein and endogenous ADMA were normalized after improving metabolic control in diabetic rats and patients [12,21]. Based on these reports, we hypothesized that AGEs-induced impairment of endothelium-dependent vasodilatation may be secondary to the elevation of endogenous ADMA as a result of decreased DDAH activity. Accordingly, it is important to search for a pharmacological approach to prevent the role of AGEs in the development of diabetic vascular complications.

Aminoguanidine not only inhibit inducible NOS but also inhibit AGEs formation, therefore preventing the development of diabetic angiopathy [22]. In addition, aminoguanidine has been reported to have antioxidant property [23]. But it is unknown whether aminoguanidine can directly protect against the impairment of endothelium-dependent relaxation induced by AGEs in vitro or in vivo, and whether improving DDAH activity and decreasing endogenous ADMA concentration contribute to this protective effect of aminoguanidine. Accordingly, this study was designed to investigate the effect of aminoguanidine on impaired endothelium-dependent relaxation of isolated rat aorta induced by exogenous glycosylated bovine serum albumin (GBSA) in vitro or in vivo, and to determine the impacts of GBSA or aminoguanidine treatment on endogenous ADMA concentration in serum and DDAH activity in aorta from rats administered with exogenous GBSA for 4 weeks.

2. Methods

2.1. Reagents

Acetylcholine, phenylephrine, sodium nitroprusside, aminoguanidine, bovine serum albumin (BSA), glucose, ADMA, antipyrine and diacetyl monoxime were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Thiobarbituric acid was obtained from Fluka (Milwaukee, WI, USA). Superoxide dismutase was purchased from Changsha Biological Pharmacological Company (Hunan, China). The kits for measuring glycosylated serum proteins, nitrite/nitrate, protein content and NOS, superoxide dismutase activity were products of the Nanjing Jiancheng Bioengineer Institute (Jiangsu, China), while the commercial kit of creatinine was a product of Zhongsheng Bioengineering Company (Beijing, China).

2.2. Preparation of GBSA

Exogenous GBSA was prepared as described previously [24,25]. Briefly, 2.5 g BSA was dissolved in 100 ml phosphate buffer saline (PBS, 0.5 mol/l, pH 7.4) with 1 mol D-glucose and incubated at 37 °C for 8 weeks under sterile and photophobic conditions. BSA was purchased from Sigma, which was of high purity and low endotoxin levels (<0.1 ng/ml). The formation of GBSA was checked by measurement of the characteristic yellow-brown pigment and fluorescence at 450 nm on excitation and 390 nm on emission with a fluorescence spectrometer [24]. Nonglycosylated BSA was prepared under the same conditions except glucose. Subsequently, both GBSA and nonglycosylated BSA were extensively dialyzed against PBS, which has been shown to remove BSA fragments and other unnecessary components [25,26]. The final preparations were stored at −70 °C for use.

2.3. Experimental animals

The study protocol was approved by the Animal Care and Use Committee of Central South University. Male Sprague–Dawley rats (weighing 220 ± 20 g) were obtained from Central South University Animal Services (Hunan, China). After an adaptation period, rats were subjected to the following experiments in vitro or in vivo.

2.4. Experiments in vitro

Rats were anaesthetized with sodium pentobarbital (30 mg/kg, i.p.). The thoracic aorta was immediately isolated and placed in 4 °C Krebs' solution. The thoracic aorta was cleaned of connective tissue and then cut into rings of 3–4 mm in length, and the rings were suspended horizontally between two stirrups in organ chambers filled with 5 ml Krebs' solution at 37 °C, aerated continuously with 95% O_2 and 5% CO_2. Endothelium-dependent relaxation of aortic rings in response to cumulative concentrations of

acetylcholine (0.03–3 μmol/l) was examined as described previously [12]. If the ring's maximal relaxation (E_{max}) to 3 μmol/l acetylcholine is more than 80%, it was considered as endothelium-intact and used in the study.

After each ring was serially washed, aortic rings of GBSA-treated groups were incubated with GBSA (1.10 and 1.70 mmol/l) for 60 min, respectively. The aortic rings from groups of aminoguanidine plus GBSA treatment were pre-incubated with various concentrations of aminoguanidine (50, 100, and 500 μmol/l) for 15 min and then exposed to 1.70 mmol/l GBSA for another 60 min in the presence of aminoguanidine, respectively. Some rings were exposed to 1.70 mmol/l nonglycosylated BSA for 60 min in the absence of any drugs to be used as BSA control, and other rings were incubated with 500 μmol/l aminoguanidine alone for 60 min to be used as the aminoguanidine control. Normal control rings were incubated with Krebs' solution for 60 min in the absence of GBSA or aminoguanidine. After these incubations, the endothelium-dependent relaxation to acetylcholine was again examined, and endothelium-independent relaxation to 10 μmol/l sodium nitroprusside was also tested at the end of the experiment. The doses of GBSA used in the experiment in vitro were chosen according to our previous studies showing that endothelium-dependent relaxation of thoracic aorta was significantly attenuated in streptozotocin-induced diabetic rats when their serum levels of glycosylated serum proteins reached a level of 1.70 mmol/l compared with control rats whose serum concentration of glycosylated serum proteins was 1.10 mmol/l [12].

2.5. Experiments in vivo

2.5.1. Animal treatment

Male Sprague–Dawley rats (weighing 220 ± 20 g) were randomly divided into the following groups. In the group of GBSA treatment, rats were given tail vein injection with 35 mg/kg/d GBSA for 4 weeks, and in the group of aminoguanidine plus GBSA treatment, the rats received tail vein infusion with GBSA followed immediately by injection of aminoguanidine (100 mg/kg/d) for 4 weeks. The other rats, respectively, received an equal volume of physiological saline or nonglycosylated BSA (35 mg/kg/d) or aminoguanidine (100 mg/kg/d) alone for 4 weeks to be used as age-matched control, BSA control, and aminoguanidine control. The doses of GBSA and aminoguanidine used in the experiment in vivo was chosen according to previous studies showing that infusion of exogenous GBSA (25–100 mg/kg/d) to normal rats for 2–4 weeks resulted in a significant inhibition of endothelium-dependent vasodilator responses to acetylcholine, and treatment the animals with aminoguanidine at the doses of 100 mg/kg/d could prevent AGEs accumulation on vessels and preserve endothelial function in rats with infusion of exogenous AGEs [27,28]. The tail vein injection of rat was performed by lightly anaesthetizing the rats and placing their tails close to a lamp to ensure enough dilation of tail vein for injection. At the first time, the injection site was selected from the distal part of the tail. In the following days, repeated injection was avoided by moving up the veins with each injection.

At the end of above treatments, rats were anaesthetized with sodium pentobarbital (30 mg/kg, i.p.). Blood samples were collected from carotid artery, centrifuged at 1500 × *g* for 20 min at 4 °C, and serum was stored at −70 °C for biochemical assays. Thoracic aorta was immediately isolated and placed in 4 °C Krebs' solution. A part of aorta was used for analysis of some enzymes activities and the other was cut into rings for measurement of endothelium-dependent and independent relaxation in the similar way to the experiment in vitro as described above.

2.5.2. Determinations of serum dimethylarginine and L-arginine

Serum (1.0 ml) was pipetted into a tube containing 20 mg 5-sulfosalicylic acid, and then the mixture was left at 4 °C for 10 min. The precipitated protein was removed by centrifugation at 2500 × *g* for 15 min (4 °C) and supernatant was used for measurement of ADMA, symmetric dimethylarginine and L-arginine by high-performance liquid chromatography (HPLC) using a method as described previously [29].

2.5.3. Measurements of serum nitrite/nitrate, glycosylated serum proteins, and malondialdehyde

The serum contents of nitrite were determined using the commercial kit to reflect the levels of nitric oxide by converting nitrate, the stable end product of NO, into nitrite catalyzed by nitrite reductase [29]. The absorbance was determined at 550 nm with a spectrophotometer.

Serum concentrations of glycosylated serum proteins were also detected using the commercial kit [12]. Briefly, an aliquot of serum (0.1 ml) was mixed with 2 ml of nitroblue tetrazolium for 15 min at 37 °C before a stabilizer of the kit was added to the tube. The absorbance of the mixture was read at 530 nm with a spectrophotometer.

In order to assess whether the changes in serum concentration of ADMA as a consequence of GBSA or aminoguanidine treatment could be related to alterations in lipid peroxide and renal clearance, serum contents of malondialdehyde, derived from lipid peroxidation, and creatinine, an index of renal function, were also determined [29]. The serum content of thiobarbituric acid reactive substance, reflecting the level of lipid peroxidation, was measured by a spectrofluorometer, and expressed as the amount of malondialdehyde. The serum levels of creatinine were determined by routine methods.

2.5.4. Assays of aortic DDAH, NOS, and superoxide dismutase activities

Aortas from each group were homogenized in ice-cold phosphate buffer solution (0.1 mol/l, pH 6.5), and centrifuged at 3500 × *g* for 30 min (at 4 °C). The supernatant was used for assay of DDAH, NOS, and superoxide dismutase activity. The DDAH activity of rat aorta was assayed by the conversion of L-citrulline from ADMA as previously described [30]. One

unit of the enzyme was defined as the amount that catalyzed formation of 1 μmol/l L-citrulline from ADMA per minute at 37 °C.

In this study, NOS activity was measured to reflect the inhibition of ADMA on it using a commercial kit [14]. An aliquot of supernatant was incubated with L-arginine, the substrate for NOS, an accelerator, and a developer for 15 min at 37 °C. Afterwards, a clearer and a stop buffer were added to the reaction volume, and then NO production was determined at 530 nm with a spectrophotometer to indirectly reflect the activity of NOS. One unit of the enzyme was defined as the amount that catalyzed formation of 1 nmol/l NO from L-arginine per min per mg protein.

To examine whether the resultant changes in endogenous ADMA concentrations and DDAH activity after GBSA or aminoguanidine treatment are related to alterations in antioxidant ability, the activity of superoxide dismutase, the scavenger of superoxide anion, was also determined by monitoring the inhibition of autoxidation of hydroxylamine by previous described method [29]. The protein content of supernatant was measured by the method of coomassie brilliant blue assay.

2.6. Statistical analysis

Results were expressed as mean ± S.E.M. The significance of differences between groups was tested with one-way ANOVA followed by the Newman–Keuls test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of treatment with GBSA and drugs in vitro on endothelium-dependent relaxation of rat aortas

Fig. 1 shows that endothelium-dependent relaxation responses to cumulative concentrations of acetylcholine (0.03–3 μmol/l) were significantly inhibited after exposure of aortic rings to 1.70 mmol/l GBSA for 60 min compared with control group ($P < 0.01$). However, neither 1.10 mmol/l GBSA nor 1.70 mmol/l nonglycosylated BSA had significant effect on endothelium-dependent relaxation.

Pretreatment of aortic rings with various concentrations of aminoguanidine (50, 100, 500 μmol/l) for 15 min and then exposure to 1.70 mmol/l GBSA for 60 min in the presence of aminoguanidine, the inhibition of endothelium-dependent relaxation induced by GBSA was significantly attenuated in a concentration-dependent manner ($P < 0.01$, Fig. 2). In order to determine whether oxidation injury is involved in the detrimental effect of GBSA on endothelium-dependent relaxation, and whether antioxidant action contribute to the benefit effect of aminoguanidine on vascular endothelial function, we compared effect of superoxide dismutase on the inhibition of GBSA in isolated aortic rings with those of aminoguanidine. In an analogous manner, pretreated with

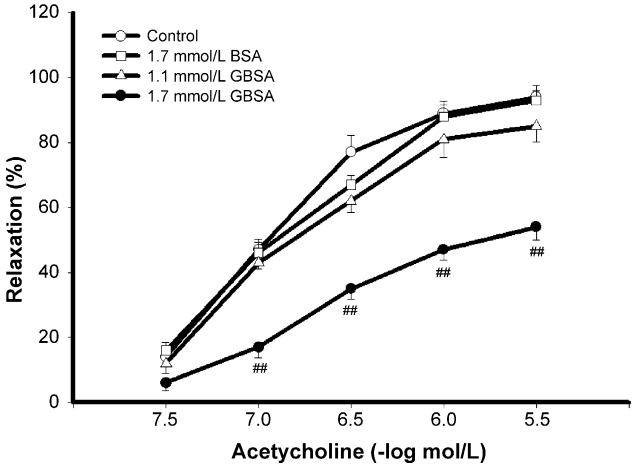

Fig. 1. Endothelium-dependent relaxation response to acetylcholine of isolated rat aortic rings after exposure to GBSA in vitro. Isolated rat aortic rings were incubated with Krebs' solution (control: ◯), 1.7 mmol/l bovine serum albumin (BSA, □), 1.1 mmol/l glycosylated bovine serum albumin (GBSA, △), and 1.7 mmol/l GBSA (●) for 60 min in organ bath chamber, respectively. The endothelium-dependent relaxation responses to accumulating concentrations of acetylcholine were measured. Data are expressed as $\bar{x} \pm$ S.E.M., $n = 6$. $^{\#\#}P < 0.01$ vs. control.

200 U/ml superoxide dismutase for 15 min and then co-treated with 1.70 mmol/l GBSA for a further 60 min also weakened the inhibition of endothelium-dependent relaxation of aortic rings induced by GBSA in vitro, which was similar to the effect of 500 μmol/l aminoguanidine ($P < 0.01$, Fig. 3). However, either aminoguanidine or superoxide dismutase per se had no effect on endothelium-dependent relaxation of aortic rings (P = NS, Figs. 2 and 3).

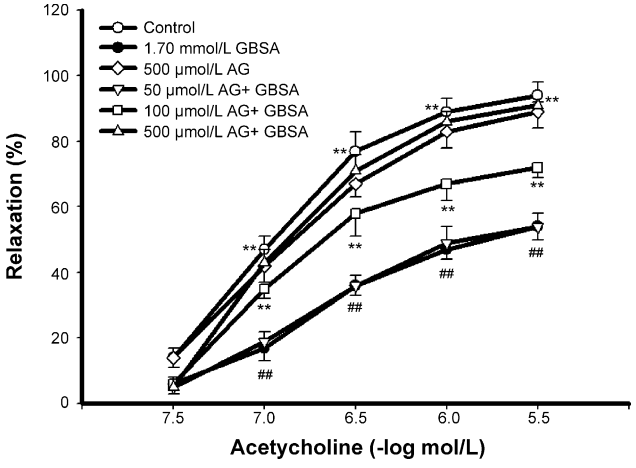

Fig. 2. Effect of aminoguanidine on the inhibition of endothelium-dependent relaxation induced by GBSA in vitro. Isolated rat aortic rings were incubated with Krebs' solution (control, ◯), 1.7 mmol/l glycosylated bovine serum albumin (GBSA, ●), and 500 μmol/l aminoguanidine (AG, ◊) alone for 60 min, respectively, or were pre-incubated with 50 μmol/l (▽), 100 μmol/l (□), 500 μmol/l (△) AG for 15 min and then co-incubated with 1.70 mmol/l GBSA for another 60 min, respectively. The endothelium-dependent relaxation responses to accumulating concentrations of acetylcholine were measured. Data are expressed as $\bar{x} \pm$ S.E.M., $n = 6$. $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GBSA group.

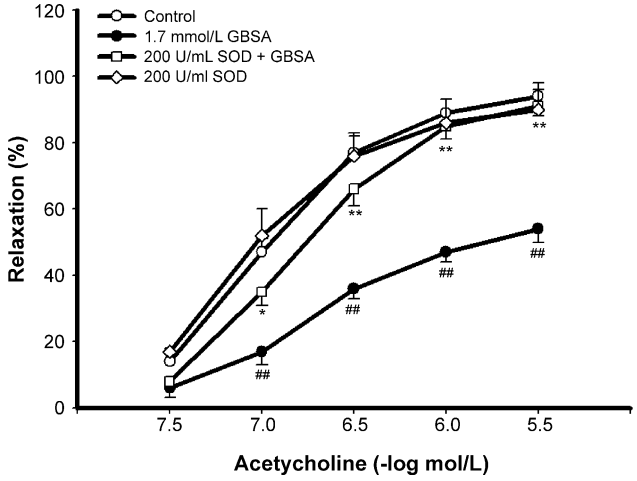

Fig. 3. Effect of SOD on the inhibition of endothelium-dependent relaxation induced by GBSA in vitro. Isolated rat aortic rings were incubated with Krebs' solution (control, ◯), 1.7 mmol/l glycosylated bovine serum albumin (GBSA, ●), 200 U/ml superoxide dismutase (SOD, ◊) alone for 60 min, respectively, or were pre-incubated with 200 U/ml SOD for 15 min and then co-incubated with 1.70 mmol/l GBSA (SOD + GBSA, □) for 60 min. The endothelium-dependent relaxation responses to accumulating concentrations of acetylcholine were measured. Data are expressed as $\bar{x} \pm$ S.E.M., $n = 6$. $^{\#\#}P < 0.01$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. GBSA group.

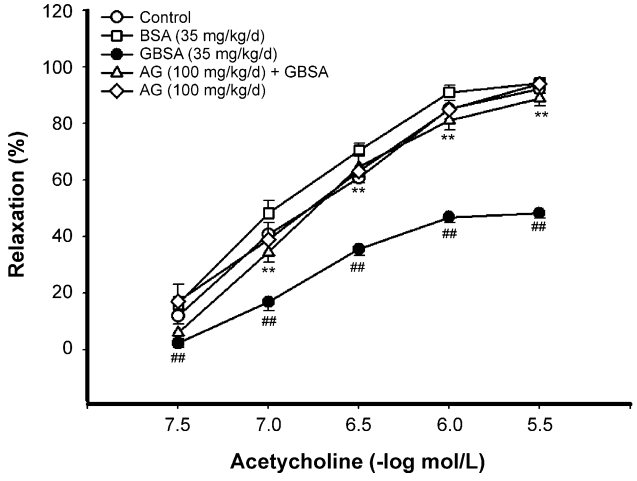

Fig. 4. Effect of chronic aminoguanidine treatment on the inhibition of endothelium-dependent relaxation of rat aortas induced by GBSA in vivo. Endothelium-dependent relaxation to acetylcholine was tested in aortic rings from rats administered with physiological saline (control, ◯), 35 mg/kg/d bovine serum albumin (BSA, □), 35 mg/kg/d glycosylated bovine serum albumin (GBSA, ●), 100 mg/kg/d aminoguanidine (AG) plus GBSA (△), and AG alone (◊) for 4 weeks. The endothelium-dependent relaxation responses to accumulating concentrations of acetylcholine were measured. Data are expressed as $\bar{x} \pm$ S.E.M., $n = 6$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control; $^{**}P < 0.01$ vs. GBSA group.

3.2. Effects of treatment with GBSA and aminoguanidine in vivo on endothelium-dependent relaxation of rat aortas

To confirm the detrimental effect of GBSA and the protective effect of aminoguanidine on endothelium-dependent relaxation of rat aorta, the experiment in vivo were performed. As shown in Fig. 4, with injection of GBSA (35 mg/kg/d) to normal rats for 4 weeks significantly impaired endothelium-dependent relaxation of isolated aortic rings to acetylcholine compared with control rats ($P < 0.01$). Aminoguanidine treatment for 4 weeks remarkably improved the impairment of endothelium-dependent relaxation induced by GBSA in vivo ($P < 0.01$). But administration of nonglycosylated BSA (35 mg/kg/d) and aminoguanidine (100 mg/kg/d) alone to normal rats had no significant effect on endothelium-dependent relaxation (P = NS, Fig. 4).

3.3. Effects of GBSA and aminoguanidine on the pathway of DDAH/ADMA/NOS/NO

In order to further explore the mechanisms by which AGEs impair endothelium-dependent relaxation of aorta and aminoguanidine antagonizes the detrimental effect of GBSA, the changes in the pathway of DDAH/ADMA/NOS/NO were determined. Table 1 illustrates the effects of GBSA and aminoguanidine treatment in vivo on serum levels of ADMA, symmetric dimethylarginine, L-arginine, nitrite/nitrate, aortic NOS and DDAH activities. Compared with control, significant decreased DDAH activity was observed in aortas from rats with injection of GBSA for 4 weeks ($P < 0.01$), and aminoguanidine treatment restored DDAH activity nearly to a normal level.

Table 1
Effects of treatment with glycosylated bovine serum albumin and aminoguanidine on the pathway of DDAH/ADMA/NOS/NO in rats

Groups	ADMA (μmol/l)	SDMA (μmol/l)	L-Arginine (μmol/l)	Nitrite/nitrate (nmol/l)	NOS activity (U/mg protein)	DDAH activity (U/g protein)
Control	1.18 ± 0.19	0.29 ± 0.01	25.81 ± 3.37	47.50 ± 5.10	0.57 ± 0.06	0.095 ± 0.002
BSA	1.57 ± 0.08	0.38 ± 0.12	33.51 ± 5.07	47.35 ± 1.70	0.50 ± 0.09	0.090 ± 0.003
GBSA	2.21 ± 0.16$^{\#\#}$	0.28 ± 0.04	36.42 ± 8.94	27.00 ± 4.50$^{\#\#}$	0.27 ± 0.05$^{\#\#}$	0.031 ± 0.004$^{\#\#}$
AG + GBSA	1.59 ± 0.18*	0.34 ± 0.09	31.79 ± 4.09	43.60 ± 4.80**	0.51 ± 0.07**	0.082 ± 0.004**
AG	1.23 ± 0.05	0.30 ± 0.03	27.40 ± 3.83	46.92 ± 3.09	0.55 ± 0.01	0.087 ± 0.003

The serum levels of asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), L-arginine, nitrite/nitrate and activities of vascular DDAH, NOS were determined in rats treated with physiological saline (control), bovine serum albumin (BSA), glycosylated bovine serum albumin (GBSA), aminoguanidine plus GBSA (AG + GBSA), and aminoguanidine alone (AG) for 4 weeks. Data are expressed as $\bar{x} \pm$ S.E.M., $n = 6$.

$^{\#\#}$ $P < 0.01$ vs. control.

* $P < 0.05$ vs. GBSA group.

** $P < 0.01$ vs. GBSA group.

Subsequently, serum levels of ADMA were significantly elevated in GBSA-treated rats compared with control or nonglycosylated BSA-treated rats ($P<0.01$). Treatment with aminoguanidine (100 mg/kg/d) for 4 weeks decreased the concentrations of endogenous ADMA compared with GBSA-treated group ($P<0.05$). On the contrary, serum contents of symmetric dimethylarginine and L-arginine remained unchanged in all groups of experiment in vivo ($P=$NS, Table 1).

As expected, NOS activity was significantly decreased in GBSA-treated rats versus age-matched control or BSA control rats ($P<0.01$). Aminoguanidine treatment completely abolished the inhibition of GBSA on the activity of NOS ($P<0.01$, Table 1).

Nitrite is the oxidative metabolite of NO and is usually used as the indicator of NO production. Administration of GBSA to normal rats for 4 weeks, serum nitrite contents were remarkably decreased compared with their age-matched control ($P<0.01$), which was in company with elevation of endogenous ADMA, while aminoguanidine treatment normalized the change of nitrite levels ($P<0.01$, Table 1). In contrast, aminoguanidine alone and nonglycosylated BSA had no significant effect on the pathway of DDAH/ADMA/NOS/NO ($P=$NS, Table 1).

3.4. Effects of GBSA and aminoguanidine on the ability of antioxidation, the level of lipid peroxidation, and the accumulation of glycosylated serum proteins

In this study, we assayed the activity of vascular superoxide dismutase to assess the antioxidant ability, determined the serum concentration of malondialdehyde to estimate the level of lipid peroxidation, and measured glycosylated serum proteins to reflect the accumulation of exogenous GBSA in rats administered with GBSA for 4 weeks. As shown in Table 2, administration of GBSA (35 mg/kg/d) to rats for 4 weeks induced significantly decreased superoxide dismutase activity as compared to control rats or nonglycosylated BSA-treated rats ($P<0.01$). Aminoguanidine treatment had favorable effects by reversing the decrease of superoxide dismutase activity induced by GBSA ($P<0.01$).

However, serum contents of malondialdehyde, the reflector of lipid peroxidation, were significantly elevated in rats administered with GBSA versus age-matched control rats or nonglycosylated BSA-treated rats ($P<0.01$). This elevation of serum malondialdehyde concentrations was prevented by treatment with aminoguanidine ($P<0.01$, Table 2).

Similarly, there was an increase in serum levels of glycosylated serum proteins after injection of GBSA to rats for 4 weeks ($P<0.05$ versus control), and this elevation was not observed in rats with administration of equal dose of nonglycosylated BSA ($P=$NS). Aminoguanidine treatment attenuated the elevation of serum glycosylated serum proteins induced by GBSA ($P<0.05$ versus GBSA group, Table 2).

Serum concentrations of creatinine were not significantly affected by treatment with GBSA or aminoguanidine and showed no difference between the groups ($P=$NS, Table 2).

4. Discussion

It is well documented that AGE formation is an important risk factor in the pathogenesis of diabetic vascular complications [5–7]. In the present study, we further demonstrated that GBSA remarkably inhibited endothelium-dependent relaxation of rat aorta when incubated with aortic ring in vitro or administered to normal rats in vivo. AGEs exert their cellular effects through interaction with specific cell surface binding proteins, termed receptor for AGE (RAGE). Although we did not determine the change of RAGE, other studies have shown that the interaction of AGEs with RAGE enhanced oxidant stress, potentially contributing to the development of vascular lesions [31]. But the precise mechanisms are not elucidated.

Accumulating evidence has suggested that elevated ADMA, an endogenous competitive inhibitor of NOS, is closely related to the development of endothelial dysfunction in diabetes mellitus [11–13,18–20]. Our previous study demonstrated that serum contents of ADMA were signifi-

Table 2
Effects of treatment with glycosylated bovine serum albumin and aminoguanidine on vascular superoxide dismutase (SOD) activity and serum contents of malondialdehyde (MDA), glycosylated serum protein (GSP), creatinine (Cr) in rats

Groups	SOD (μU/g protein)	MDA (μmol/l)	GSP (mmol/l)	Cr (μmol/l)
Control	490.00 ± 35.00	1.30 ± 0.15	1.20 ± 0.12	57.80 ± 1.40
BSA	483.05 ± 19.46	1.20 ± 0.10	1.10 ± 0.14	55.10 ± 3.20
GBSA	365.30 ± 31.0##	2.20 ± 0.15##	2.00 ± 0.18#	66.40 ± 7.50
AG + GBSA	442.20 ± 34.30**	1.20 ± 0.09**	1.50 ± 0.18*	67.20 ± 5.50
AG	486.03 ± 23.73	1.18 ± 0.08	1.30 ± 0.01	57.18 ± 2.42

The activity of vascular superoxide dismutase (SOD) and serum contents of malondialdehyde (MDA), glycosylated serum protein (GSP), creatinine (Cr) were measured in rats treated with physiological saline (control), bovine serum albumin (BSA), glycosylated bovine serum albumin (GBSA), aminoguanidine plus GBSA (AG + GBSA), and aminoguanidine alone (AG) for 4 weeks. Data are expressed as $\bar{x} \pm$ S.E.M., $n=6$.

# $P<0.05$ vs. control.

$P<0.01$ vs. control.

* $P<0.05$ vs. GBSA group.

** $P<0.01$ vs. GBSA group.

cantly elevated in diabetic rats with poor metabolic control, which was in parallel with the increase of serum contents of glycosylated serum protein, and chronic insulin treatment not only normalized the contents of glycosylated serum protein but also prevented the elevation of serum ADMA and improved the impaired endothelium-dependent vasodilatation [12]. Furthermore, Asagami et al. also showed that plasma ADMA concentrations were decreased in type 2 diabetic patients with improved glycemic control following the metformin treatment [21]. These results indicate that the elevation of ADMA in diabetes mellitus may be associated with the formation of AGEs. The present study provided the first direct evidence that infusion of exogenous GBSA to normal rats remarkably elevated serum ADMA levels, concomitantly decreased NOS activity and nitrite/nitrate concentrations, resulting in impairment of NO-mediated and endothelium-dependent vasodilatation function.

Being that the major metabolic pathway for endogenous ADMA is degradation by DDAH to dimethylamine and L-citrulline [16,17,32], we postulate that the elevation of endogenous ADMA and the impairment of endothelium-dependent relaxation induced by GBSA may be secondary to inhibition of DDAH activity. This hypothesis was tested in the present study by determining DDAH activity in rat aorta, and it was found that DDAH activity of aorta was significantly decreased in rats administered with exogenous GBSA. Taken together, these results suggest that inhibition of DDAH activity may be responsible for the elevation of endogenous ADMA and endothelial dysfunction in rats induced by GBSA.

Recent study has identified that there is a highly reactive cysteine residue (Cys-249) in the active site of DDAH, and its sulfhydryl group predisposes DDAH to easy oxidation or nitrosation to lose its activity [33]. Antioxidants polyethylene glycol-conjugated superoxide dismutase and pyrrolidine dithiocarbamate have been recently reported to preserve DDAH activity in endothelial cells after exposure to high glucose [20] and homocysteine [34]. These studies indicate that DDAH could be modulated by cardiovascular risk factors in a reactive oxygen species-sensitive manner. It is established that AGEs could enhance oxidative stress and promote the inactivation of NO [6,8,31]. The present study showed that injection of exogenous GBSA to rats significantly increased serum content of malondialdehyde, the reflector of lipid peroxidation and decreased activity of vascular superoxide dismutase, and that exogenous superoxide dismutase treatment could prevent the inhibition of endothelium-dependent relaxation in rat aortas induced by GBSA in experiments in vitro. Accordingly, it is reasonable to speculate that GBSA might enhance oxidative stress and decrease antioxidant defense, subsequently resulting in inhibition of DDAH activity via oxidative attack on the sulfhydryl in its active site and accumulation of endogenous ADMA and pathological inhibition of NO synthesis in endothelial cells.

Since endothelial dysfunction is an early stage in developing diabetes mellitus, reversion of this condition may prevent the onset or retard the progression of diabetic vascular complications. Aminoguanidine, an inhibitor of AGEs formation, has been shown to slow the progression of diabetic complications in experimental models and diabetic individuals [22,23]. In the present study, we provided the first evidence that aminoguanidine directly protected against impairment of endothelium-dependent vasodilatation induced by GBSA in vitro and in vivo. In addition, we confirmed that aminoguanidine treatment decreased the accumulation of glycosylated serum proteins in serum of rats administered with exogenous GBSA, which is not due to its effect of inhibiting AGEs formation but may be due to its property as a cross-linking inhibitor to inhibit the covalent attachment and facilitate the renal clearance of exogenous AGEs [28]. Aminoguanidine is well known as a selective inhibitor of inducible NOS [35]. We therefore assayed total NOS and iNOS activity in rat aorta to evaluate the effects of GBSA and aminoguanidine treatment on NOS activity. In agreement with previous studies [36], we found that total NOS activity in GBSA-treated rats was significantly inhibited compared with control rats. Aminoguanidine treatment completely abolished the inhibition of total NOS activity induced by GBSA. However, iNOS activity was not altered by GBSA or aminoguanidine treatment in our study. This result was consistent with others' finding in diabetic rats [37]. To our knowledge, there is no direct evidence about the alterations of iNOS activity in rats treated with GBSA or aminoguanidine to be reported yet. Sugimoto et al. demonstrated that iNOS expression in diabetic rats was not apparent until 26 weeks after induction of diabetes mellitus, while serum AGEs levels were significantly elevated at the early stage of diabetes mellitus (2–4 weeks) [37]. Similar results were reported by Soulis et al. that neither change in iNOS expression were detected in experimental diabetes nor did aminoguanidine affect iNOS expression [38]. Two other NOS inhibitors, L-NAME or methylguanidine could not reproduce the beneficial effect of aminoguanidine on endothelium-dependent relaxation in experimental diabetes [39], indicating that the protective effect of aminoguanidine on vascular endothelial function is not mediated predominantly by iNOS inhibition. This condition appears to be in the case of our experiment. Aminoguanidine has been shown to have direct antioxidant properties [23,40]. Our study also showed that the protective effect of aminoguanidine on the inhibition of endothelial function induced by GBSA in vitro was similar to that of antioxidant superoxide dismutase. In the experiment in vivo, we demonstrated for the first time that chronic aminoguanidine treatment not only significantly increased vascular DDAH activity and decreased endogenous ADMA accumulation but also decreased serum level of lipid peroxidation products and increased vascular superoxide dismutase activity in rats administered with GBSA. Taken considering together, our results indicate that the beneficial effect of aminoguanidine on the GBSA-induced vascular endothelium damage may be ascribed to its antioxidant properties, which may preserve DDAH activity from oxidative modification and subsequently decrease endogenous ADMA

accumulation on the one hand, and prevent the oxidative degradation of NO on the other hand. And these two pathways together enhance the bioavailability of NO, improving the impairment of endothelium-dependent relaxation induced by GBSA.

In conclusion, the present study provides the first evidence that the inhibition of DDAH activity and accumulation of endogenous ADMA may contribute to the endothelial dysfunction of rat induced by GBSA, and aminoguanidine is an effective pharmacological approach in preserving DDAH activity, attenuating ADMA accumulation, and improving endothelial function.

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