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RESEARCH PAPER

Alagebrium attenuates acute methylglyoxal-induced glucose intolerance in Sprague-Dawley rats

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Background and purpose: Alagebrium is a breaker of cross-links in advanced glycation endproducts. However, the acute effects of alagebrium on methylglyoxal (MG), a major precursor of advanced glycation endproducts have not been reported. MG is a highly reactive endogenous metabolite, and its levels are elevated in diabetic patients. We investigated whether alagebrium attenuated the acute effects of exogenous MG on plasma MG levels, glucose tolerance and distribution of administered MG in different organs in Sprague-Dawley rats.
Experimental approach: We measured MG levels (by HPLC), glucose tolerance, adipose tissue glucose uptake, GLUT4, insulin receptor and insulin receptor substrate 1 (IRS-1) protein expression, and phosporylated IRS-1 in rats treated with MG at doses of either 17.25 mg·kg^{-1} i.p. (MG-17 i.p.) or 50 mg·kg^{-1} i.v. (MG-50 i.v.) with or without alagebrium, 100 mg·kg^{-1} i.p.
Key results: Alagebrium attenuated the increased MG levels in the plasma, aorta, heart, kidney, liver, lung and urine after MG administration. In MG-treated rats, glucose tolerance was impaired, plasma insulin levels were higher and insulin-stimulated glucose uptake by adipose tissue was reduced, relative to the corresponding control groups. In rats treated with MG-50 i.v., GLUT4 protein expression and IRS-1 tyrosine phosphorylation were decreased. Alagebrium pretreatment attenuated these effects of MG. In an *in vitro* assay, alagebrium reduced the amount of detectable MG.
Conclusions and implications: Alagebrium acutely attenuated MG-induced glucose intolerance, suggesting a possible preventive role for alagebrium against the harmful effects of MG.
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Keywords: alagebrium; methylglyoxal; glucose intolerance; diabetes

Abbreviations: AGEs, advanced glycation endproducts; AUC, area under the curve; GSH, reduced glutathione; GSSG, oxidized glutathione; IR, insulin receptor; IRS-1, insulin receptor substrate 1; IVGTT, intravenous glucose tolerance test; MG, methylglyoxal; *o*-PD, *o*-phenylenediamine; PI3K, phosphatidylinositol 3-kinase; PTB, phenacylthiazolium bromide; SD, Sprague-Dawley

Introduction

Alagebrium (4,5-dimethylthiazolium) (formerly known as ALT-711) (Figure 1) is a novel breaker of cross-links in advanced glycation endproducts (AGEs) and has been studied mainly for its chronic effects on AGEs (Ulrich and Zhang, 1997; Wolffenbuttel *et al.*, 1998; Susic *et al.*, 2004; Thallas-Bonke *et al.*, 2004; Little *et al.*, 2005; Peppa *et al.*, 2006; Coughlan *et al.*, 2007; Zieman *et al.*, 2007; Guo *et al.*, 2009). The first AGEs cross-link breaking compound discovered in 1996 was phenacylthiazolium bromide (PTB), which reacts with and cleaves covalent cross-links of AGEs-derived proteins. PTB degrades rapidly and hence a more stable derivative, alagebrium, was developed. This compound (210 mg·kg^{-1} twice a day for 8 weeks) given to patients with systolic hypertension reduced vascular fibrosis and markers of inflammation (Zieman *et al.*, 2007). I.p. injection of alagebrium (1 mg·kg^{-1}) daily for 1 or 3 weeks reversed diabetes-induced increase of arterial stiffness measured by *in vivo* and *in vitro* parameters in streptozotocin-induced diabetic rats, and improved impaired cardiovascular function in older rhesus monkeys (Ulrich and Zhang, 1997; Wolffenbuttel *et al.*, 1998). Alagebrium (10 mg·kg^{-1} for 16 weeks) also increased glutathione peroxidase and superoxide dismutase activities in aging rats and reduced oxidative stress (Guo *et al.*, 2009). However, the acute effects of alagebrium against precursors of AGEs, such as methylglyoxal (MG) and glyoxal, are presently not known.

Methylglyoxal, a highly reactive dicarbonyl compound, is a normal product of glucose, fatty acid and protein metabolism (Thornalley, 1996; Desai and Wu, 2007). The clinical significance of MG lies in the fact that it reacts with and modifies certain proteins to form AGEs (Thornalley, 1996; Desai and

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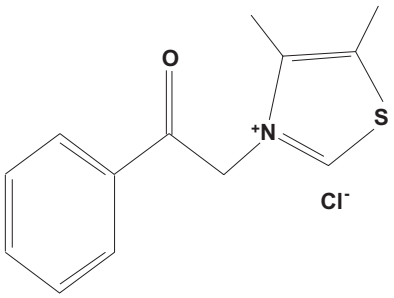

Figure 1 Chemical structure of alagebrium (4,5-dimethylthiazolium).

Wu, 2007; Vlassara and Palace, 2002) and AGEs are implicated in the pathogenesis of vascular complications of diabetes (Vlassara and Palace, 2002). Plasma MG levels in healthy humans are 1 μM or less but are elevated to 2–6 μM in diabetic patients, with a positive correlation to the degree of hyperglycaemia (Wang *et al.*, 2007; McLellan *et al.*, 1994). Sprague-Dawley (SD) rats fed chronically with fructose develop insulin resistance (Hwang *et al.*, 1987; Jia and Wu, 2007). We have shown that incubation of vascular smooth muscle cells with 25 mM glucose or fructose for 3 h increases MG production about fourfold and increases oxidative stress (Dhar *et al.*, 2008). MG modifies the structure of the insulin molecule *in vitro*, in a way that impairs insulin-mediated glucose uptake in adipocytes (Jia *et al.*, 2006). In cultured 3T3-L1 adipocytes MG (20 μM) decreased insulin-induced insulin-receptor substrate-1 (IRS-1) tyrosine phosphorylation and phosphatidylinositol (PI) 3-kinase (PI3K) activity (Jia and Wu, 2007). Incubation of cultured L6 muscle cells with high concentrations of MG (2.5 mM) for 30 min impaired insulin signalling (Riboulet-Chavey *et al.*, 2006), and a very high dose of MG (500 $mg \cdot kg^{-1}$ i.p.) elevated plasma glucose level in cats, by releasing glucose from the liver *via* an adrenergic mechanism (Jerzykowski *et al.*, 1975). In spite of these cellular and molecular studies on MG and insulin signalling, the *in vivo* effect of exogenous MG, especially in pathologically relevant plasma MG concentrations, on glucose tolerance is not known.

Numerous studies have been carried out to study the toxicity of high concentrations of MG *in vitro* (up to 20 mM) (Sheader *et al.*, 2001) and *in vivo* (100 $mg \cdot kg^{-1}$ to 1 $g \cdot kg^{-1}$, i.p. or i.v.). Similar high concentrations of exogenous MG have been employed in most *in vivo* and *in vitro* studies, which raises concern of whether these studies bear physiological or pathological relevance (Golej *et al.*, 1998; Kalapos, 1999; Berlanga *et al.*, 2005; Ghosh *et al.*, 2006; Riboulet-Chavey *et al.*, 2006; Cantero *et al.*, 2007). Under physiological conditions the highly efficient glyoxalase system degrades MG into D-lactate (Thornalley, 1996) and keeps plasma MG levels at around 1 μM or less (McLellan *et al.*, 1994; Wang *et al.*, 2007). The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II that require catalytic amounts of reduced glutathione (GSH) for its activity (Thornalley, 1996).

In the present study we have determined an appropriate dose and route for administration of exogenous MG that would result in pathologically relevant plasma concentrations of MG in experimental animals. We used this dose to investigate the tissue distribution of exogenous MG in these animals and the effects of the consequent acutely elevated levels of plasma MG on glucose tolerance and plasma insulin levels. In adipose tissue from MG-treated rats, glucose uptake, GLUT4, insulin receptor (IR), insulin receptor substrate-1(IRS-1) protein expression and IRS-1 tyrosine phosphorylation were studied. More importantly, we examined whether alagebrium could prevent or attenuate these effects of exogenous MG.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Canadian Council on Animal Care. Male 11-week-old SD rats from Charles River Laboratories (Quebec, Canada) were used. After 1 week of acclimatization, the rats were fasted overnight before the experiments.

In vitro *incubation of alagebrium with MG*

Methylglyoxal (10 μM) was incubated with or without alagebrium (100 μM) for different times at 37°C. After the given incubation time, the sample was analysed for MG by HPLC as described below.

Determination of an appropriate dose and route of administration of MG: effects of pretreatment with alagebrium

In view of the inherent bioavailability barriers associated with the oral route, administering MG in drinking water or by gavage was not considered suitable for acute administration of a single dose to achieve consistent plasma levels. We chose the i.p. and i.v. routes to get consistent plasma levels of MG. In order to achieve a pathologically relevant plasma concentration of 2–5 μM MG (McLellan *et al.*, 1994; Baynes and Thorpe, 1999; Wang *et al.*, 2007; Wang *et al.*, 2008), we calculated a dose based on an average blood volume of 6 mL per 100 g body weight (Lee and Blaufox, 1985) for a 300 g rat and assumed complete absorption from the i.p. injection site into the circulation. We gave 17.25 $mg \cdot kg^{-1}$ (240 $\mu mol \cdot kg^{-1}$) by a single i.p. injection (described hereafter as MG-17 i.p.) or 6.48 $mg \cdot kg^{-1} \cdot h^{-1}$ (90 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) by i.v. infusion for 2 h (12.96 or about 13 $mg \cdot kg^{-1}$, MG-13 i.v.) with or without alagebrium (100 $mg \cdot kg^{-1}$ i.p.). Alagebrium was administered 15 min before the administration of MG (described hereafter as pretreatment).

The continuous i.v. infusion was chosen to deliver a constant low dose of MG in the circulation and compare its plasma levels with those resulting from the i.p. injection. In another group of rats, MG (50 $mg \cdot kg^{-1}$ i.v., described hereafter as MG-50 i.v.) was given as a bolus injection in order to achieve higher plasma MG level. The rats were anaesthetized with thiopental sodium (100 $mg \cdot kg^{-1}$ i.p.). The trachea was cannulated to allow spontaneous respiration, and the left jugular vein and right carotid artery were also cannulated. Blood samples were collected at 5, 15, 30, 60 and 120 min into tubes containing EDTA. Plasma MG levels were determined by HPLC.

In vivo *distribution of MG after treatment with exogenous MG*

In rats treated with saline (control), MG-17 i.p. or MG-17 i.p. + alagebrium, selected tissues and urine were collected 3 h

after administration of the test compounds and frozen in liquid nitrogen. The tissues were finely ground and homogenized in liquid nitrogen and reconstituted in sodium phosphate buffer (pH 4.5) and sonicated (30 s, three times). The samples were assayed for MG by HPLC, as described below, and for protein measurement.

Intravenous glucose tolerance test (IVGTT)
After overnight fasting, the IVGTT was performed as described previously (Laight *et al.*, 1999). Briefly, the trachea, left jugular vein and right carotid artery were cannulated in anaesthetized rats. After collecting a basal blood sample (0.5 mL), rats were treated with saline, MG or MG + alagebrium. After 2 h, a zero-time blood sample (0.3 mL) was taken and a bolus dose of glucose (0.5 $g \cdot kg^{-1}$) was given i.v. and further blood samples (0.2 mL each) were collected at 1, 3, 6, 12 and 24 min, from the carotid artery. Each sample was replaced with an equal volume of saline. Plasma glucose levels were determined using a glucose assay kit (BioAssay Systems, Hayward, CA, USA), and insulin levels were measured with a rat insulin assay kit (Mercodia Rat Insulin ELISA). The IVGTT result was calculated as the area under the curve (AUC) for both plasma glucose and insulin levels between zero time and 24 min and expressed as arbitrary units.

Glucose uptake
Insulin sensitivity of adipose tissue was evaluated by measuring insulin-induced 2-Deoxy-[^{3}H] glucose (2-DOG) uptake as described previously (Jia and Wu, 2007). Briefly, abdominal visceral adipose tissue was chopped and digested in Dulbecco's modified Eagle's medium (DMEM) base (no glucose, no serum) with collagenase (1.5 $mg \cdot mL^{-1}$) at 37°C for 20 min. The mixture was filtered, centrifuged, supernatant discarded, and the pellet was resuspended in the same DMEM. Thereafter, the cells were exposed to 100 nM insulin for 30 min and continuously incubated for another 20 min after the addition of [^{3}H]-2-DOG (0.1 μCi per 500 μL) with glucose (50 μM) to the medium. The incubation was stopped by washing cells three times with ice-cold glucose-free phosphate buffer. The cells were lysed in 0.1% sodium dodecyl sulphate (SDS) and 1 N NaOH and transferred into scintillation vials for counting (Beckman LS 3801 scintillation counter).

Preparation of total membrane fraction from adipose tissue for GLUT4
Abdominal visceral adipose tissue isolated from rats was homogenized in buffer B [10 mM Tris–HCl, 1 mM EDTA, 250 mM sucrose and 0.1 mM phenylmethylsulphonyl fluoride (PMSF, pH 7.4)] using a Polytron homogenizer. The homogenate was centrifuged at 1700× *g* for 10 min at 4°C, and the resulting supernatant was centrifuged at 8600× *g* for 10 min at 4°C. The supernatant was then centrifuged at 185 000× *g* for 60 min at 4°C and stored at −70°C before use (Furuta *et al.*, 2002). The protein concentration of the supernatant was determined by the bicinchoninic acid protein assay reagent.

Immunoprecipitation and Western blotting
For immunoprecipitation abdominal visceral adipose tissue was lysed in an ice-cold radioimmunoprecipitation assay buffer containing 30 mM HEPES (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 0.5% sodium deoxycholate, 8 mM Na_3VO_4, 1 mM NaF and 2 mM protease inhibitor (Jia and Wu, 2007). Tissue homogenates were incubated with IRS-1 antibody for 2 h at 4°C, followed by incubation with Protein A/G-Agarose for further 2 h at 4°C. Immunoprecipitates were separated using spin-collection filters and washed once with radioimmunoprecipitation assay buffer and three times with phosphate-buffered saline. For Western blotting, cell lysates or membrane fractions (50 μg) were boiled with sample buffer for 5 min, resolved by 10–12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked and incubated with the anti-IR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-GLUT4 (Santa Cruz Biotechnology Inc.) and anti-*β*-actin antibodies (Santa Cruz Biotechnology Inc.), respectively, followed by incubation with horseradish peroxidase conjugated secondary antibodies (Upstate, Biotech, Billerica, MA, USA). The proteins were then visualized with chemiluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) and exposed to X-ray film (Kodak Scientific Imaging film, X-Omat Blue XB-1).

MG assay
Methylglyoxal was measured by a specific and sensitive HPLC method as described previously (Dhar *et al.*, 2008) with some modifications to the original protocol (Chaplen *et al.*, 1998). MG was derivatized with *o*-phenylenediamine (*o*-PD) to specifically form 2-methylquinoxaline. The samples were incubated in the dark for 24 h with 0.45 N perchloric acid and 10 mM *o*-PD at room temperature. Samples were centrifuged at 15 000× *g* for 10 min. 2-Methylquinoxaline and quinoxaline internal standard (5-methylquinoxaline) were quantified on a Hitachi D-7000 HPLC system (Hitachi, Ltd., Mississauga, ON, Canada) via Nova-Pak® C18 column (3.9 × 150 mm, and 4 μm particle diameter, MA, USA).

Glutathione and D-lactate assays
Reduced glutathione was measured by derivatization with 5,5′-dithio-bis(2-nitrobenzoic acid), and reverse-phase HPLC using ultraviolet detection (Wang *et al.*, 2008) whereas D-lactate was measured by an assay kit (BioAssays Systems, Hayward, CA, USA).

Statistical analysis
Data are expressed as mean ± SEM and analysed using one-way ANOVA and *post hoc* Dunnett's test. *P*-value less than 0.05 was considered significant. Data on tissue distribution of MG (Figure 2) were analysed with two-way ANOVA with treatment and tissue as two variables.

Materials
All chemicals were of analytical grade. MG and *o*-PD were purchased from Sigma Aldrich, Oakville, ON, Canada. Alagebrium (formerly known as ALT-711) was a generous gift from Synvista Therapeutics, Inc. (Montvale, NJ, USA).

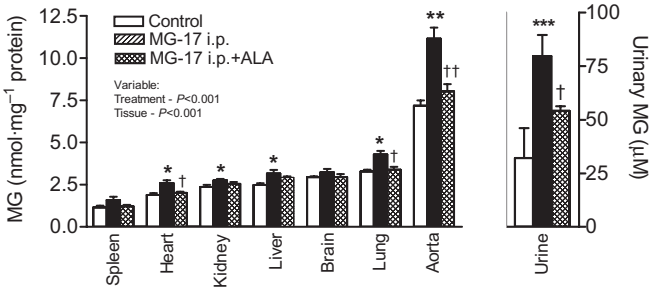

Figure 2 Distribution of methylglyoxal (MG) in different tissues and urine in Sprague-Dawley rats after i.p. administration. Saline (control), MG (17.25 mg·kg^{-1} i.p., MG-17 i.p.) or MG-17 i.p. + alagebrium (ALA; 100 mg·kg^{-1} i.p.) were administered to three groups of rats (n = 6 each). The tissues and urine were collected 3 h after administration of MG. Data were analysed with two-way ANOVA with treatment and tissue as variables. *P < 0.05, **P < 0.01, ***P < 0.001 versus corresponding control group, $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ < 0.01 versus MG-17 i.p. group.

Table 1 ALA reduces detectable MG

Time of in vitro *incubation*	*MG (10 μM) alone*	*MG (10 μM) + ALA (100 μM)*	*ALA (100 μM) alone*
	Amount of MG detected by HPLC		
15 min	9.5 ± 0.7	6.9 ± 0.1*	0
30 min	9.4 ± 0.7	6.8 ± 0.3*	0
1 h	9.3 ± 0.7	5.8 ± 0.1**	0
2 h	9.4 ± 0.7	5.2 ± 0.9*	0
24 h	9.4 ± 0.7	5.0 ± 0.1**	0

MG was incubated with ALA at 37°C for different times. The solution was analysed for MG by HPLC after the given incubation period. The values are mean ± SEM (n = 4 each).
*P < 0.05, **P < 0.01 versus MG alone.
ALA, alagebrium; MG, methylglyoxal.

Results

Incubation of alagebrium with MG in vitro

Incubation of MG (10 μM) with alagebrium (100 μM) for different times resulted in a significant reduction in the amount of MG detected by HPLC with increasing time of incubation. Even after 15 min of incubation, the amount of MG detected was significantly reduced suggesting an acute effect of alagebrium (Table 1).

Alagebrium attenuates increase in plasma MG levels following exogenous MG administration

After acute administration of MG-17 i.p. the plasma level of MG peaked at 15 min to reach 2.5 μM, 2.6-fold higher than the basal value (Figure 3A). The MG level declined to a plateau after 1 h but was still higher (2.1-fold) than the basal value even after 2 h. In another group of rats, after i.v. infusion of MG (6.48 mg·kg^{-1}·h^{-1} or 90 μmol·kg^{-1}·h^{-1} for 2 h) the plasma MG level peaked at 5 min to reach 2.7 μM, 2.7-fold higher than the basal value (Figure 3B). Similarly, the plasma level of MG in this group declined gradually and was still significantly (2.2-fold) higher than the basal value after

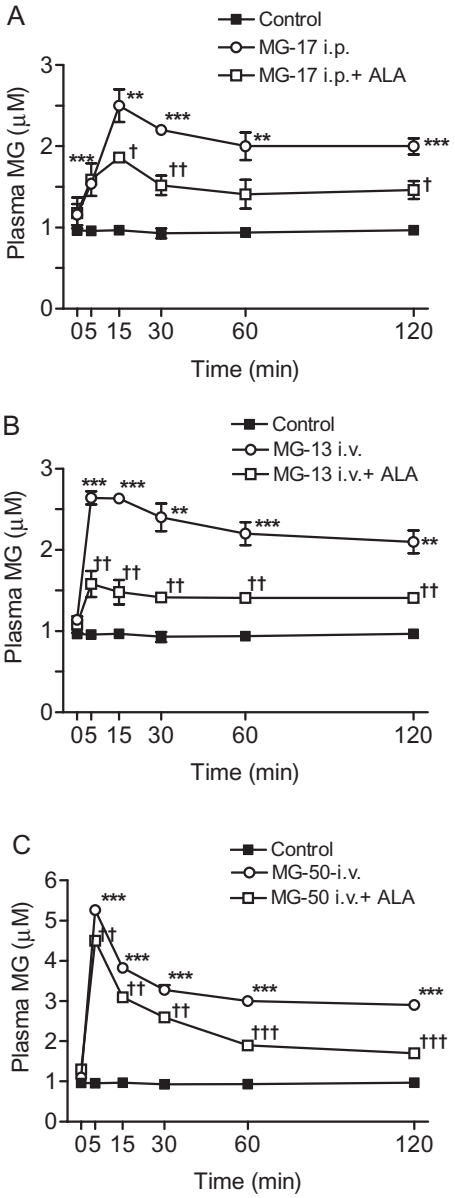

Figure 3 Plasma methylglyoxal (MG) levels after (A) i.p. or (B,C) i.v. administration of MG in Sprague-Dawley rats. n = 6 for each group. Control: saline injection; MG-17 i.p.: MG 17.25 mg·kg^{-1} i.p.; MG-13 i.v.: MG 6.48 mg·kg^{-1}·h^{-1} i.v. infusion for 2 h; MG-50 i.v.: MG 50 mg·kg^{-1} i.v. slow bolus injection; alagebrium (ALA; 100 mg·kg^{-1} i.p.) was given 15 min before the administration of MG in (A–C). **P < 0.01, ***P < 0.001 compared with control at same time point, $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ < 0.01, $^{\dagger\dagger\dagger}P$ < 0.001 compared with corresponding values for MG-17 i.p.-, MG-13 i.v.- or MG-50 i.v.-treated groups, at the same time point.

2 h. Pretreatment with alagebrium (100 mg·kg^{-1}, i.p.) significantly prevented the increase in plasma MG after both i.p. and i.v. administration of MG (Figure 3A,B), which most probably could be due to scavenging of MG by alagebrium. Thus, both routes of administration (i.p. and i.v.) can increase plasma MG levels in similar pattern to a level comparable to that under various pathological conditions (McLellan *et al.*, 1994; Baynes and Thorpe, 1999; Wang *et al.*, 2007; 2008). Therefore, MG-17 i.p. was chosen for

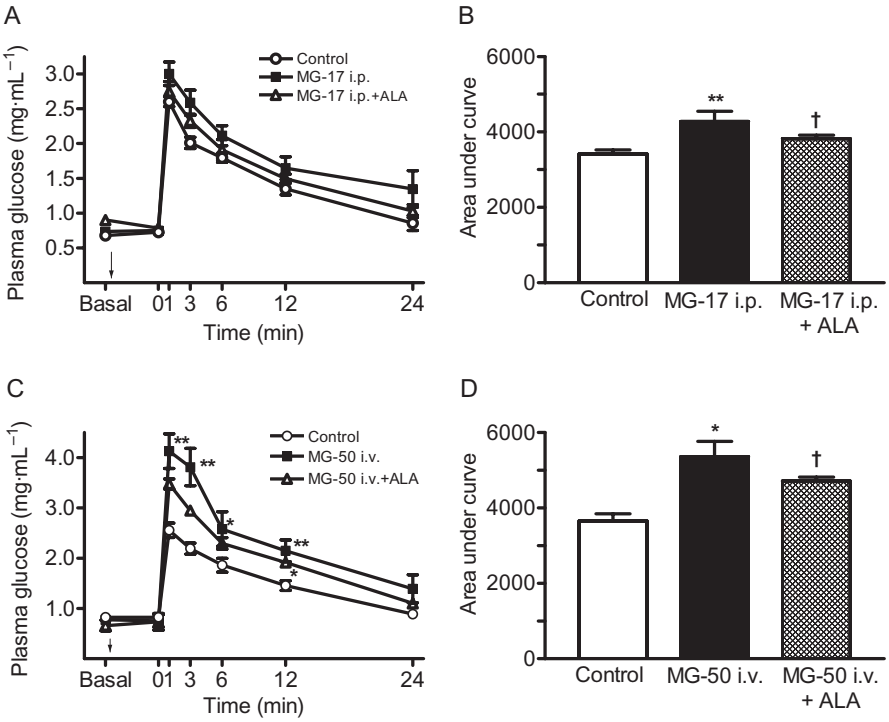

Figure 4 Intravenous glucose tolerance test (IVGTT) in methylglyoxal (MG)-treated Sprague-Dawley rats, effect of alagebrium. Basal plasma glucose levels were determined before any treatment. The plasma glucose levels (A) and area under curve (B) were evaluated in rats for 24 min during an IVGTT that was performed 2 h after treatment with saline (control), MG-17 i.p. or MG-17 i.p. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). The plasma glucose levels (C) and area under curve (D) were evaluated in rats for 24 min during an IVGTT that was performed 2 h after treatment with saline (control), MG-50 i.v. or MG-50 i.v + alagebrium (ALA; 100 mg·kg^{-1} i.p.). Two hours after saline or drugs, a zero-time plasma sample was obtained before giving a glucose load (0.5 g·kg^{-1} i.v.) to perform the IVGTT. (C) *$P < 0.05$, **$P < 0.01$ compared with control group at the same time point, (B,D) *$P < 0.05$, **$P < 0.01$ compared with corresponding control group, $^{\dagger}P < 0.05$ compared with respective MG-treated group. $n = 9$ in each group.

most of the following studies. Administration of a higher dose of MG-50 i.v. resulted in significantly higher plasma MG levels than with MG-17 i.p. or MG-13 i.v., and alagebrium attenuated the increase in plasma MG (Figure 3C). MG-50 i.v. was administered to some groups of rats to assess dose-related severity of effects of MG on glucose tolerance and plasma insulin levels.

Alagebrium attenuates distribution of MG in rats after treatment with exogenous MG

At 3 h after administration of MG-17 i.p. MG levels were increased significantly in the aorta (1.6-fold), heart (1.4-fold), liver (1.3-fold), lungs (1.3-fold) and kidney (1.2-fold) compared with the basal levels in the control group (Figure 2). The aorta had the greatest increase in the level of MG, compared with control and had the highest levels among the tissues tested (Figure 2). Urinary MG level was also significantly higher (2.5-fold) in the MG-17 i.p. group compared with the control group (Figure 2). The increased MG levels in rats treated with MG-17 i.p. were significantly attenuated by pretreatment with alagebrium (Figure 2). The urinary MG levels (mean ± SEM, μM) in the MG-50 i.v. group were as follows: control (saline), 25 ± 4 ($n = 9$); MG-50 i.v., 232 ± 45*** ($n = 6$); MG-50 i.v. + alagebrium, 134 ± 41** ($n = 4$); **$P < 0.01$, ***$P <$ 0.001 versus control group. There was no significant increase in MG levels in spleen and brain of rats after MG-17 i.p. administration. Inter-tissue variation in MG levels before and after MG or MG + alagebrium administration was significantly different as analysed by two-way ANOVA (Figure 2).

Impairment of glucose tolerance and glucose uptake in MG-treated rats is prevented by alagebrium

After treatment with MG-17 i.p. or MG-50 i.v., plasma glucose and AUC were measured (Figure 4). MG-17 i.p. significantly impaired glucose tolerance with increased AUC, which was attenuated by pretreatment with alagebrium (Figure 4A,B). The impairment of glucose tolerance was significantly greater in the MG-50 i.v.-treated group than its control group (Figure 4C,D). Pretreatment with alagebrium significantly attenuated impairment of glucose tolerance by MG and reduced the AUC (Figure 4D).

Insulin-stimulated glucose uptake was evaluated in abdominal visceral adipose tissue freshly isolated from rats 2 h after administration of MG-17 i.p. or MG-50 i.v. or saline (control) in separate groups of rats. There was a significant decrease in insulin-stimulated glucose uptake in MG-17 i.p.-treated rats, and it was more severe in MG-50 i.v.-treated rats compared with control. The reduced glucose uptake, by either dose of MG, was prevented by pretreatment with alagebrium (Figure 5).

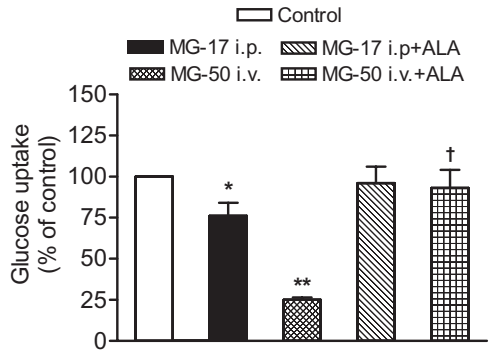

Figure 5 Glucose uptake in adipose tissue from methylglyoxal (MG)-treated Sprague-Dawley rats. Glucose uptake by adipose tissue was evaluated in five groups of rats 2 h after treatment with saline (control); MG 17.25 mg·kg^{-1} i.p. (MG-17 i.p.); MG-17 i.p. + alagebrium (ALA; 100 mg·kg^{-1} i.p. given 15 min before the administration of MG); MG 50 mg·kg^{-1} i.v. slow bolus injection (MG-50 i.v.) and MG-50 i.v. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). Visceral adipose tissue was removed from the abdomen and tested for insulin-stimulated glucose uptake *in vitro*. *$P < 0.05$, **$P < 0.01$ compared with control, $^{\dagger}P < 0.05$ compared with MG-50 i.v.-treated group. $n = 4$ for each group.

Increased plasma insulin levels in MG-treated rats are attenuated by alagebrium

The basal plasma insulin levels were not different among the control, MG-treated and MG + alagebrium groups. Following an IVGTT, the plasma insulin levels were higher in rats treated with MG-17 i.p. and MG-50 i.v. (Figure 6A,C). The AUC for plasma insulin levels after the IVGTT was significantly greater in MG-17 i.p.- and MG-50 i.v.-treated rats compared with the respective controls (Figure 6B,D). Pretreatment with alagebrium significantly attenuated the increase in plasma insulin levels and AUC values induced by MG-17 i.p. (Figure 6A,B).

Alagebrium prevents decreased plasma GSH levels in MG-treated rats

Rats treated with MG-17 i.p. had significantly lower plasma GSH levels than the control rats (Table 2). Co-administration of alagebrium (100 mg·kg^{-1} i.p.) with MG-17 i.p. significantly reversed the decrease in plasma GSH induced by MG-17 i.p. (Table 2).

Effects of MG and alagebrium on plasma and aortic D-lactate levels

D-lactate is a metabolite of MG (Desai and Wu, 2007). Plasma D-lactate levels were significantly elevated after MG-50 i.v. and even further elevated after MG-17 i.p. + alagebrium and MG-50 i.v. + alagebrium (Table 3). Aortic D-lactate levels (μmol·mg^{-1} protein, $n = 3$ each group) were also significantly elevated after MG-17 i.p. (7.7 ± 0.8*) and further elevated after MG-17 i.p. + alagebrium (9.6 ± 1.0**) compared with the control group (4.0 ± 0.6) (*$P < 0.05$, **$P < 0.01$ vs. control group).

Effects of MG on insulin signalling pathway in adipose tissue are attenuated by alagebrium

In order to confirm the possible mechanism of MG-induced glucose intolerance and reduced glucose uptake, the protein expression of GLUT4 (Figure 7), IR, IRS-1 (Figure 8) and tyrosine phosphorylation of IRS-1 (Figure 9) was examined in MG-50 i.v.-treated rats. There was significant decrease in GLUT4 protein expression in abdominal visceral adipose tissue from MG-50 i.v.-treated rats, compared with that from control rats (Figure 7). There was no change in the protein expression of IR and IRS-1 (Figure 8). However, insulin-induced tyrosine phosphorylation of IRS-1 was significantly reduced in MG-50 i.v.-treated rats, and this reduction was attenuated by pretreatment with alagebrium (100 mg·kg^{-1} i.p.) (Figure 9).

Discussion

In the present study we report for the first time that alagebrium has acute preventive effects against the harmful effects of the AGEs precursor, MG, *in vivo*. Alagebrium is a well-documented breaker of AGEs cross-links in chronic studies (Ulrich and Zhang, 1997; Wolffenbuttel *et al*., 1998; Susic *et al*., 2004; Thallas-Bonke *et al*., 2004; Little *et al*., 2005; Peppa *et al*., 2006; Coughlan *et al*., 2007; Zieman *et al*., 2007; Guo *et al*., 2009). We also show for the first time that a single dose of MG adversely affects glucose tolerance in SD rats. When MG was administered in a lower dose (17.25 mg·kg^{-1}, i.p.: MG-17 i.p.) the plasma MG levels were elevated to the pathologically relevant concentrations observed in diabetic patients (McLellan *et al*., 1994; Wang *et al*., 2007), for more than 2 h. With this acute elevation of circulating MG, glucose tolerance of the rats was impaired, glucose-stimulated plasma insulin level increased, insulin-stimulated glucose uptake in the adipose tissue was reduced, and urinary MG levels and aortic tissue content of MG increased. To achieve a higher plasma level, MG was given at a higher dose (50 mg·kg^{-1} i.v.: MG-50 i.v.) in separate groups of rats (Figure 3C), and this dose also significantly impaired glucose tolerance, increased plasma insulin levels, reduced insulin-stimulated glucose uptake in adipose tissue along with a significant reduction in GLUT4 protein expression and tyrosine phosphorylation of IRS-1. Alagebrium, administered i.p. 15 min prior to MG, attenuated all of these acute effects of MG and the increase in plasma levels following MG administration. *In vitro* incubation of alagebrium with MG for different times, starting with 15 min, significantly reduced the amount of MG detected in the sample (Table 1) possibly suggesting binding (scavenging) of MG by alagebrium. The attenuation by alagebrium of increased plasma MG levels following exogenous MG also suggests a scavenging or binding effect of alagebrium on MG. To the best of our knowledge, an acute scavenging or binding effect of alagebrium on MG has not been reported before. PTB was the first AGEs cross-link breaking compound reported, but it degrades rapidly (Vasan *et al*., 1996). Alagebrium is a more stable thiazolium derivative (Figure 1) (Desai and Wu, 2007) and was developed based on an earlier observation that the carbon–carbon bond of α-diketones can be selectively cleaved with some thiazolium salts (Vasan *et al*., 1996). Thus, our results show that alagebrium has additional acute upstream effects that can prevent formation of AGEs from MG, which can be useful for prevention of AGEs-related disorders.

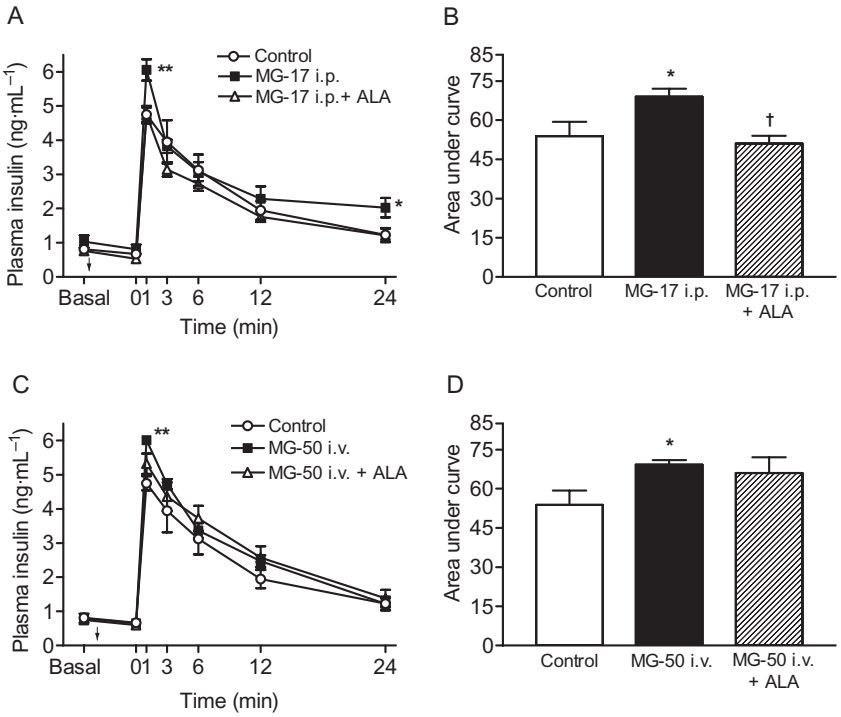

Figure 6 Plasma insulin levels in methylglyoxal (MG)-treated Sprague-Dawley rats, effect of alagebrium. Basal plasma insulin levels were determined before any treatment. The plasma insulin levels (A) and area under curve (B) were evaluated in the rats for 24 min during an intravenous glucose tolerance test (IVGTT) that was performed 2 h after treatment with saline (control), MG-17 i.p. or MG-17 i.p. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). The plasma insulin levels (C) and area under curve (D) were evaluated in the rats for 24 min during an IVGTT that was performed 2 h after treatment with saline (control), MG-50 i.v. or MG-50 i.v. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). Two hours after saline or drugs, a zero-time plasma sample was obtained before giving a glucose load (0.5 g·kg^{-1} i.v.) to perform the IVGTT. *P < 0.05, **P < 0.01 compared with corresponding control group, †P < 0.05 compared with MG-17 i.p. group. n = 9 for each group.

Table 2 ALA partially reverses MG-induced depletion of GSH in rats

	Control	*MG*	*MG + ALA*
Plasma GSH (μM)	111 ± 5	35 ± 1***	61 ± 5***†

Sprague-Dawley rats were treated with saline (control), MG-17 i.p. (MG, 17.28 mg·kg^{-1} i.p) and MG + ALA (100 mg·kg^{-1} i.p.). Plasma levels of GSH were measured 3 h after treatment with MG. The values are mean ± SEM (n = 6 each).
***P < 0.01 versus control group, †P < 0.05 versus MG group.
ALA, alagebrium; GSH, reduced glutathione; MG, methylglyoxal.

The *in vivo* fate of exogenous MG is unknown. Our results show for the first time that the majority of administered MG-17 i.p. is excreted in the urine, an effect attenuated by alagebrium (Figure 2). As proteins are retained in the glomerular capillaries, the presence of MG in the urine indicates that most of the MG is likely to be in free in the plasma, at least initially, and thus filtered into the urine. When alagebrium is present, the free MG probably binds to alagebrium and urinary excretion of free MG is reduced. We have observed that when MG is incubated with bovine serum albumin at 37°C, more than 90% is free, that is, not protein bound, up to the first 15 min of incubation (Dhar *et al.*, 2009).

After treatment with MG-17 i.p., the aortic content of MG increased significantly more compared with the other six organs investigated, including the heart and lungs. This increase in MG level was attenuated by alagebrium. The high basal, as well as post-MG, levels of aortic MG are of great pathological significance in terms of development of MG-induced AGEs and atherogenesis, and endothelial dysfunction over the longer term (Thornalley, 1996; Vlassara and Palace, 2002; Desai and Wu, 2007). There was no significant increase in MG levels in the spleen and the brain as compared with control. The reasons for the increased basal as well as post-administration MG in the aorta and the uneven organ distribution needs further separate studies.

The plasma levels of MG are around 1 μM in normal SD rats (Figure 3) (Wang *et al.*, 2008) and 1 μM or less in healthy humans (McLellan *et al.*, 1994; Wang *et al.*, 2007). Under physiological conditions, the glyoxalase system rapidly degrades MG into D-lactate, which minimizes its reaction with proteins and other cellular components to form AGEs. GSH is an essential component of the glyoxalase system (Thornalley, 1996; Baynes and Thorpe, 1999; Desai and Wu, 2007). We found lower GSH levels in rats treated with MG-17 i.p. (Table 2). Also, in hyperglycaemia and diabetic patients, the plasma MG levels are elevated to between 2 and 6 μM (McLellan *et al.*, 1994; Wang *et al.*, 2007) and associated with oxidative stress and decreased GSH levels (Baynes and Thorpe, 1999). The enzymes glutathione reductase and glutathione peroxidase play a key role in the recycling of glutathione between its reduced (GSH) and oxidized (GSSG) forms. Glutathione peroxidase removes hydrogen peroxide through the oxidation of GSH to GSSG. Glutathione reductase acts as an antioxidant by converting GSSG to GSH. MG can increase

Table 3 Effects of MG and alagebrium on D-lactate levels in rats

	Treatment group				
	Control	*MG-17 i.p.*	*MG-17 i.p. + ALA*	*MG-50 i.v.*	*MG-50 i.v. + ALA*
Plasma D-lactate (mM)	4.6 ± 0.3	6.0 ± 0.9	7.8 ± 1.0*	7.5 ± 1.0*	8.9 ± 0.5***

Sprague-Dawley rats were treated with saline (control), MG-17 i.p. (MG, 17.28 mg·kg^{-1} i.p.), MG-50 i.v. (MG, 50 mg·kg^{-1} i.v.) or MG + ALA (100 mg·kg^{-1} i.p.). Plasma D-lactate levels were measured 3 h after MG treatment. The values are mean ± SEM (n = 4 each).
*P < 0.05, ***P < 0.01 versus control group.
ALA, alagebrium; MG, methylglyoxal.

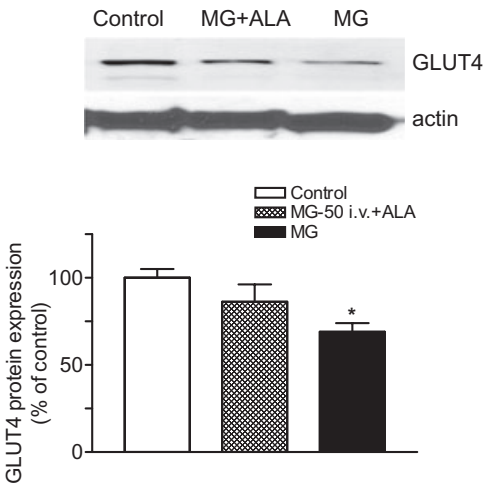

Figure 7 GLUT4 protein expression in methylglyoxal (MG)-treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). After 2 h the abdominal adipose tissue was removed and processed for determination of GLUT4 protein expression by Western blotting. *P < 0.05 compared with control. n = 4 for each group.

oxidative stress by causing glycation of glutathione reductase and glutathione peroxidase and thus inactivating them (Desai and Wu, 2008). MG has also been shown to directly deplete GSH in various cell types so that the cell becomes more sensitive to oxidative stress. Reduced availability of GSH will affect the glyoxalase system and impair degradation of MG. This establishes a vicious cycle that leads to increased levels of MG (Desai and Wu, 2008). A direct interaction of alagebrium and GSH has not been reported. However, in a recent study, alagebrium given for 16 weeks to aging rats increased glutathione peroxidase and reduced oxidative stress (Guo *et al.*, 2009); however, GSH was not measured in this study. Alagebrium, by scavenging MG, can potentially prevent the interaction between MG and GSH. Thus, alagebrium can prevent the decrease in GSH caused by MG that was observed in our study. An increased availability of GSH in the alagebrium-treated group can potentially lead to increased degradation of MG by the glyoxalase system with a consequent increase in D-lactate levels. This mechanism can explain the increase in plasma and aortic D-lactate levels that was found in MG + alagebrium-treated groups. The elevated D-lactate levels observed in the groups treated with MG alone can be explained by increased metabolism of MG by the glyoxalase system until this enzyme is saturated.

In chronically fructose-fed SD rats, the serum MG levels are elevated to around 4 μM along with development of insulin resistance-like syndrome (Jia and Wu, 2007). This raises an important question of whether MG is the cause or the effect of type 2 diabetes mellitus. Glucose and fructose are the major precursors of MG formation (Thornalley, 1996; Desai and Wu, 2007; Dhar *et al.*, 2008). Thus, a regular high intake of carbohydrates in normal people can result in increased MG formation, which can eventually lead to the development of insulin resistance and type 2 diabetes mellitus. Our results showing impaired glucose tolerance after the MG-17 i.p. or MG-50 i.v. treatments *in vivo* (Figure 4) point to the beginnings of insulin resistance. Abdominal visceral adipose tissue isolated from rats treated *in vivo* with MG-17 i.p. and MG-50 i.v. showed reduced insulin-stimulated glucose uptake (Figure 5). These results provide further insight into the mechanisms behind the *in vivo* observations. In adipose tissue, glucose transport is insulin-dependent and is mediated by GLUT4. The acute effects of MG that might have an implication for the development of insulin resistance and diabetes have mostly been studied *in vitro* in cultured cells. Thus, incubation of cultured 3T3-L1 adipocytes with MG (20 μM) reduced glucose uptake, decreased insulin-induced IRS-1 tyrosine phosphorylation and decreased the activity of PI3K (Jia and Wu, 2007). Incubation of cultured L6 muscle cells with high concentrations of MG (2.5 mM) for 30 min impaired insulin signalling (Riboulet-Chavey *et al.*, 2006). Incubation of insulin with MG modifies the structure of the insulin molecule in a way that impairs insulin-mediated glucose uptake in adipocytes (Jia *et al.*, 2006). To the best of our knowledge the effects of acute MG *in vivo* on glucose tolerance have not been reported previously. In a genetic model of diabetes such as the Zucker obese rat a defect of glucose transport in muscle has been reported (Sherman *et al.*, 1988). Protein kinase Akt2 (protein kinase B) plays a vital role in insulin signalling in muscle and liver, and mice lacking Akt2 develop insulin resistance and a diabetes mellitus-like syndrome (Cho *et al.*, 2001). Our study reveals a reduced insulin-mediated glucose uptake in adipose tissue from MG-treated rats, which could be due to reduced GLUT4-mediated glucose uptake into the cells, as indicated by reduced GLUT4 protein expression (Figure 7). One or more steps in the insulin signalling pathway may also be impaired as indicated by reduced IRS-1 tyrosine phosphorylation in MG-50 i.v.-treated rats (Figure 9) (Baynes and Thorpe, 1999; Birnbaum, 2001; Cho *et al.*, 2001; Jia and Wu, 2007). Along with plasma glucose, the plasma insulin AUC was significantly higher after i.v. glucose load in the MG-treated rats than in its control group (Figure 6B,D), indicating insulin

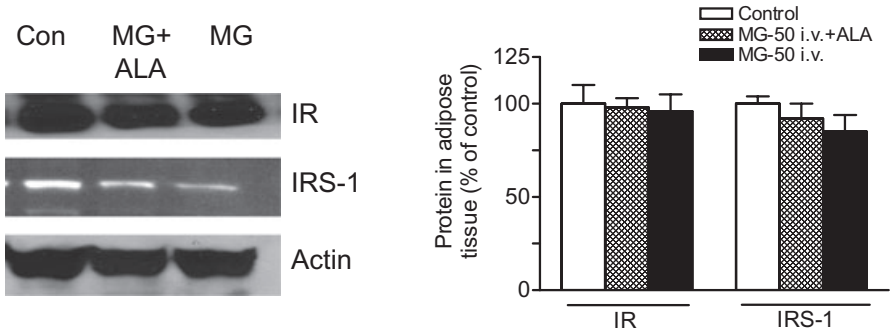

Figure 8 Insulin receptor (IR) and insulin receptor substrate 1 (IRS-1) protein expression in methylglyoxal (MG)-treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). After 2 h, the abdominal adipose tissue was removed and processed for determination of IR and IRS-1 protein expression by Western blotting. $n = 4$ for each group.

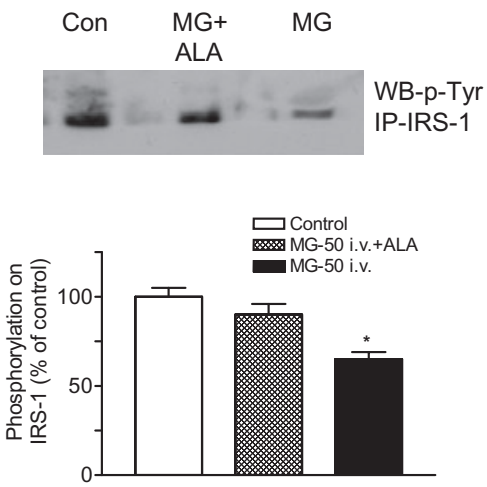

Figure 9 Insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation in methylglyoxal (MG)-treated rats. Groups of rats were treated with saline (Control), MG-50 i.v. or MG-50 i.v. + alagebrium (ALA; 100 mg·kg^{-1} i.p.). After 2 h, the abdominal adipose tissue was removed, and tissue lysates were subjected to immunoprecipitation (IP) with IRS-1 antibody. The immunoprecipitates were then subjected to Western blotting (WB) using anti-pTyr. The immunoreactivity level was compared with the control level of IRS-1 phosphorylation. *$P < 0.05$ versus control rats, $n = 4$ for each group.

resistance. Alagebrium pretreatment attenuated the acute effects of MG on glucose tolerance that cannot be due to the AGEs cross-link breaking property of alagebrium, as AGEs are formed slowly by reaction of MG with certain proteins a process that requires more than 24 h and up to many weeks (Thornalley, 1996; Desai and Wu, 2007). Therefore, the attenuation of acute effects of MG seems to be most likely due to binding of alagebrium with MG.

Plasma MG levels remained significantly elevated for at least 2 h after a single i.v. or i.p. injection of MG (Figure 3) indicating a long half-life of more than 10 h (data not shown), which may lead to cumulative toxicity when MG is given daily (Slavik *et al.*, 1983). We have established doses for i.p. and i.v. administration of MG that result in pathologically relevant concentrations in the plasma (Figure 3).

In recent years, Western diets have increasing amounts of carbohydrates, and the rapid rise in the incidence of childhood obesity and type 2 diabetes mellitus has become a major health concern (Birnbaum, 2001; Van Dam *et al.*, 2002). In the absence of a genetic predisposition, the link between high carbohydrate intake and the development of type 2 diabetes mellitus is unknown from a mechanistic perspective (Van Dam *et al.*, 2002). Carbohydrates are a major metabolic source of MG (Thornalley, 1996; Desai and Wu, 2007; Dhar *et al.*, 2008), and it would be interesting to examine the effects of alagebrium on chronic administration of high glucose or MG and the development of insulin resistance. The attenuation of acute effects of MG on glucose tolerance by alagebrium can be a promising strategy to prevent the chronic harmful effects of high glucose intake.

In summary, we have achieved pathologically relevant plasma levels of MG in normal SD rats with exogenous MG, given i.p. or i.v. The elevated MG induced glucose intolerance, and alagebrium attenuated these effects of MG, an acute *in vivo* effect of alagebrium against MG, possibly due to scavenging. Our study suggests a pathogenetic mechanism linking high carbohydrate intake and development of glucose intolerance through increased formation of MG.

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Conflicts of interest

None.

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