Arginine attenuates methylglyoxal- and high glucose-induced endothelial dysfunction and oxidative stress by an eNOS-independent mechanism

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Abbreviations: ACh, acetylcholine; AGEs, advanced glycation endproducts; D-Arg, D-arginine; L-Arg, L-arginine; CEL, Nε-carboxyethyl lysine; GSH, glutathione; eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells; MG, methylglyoxal; L-NAME, Nω-nitro-L-arginine methyl ester; ROS, reactive oxygen species; VSMCs, vascular smooth muscle cells.

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Methylglyoxal (MG), a reactive metabolite of glucose, has high affinity for arginine and is a precursor of advanced glycation endproducts (AGEs). We tested the hypothesis that L-arginine, and its inactive isomer D-arginine, can efficiently scavenge MG, administered exogenously or produced endogenously from high glucose, and attenuate its harmful effects including endothelial dysfunction and oxidative stress by an endothelial nitric oxide synthase (eNOS)-independent mechanism. We used isolated aortic rings from 12 week old male Sprague-Dawley rats and cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs). Both D-arginine and L-arginine prevented the attenuation of acetylcholine-induced endothelium-dependent vasorelaxation by MG and high glucose. However, the inhibitory effect of the NOS inhibitor, \( \text{N} \omega \)-nitro-L-arginine methyl ester, on vasorelaxation was prevented only by L-arginine, but not by D-arginine. MG and high glucose increased protein expression of arginase, a novel finding, and also of NADPH oxidase 4 and nuclear factor kappa B, and production of reactive oxygen species in HUVECs and VSMCs, which were attenuated by D- and L-arginine. However, D- and L-arginine did not attenuate MG and high glucose-induced increased arginase activity in VSMCs and the aorta. D- and L-arginine also attenuated the increased formation of the MG-specific AGE, \( \text{N}\epsilon \)-carboxyethyl lysine, caused by MG and high glucose in VSMCs. In conclusion, arginine attenuates the increased arginase expression, oxidative stress, endothelial dysfunction and AGEs formation induced by MG and high glucose, by an endothelial NOS independent mechanism. The therapeutic potential of arginine against MG and high glucose-induced pathology merits further investigation.
**Introduction**

L-arginine (L-Arg), but not D-arginine (D-Arg), is a substrate for nitric oxide synthase (NOS), which catalyzes the formation of nitric oxide (NO) and L-citrulline (Palmer et al., 1988). Endothelial nitric oxide synthase (eNOS), one of the three isoforms of NOS found mainly in the endothelial cells mediates endothelium-dependent agonist induced vessel relaxation (Moncada et al., 1991). Reduced production or availability of NO causing reduced endothelium-dependent vascular relaxation and endothelial dysfunction is a common feature of diabetes, atherosclerosis, hypertension and several other conditions (Potenza et al., 2009).

L-Arg, but not D-Arg, is also a substrate for arginase, an enzyme of the urea cycle that catalyzes the formation of urea and ornithine. There are two isoforms, arginase I and arginase II (Haraguchi et al., 1987; Morris et al., 1997). In blood vessels, arginases are mainly expressed in the endothelium and at low levels in vascular smooth muscle cells (VSMCs) (Wei et al., 2000). Endothelial arginase can affect eNOS function and cause endothelial dysfunction (Berkowitz et al., 2003; Zhang et al., 2001). High glucose (25 mM) has been shown to increase arginase I activity, but not protein expression, in bovine coronary endothelial cells, and arginase I is increased in the aorta of streptozotocin diabetic rats (Romero et al., 2008), causing endothelial dysfunction (Ishizaka et al., 2007; Romero et al., 2008).

Methylglyoxal (MG), a reactive dicarbonyl molecule, produced during glucose, fatty acid and amino acid metabolism to varying degrees (Thornalley, 1996), is a major precursor for the formation of advanced glycation end products (AGEs) (Kilhovd et al., 2003). Under physiological conditions the MG produced in the body is efficiently degraded by the glyoxalase enzymes and reduced glutathione (GSH) into D-lactate (Thornalley, 1993; Vander Jagt et al., 2005).
Increased MG formation in diabetic patients increases plasma MG levels 3-4 fold (McLellan et al., 1994; Thornalley, 1988; Thornalley et al., 1989; Wang et al., 2007).

Incubation of VSMCs with 25 mM glucose or fructose for 3 h increased MG production 3.5 or 3.9 fold, respectively, and increased oxidative stress (Dhar et al., 2008). We have recently shown that in cultured rat aortic and human umbilical vein endothelial cells (HUVECs) MG and high glucose reduced basal and bradykinin-stimulated NO production, and serine-1177 phosphorylation and activity of eNOS, without affecting threonine-495 and Akt phosphorylation, and total eNOS protein (Dhar et al., 2010b). Chronic treatment of Sprague-Dawley rats with MG for 4 weeks induced features characteristic of type 2 diabetes mellitus (Dhar et al., 2011).

Evidently, MG scavengers have the potential to prevent different pathological conditions, such as endothelial dysfunction (Dhar et al., 2010b), type 2 diabetes (Dhar et al., 2010a; Dhar et al., 2011) and AGEs formation (Dhar et al., 2011; Wang et al., 2008), caused by elevated MG levels in the body in hyperglycemic and other conditions. Currently, there is a lack of specific MG scavengers, and the available compounds have other actions and some of them can even produce toxicity. These compounds include aminoguanidine (Brownlee et al., 1986; Edelstein et al., 1992), alagebrium (Dhar et al., 2010a; Wolffsbuttel et al., 1998), N-acetyl cysteine (NAC) (Jia et al., 2007; Vasdev et al., 1998) and metformin (Beisswenger et al., 2003; Ruggiero-Lopez et al., 1999; Wang et al., 2008). MG has been reported to have high affinity for arginine (Lo et al., 1994; Takahashi, 1977). The Ka (affinity constant) of MG for arginine is $1.9 \times 10^3 \text{ M}^{-1}$. The Km of L-Arg for eNOS is in the micromolar range (~2.9 µM) (Pollock et al., 1991). Since the intracellular concentration of L-Arg is in the millimolar range (0.8 – 2 mM), it is generally believed that substrate availability is unlikely to limit NO production (Goumas et al., 2001). Thus, MG is unlikely to outcompete NOS for binding to L-Arg and an excess of L-Arg can
safely bind MG and inactivate it without affecting NOS activity. Therefore, we tested the hypothesis that L-Arg, and its inactive isomer D-Arg, can efficiently scavenge MG, administered exogenously or produced endogenously from high glucose, and attenuate its harmful effects including endothelial dysfunction, increased oxidative stress and AGEs formation. We also examined the effects of MG on arginase expression and activity, which has not been reported previously.
Methods

Animals

A total of 36 male 11-week old Sprague-Dawley rats from Charles River Laboratories (Quebec, Canada) were used according to a protocol approved by the Animal Care Committee at The University of Saskatchewan, following guidelines of the Canadian Council on Animal Care. The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). After one week of acclimatization, the rats were anesthetized with an inhalation of isoflurane (Forane, 4% in oxygen), delivered through a precision vaporizer and circle absorption breathing system (Ohio, 30/70 Proportioner Anesthesia Machine, Madison, Wisconsin). When anesthesia reached sufficient depth as determined by the absence of the leg flexor response and the eyelid reflex, the thorax was quickly opened by a midline incision and the rat was killed by cutting the heart and exsanguination. The aorta was quickly removed without damaging the endothelium and placed in a beaker filled with Kreb’s solution and bubbled with 95% O₂ + 5% CO₂.

Isometric tension studies on aortic rings

A group of 24 Sprague-Dawley rats was used. Isometric tension studies were carried out on rat aortic rings as described previously (Dhar et al., 2010b). Another group of 12 rats was used to isolate the aorta for incubation studies with MG and high glucose for 3 h. Briefly, 3-4 mm thoracic aortic rings were mounted under a 2 g load in four separate 10 mL organ baths containing Krebs solution with 5 mM glucose and maintained at 37° C and bubbled with 95% O₂ + 5% CO₂. After a 90 min equilibration period the rings were pre-contracted with phenylephrine (1 µM) and cumulative concentration-dependent relaxation in response to acetylcholine (ACh)
was obtained before (Control) and 2 h after incubation with either glucose (25 mM) or MG (100 µM) (Dhar et al., 2010b), or the nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME, 10 µM). In initial experiments, the responses to ACh were repeated before and 2 h after incubation with normal Kreb’s solution to confirm reproducibility of responses to ACh. Some sets of rings were co-incubated with either D-Arg (300 µM) or L-Arg (300 µM) (Desai et al., 1991; Rees et al., 1989; Schulz et al., 1991) for 2 h. Treatment with each compound was tested in rings from at least 6 different rats. Isometric tension was measured with isometric force transducers with the ‘Chart’ software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

**In vitro methylglyoxal binding assay**

MG (30 µM) was co-incubated with either L-Arg (300 µM), D-Arg (300 µM) or NAC (600 µM) in phosphate buffered saline (PBS) at 37º C for different times of 15, 30, 60 min, 3h and 24 h. After the prescribed incubation time the free MG in the sample was measured by HPLC (Dhar et al., 2009).

**Methlyglyoxal assay**

MG was measured by a specific and sensitive HPLC method as described previously (Dhar et al., 2009). MG was derivatized with o-phenylenediamine 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-phenylenediamine at room temperature, and then centrifuged at 12000 rpm 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).

**Cell culture**

Rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from American Type Culture Collection and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37° C in a humidified atmosphere of 95% air and 5% CO₂, as described in our previous studies (Dhar et al., 2008). A-10 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10⁶/ml) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on cover glass slides (2×10⁶/ml). Cells were starved in FBS-free DMEM for 24 h before exposure to different metabolic precursors.

HUVECs from American Type Culture Collection were cultured in Kaighns F12K medium containing 10% FBS, 0.1 mg/mL heparin and 0.03-0.05 mg/mL endothelial cell growth supplement (Dhar et al., 2010b).

**Western blotting**

Western blotting was performed on cell lysates as described previously (Dhar et al., 2010b; Dhar et al., 2011) and the protein concentration in the supernatant was determined by the BCA Protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates (50 μg of protein each) were separated on 7.5-10% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-Tween buffer for 2 h at room temperature, and incubated overnight at 4° C with the primary antibodies to arginase I,
arginase II, nuclear factor kappa B (NF-κB) or NADPH oxidase 4 (NOX4) (all from Santa Cruz, CA, USA), and then with horseradish peroxidase conjugated secondary antibody (Santa Cruz, CA, USA) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA) (Dhar et al., 2010b; Dhar et al., 2011).

**Arginase activity assay**

Arginase activity was measured using an arginase assay kit (BioAssays Systems, CA, USA). Arginase catalyses the conversion of arginine to ornithine and urea. Briefly cell lysates or aortic tissue homogenates were mixed with substrate arginine buffer and incubated for 2 h. Urea reagent was added to stop the reaction and optical density measured at 430 nm.

**Measurement of reactive oxygen species**

Confluent cells were loaded with a membrane-permeable, nonfluorescent probe 2’,7’-dichlorofluorescin diacetate (CM-H2DCFDA, 5 μM) for 2 h at 37°C in FBS-free medium in the dark. The cells treated with MG (30 μM) or glucose (25 mM) for 3 h or 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of reactive oxygen species (ROS) as described previously (Dhar et al., 2008; Dhar et al., 2010b). The protein content of the homogenate was measured by BCA Protein assay (Bio-Rad, Hercules, CA, USA).

**Immunocytochemistry**

A-10 cells were seeded on glass cover slips followed by incubation with different test compounds for 24 h, and subjected to staining for the MG-induced AGE, Nε-carboxyethyl lysine
(CEL). As described previously (Dhar et al., 2008; Dhar et al., 2011), the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N phosphate buffered saline (PBS). After permeation with 0.1% Triton X-100 for 5 min and two washes with PBS, the cells were incubated with normal goat serum (diluted 1:30 in 0.1 N PBS) for 1 h to block non-specific binding sites. After shaking off the goat serum the slides were incubated with the CEL antibody (1:100; a generous gift from Novo Nordisk, A/S, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocynate (FITC) conjugated anti-CEL antibody (Molecular Probes) for 2 h. After washing thrice with PBS the slides were mounted in glycerol:PBS (3:7), coverslipped and observed under a fluorescence microscope. Staining intensity was quantified using the Metapmorph image analysis software (v. 7, Molecular Devices). Slides from four different experiments were analyzed with 5 fields per slide observed and averaged.

Chemicals and Statistical analysis

All chemicals were of analytical grade. Methylglyoxal (pyruvoyl group, pyruvaldehyde), D-glucose [(2R,3S,4R,5R)-2,3,4,5,6-Pentahydroxyhexanal], L-arginine [(S)-2-Amino-5-guanidinopentanoic acid], D-arginine [(S)-2-Amino-5-guanidinopentanoic acid], and N-acetyl cysteine (2-Acetamido-3-sulfanylpropanoic acid) were purchased from Sigma Aldrich, Oakville, ON, Canada. Cell culture media and reagents were purchased from Invitrogen Canada Inc., Burlington, ON, Canada. Data are expressed as mean ± SEM and analyzed using one way ANOVA and post hoc Bonferroni’s test. The P value was considered significant when it was less than 0.05.
Results

L-arginine, D-arginine and N-acetyl cysteine bind MG in an in vitro assay

Incubation of MG (30 µM) with either L-Arg (300 µM), D-Arg (300 µM) or NAC (600 µM) for different times ranging from 15 min to 24 h showed progressive binding of MG by all three compounds. Thus, almost 50% of MG was bound by L-Arg and D-Arg within 15 min and more than 80% was bound within 3 h of co-incubation (Table 1).

The antagonistic effect of D-arginine and L-arginine on methylglyoxal and high glucose induced reduction of endothelium-dependent vasorelaxation

In rat aortic rings precontracted with phenylephrine (1 µM), MG (100 µM, Fig. 1A, B) as well as high glucose (25 mM, Fig. 1C, D) incubated for 2 h in the bath caused significant inhibition of ACh-induced endothelium-dependent relaxation, which was prevented by coincubation of L-Arg (300 µM, Fig. 1A, C) or D-Arg (300 µM, Fig. 1B, D) with MG (Fig. 1A, B) or high glucose (Fig. 1C, D). Incubation of the eNOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME 10 µM, Fig. 1E, F) prevented ACh-induced relaxation which was restored by co-incubation of L-Arg (300 µM, Fig. 1E), but not D-Arg (300 µM, Fig. 1F), with L-NAME.

D-Arg and L-Arg (300 µM) alone did not affect ACh-induced relaxation of the rings (data not shown). MG and glucose, incubated for 2 h, did not affect endothelium-independent relaxation of aortic rings induced by sodium nitroprusside (data not shown).

We have shown earlier that NAC, which has been used as a positive control MG in this study, also attenuates the reduced endothelium-dependent relaxation caused by MG and high glucose in rat isolated aortic rings (Dhar et al., 2010b).
D-arginine and L-arginine prevent high glucose-induced elevation of methylglyoxal levels in vascular smooth muscle cells and rat isolated aorta

Incubation of VSMCs with MG (30 µM) (Fig. 2A) or high glucose (25 mM) (Fig. 2B) for 24 h significantly increased the level of cellular MG to a similar extent, which was prevented by coincubation with D-Arg or L-Arg (300 µM) (Fig. 2A, B). D-Arg or L-Arg alone did not affect basal MG levels in VSMCs (Fig. 2A, B). Incubation of freshly isolated aorta from the rat with MG (30 µM) (Fig. 2C) or MG (100 µM) (Fig. 2D) or high glucose (25 mM) (Fig. 2C) for 3 h significantly increased the level of cellular MG. The increase of cellular MG was similar between MG (30 µM) and glucose 25 mM treatments (Fig. 2C). The increase of cellular MG was greater after incubation with MG (100 µM) (Fig. 2D), which was significantly attenuated by coincubation with D-Arg or L-Arg (300 µM) or NAC (600 µM) (Fig. 2D). D-Arg or L-Arg alone (100 or 300 µM) or NAC (600 µM) did not affect basal MG levels in the isolated aorta (Fig. 2C, D).

Methylglyoxal and high glucose increase arginase I and II expression and activity

Incubation of HUVECs (Fig. 3A, C) or VSMCs (Fig. 3B, D) with MG (30 µM) (Fig. 3A, B) or high glucose (25 mM) (Fig. 3C, D) for 24 h significantly increased the protein expression of arginase I and II in both cell types, which was attenuated by co-incubation with D-Arg (300 µM) or L-Arg (300 µM).

Incubation of VSMCs with MG (30 µM) or high glucose (25 mM) for 24 h significantly increased total arginase activity (Fig. 3E), which was further increased by D-Arg (300 µM) or L-Arg (300 µM) co-incubated with MG or high glucose (Fig. 3E). However, D-Arg (300 µM) or L-Arg (300 µM) alone had no effect on arginase activity (Fig. 3E).
Incubation of freshly isolated aorta from the rat with MG (30 μM) (Fig. 4A) or (100 μM) (Fig. 4B), or glucose (25 mM) (Fig. 4A) for 3 h significantly increased total arginase activity, which was further increased by D-Arg (300 μM), L-Arg (300 μM) or NAC (600 μM) co-incubated with MG (100 μM) (Fig. 4B), but not by D-Arg (100 μM) or L-Arg (100 μM) co-incubated with MG (30 μM) or glucose 25 mM (Fig. 4A). However, D-Arg (100 or 300 μM) or L-Arg (100 or 300 μM) or NAC (600 μM) alone had no effect on arginase activity (Fig. 4).

**D-arginine and L-arginine prevent increased expression of nuclear factor κB and NADPH oxidase 4 caused by methylglyoxal and high glucose**

Incubation of cultured HUVECs (Fig. 5A, C) or VSMCs (Fig. 5B, D) with MG (30 μM) (Fig. 5A, B) or with high glucose (25 mM) (Fig. 5C, D) for 24 h significantly increased the protein expression of NF-κB and NOX4 in both cell types, which was attenuated by co-incubation with D-Arg (300 μM) or L-Arg (300 μM) (Fig. 5).

**D-arginine and L-arginine prevent methylglyoxal and high glucose-induced reactive oxygen species production**

Incubation of cultured HUVECs (Fig. 6A, C, E) or VSMCs (Fig. 6B, D, F) with MG (30 μM or 100 μM) (Figs. 6A, B, C, D), or with high glucose (25 mM) (Figs. 6E, F) for 3 h or 24 h significantly increased ROS, measured as oxidized DCF, which was attenuated by D-Arg (300 μM) and L-Arg (300 μM) co-incubated with MG or glucose (Fig. 6). D-Arg and L-Arg alone did not affect basal DCF levels (data not shown).

**D-arginine, L-arginine and N-acetyl cysteine prevent methylglyoxal and high glucose-induced increased formation of Nε-carboxyethyl lysine**
Incubation of cultured VSMCs with MG (30 µM) or with high glucose (25 mM) for 24 h significantly increased the formation of the MG-specific AGE, CEL (bright green stain) (Fig. 7). Co-incubation with D-Arg (300 µM) or L-Arg (300 µM) or NAC (600 µM) attenuated the formation of CEL (Fig. 7). L-Arg, D-Arg and NAC alone did not cause any AGE (CEL) formation (data not shown).
Discussion

We investigated the potential of L-Arg and its inactive stereoisomer, D-Arg, as MG scavengers by focusing on endothelial dysfunction induced by MG and high glucose. We have reported the molecular mechanisms of eNOS dysfunction caused by MG and high glucose (Dhar et al., 2010b), so the isolated aortic ring was a good in vitro model to investigate whether D-Arg and L-Arg can prevent the endothelial dysfunction caused by MG and high glucose. Additionally and importantly, we also investigated arginase which can contribute to endothelial dysfunction (Berkowitz et al., 2003; Ishizaka et al., 2007) and also uses L-Arg as a substrate (Morris, 2009). We report the following novel findings: 1. MG up regulates arginase I and arginase II protein expression in cultured HUVECs and VSMCs; 2. MG increases arginase activity in VSMCs and the aorta; 3. High glucose up regulates arginase I and II protein expression in VSMCs and activity in VSMCs and the aorta; 4. D-Arg, L-Arg and NAC attenuate the effects of MG and high glucose in rat isolated aortic rings and cultured HUVECs and VSMCs for the parameters tested except for increased arginase activity, and the effect of D-Arg against MG and high glucose is most likely independent of eNOS, whereas for L-Arg it may be partly eNOS independent and partly eNOS dependent.

Endothelial dysfunction, which is a hallmark of diabetes (Su et al., 2008) (Potenza et al., 2009), and a precursor of cardiovascular complications of diabetes (Potenza et al., 2009; Su et al., 2008), has been widely ascribed to reduced NO availability (Dhar et al., 2010b; Potenza et al., 2009). We have shown that MG and high glucose reduce serine 1177 phosphorylation and activity of eNOS, NO production and endothelium-dependent relaxation, which was prevented by the MG scavengers aminoguanidine and NAC (Dhar et al., 2010b).
D-Arg as well as L-Arg attenuated the reduced endothelium dependent relaxation of the aortic rings caused by MG and high glucose, but only L-Arg prevented the inhibition of ACh-induced relaxation by the NOS inhibitor, L-NAME (Fig. 1). L-Arg, but not D-Arg, is a substrate of NOS (Palmer et al., 1988) and since D-Arg also prevented MG and high glucose-induced reduced relaxation but not that caused by L-NAME, it strongly suggests that arginine, especially D-Arg, prevents MG and high glucose-induced reduced relaxation by an eNOS independent mechanism.

Arginase can cause endothelial dysfunction, mainly by competing with eNOS for the common substrate, L-Arg (Berkowitz et al., 2003; Romero et al., 2008). If L-Arg and D-Arg are to be used as drugs or supplements then their effects on arginase have to be considered. High glucose has been reported to increase expression of arginase in endothelial cells (Romero et al., 2008). The effect of MG on arginase has not been studied. We report, for the first time, that MG increases arginase I and II expression in endothelial cells and VSMCs, and that high glucose also increases arginase I and II expression in VSMCs (Fig. 3) besides endothelial cells, and increases arginase activity in cultured endothelial cells, VSMCs and the rat aorta. D-Arg and L-Arg attenuated the increased arginase expression caused by MG and high glucose in both cell types (Fig. 3), possibly by scavenging MG. MG and high glucose also increased the activity of arginase (Figs. 3, 4), which seems to be independent of increased protein expression because, even when the protein expression of arginase was normalized by co-treatment with MG or high glucose and D-Arg or L-Arg, we still observed increased arginase activity (Fig. 3). Moreover, D-Arg and L-Arg alone did not increase arginase activity. While, the increased arginase activity seen with MG or high glucose plus L-Arg (300 µM) can be explained by increased substrate availability, the increased arginase activity seen with MG or high glucose plus D-Arg (300 µM)
is surprising and needs further evaluation. Alternatively, the increased arginase activity could be an artifact of a higher concentration of 300 µM of L-Arg and D-Arg, since a lower concentration of 100 µM of L-Arg or D-Arg co-incubated with MG or high glucose did not further increase arginase activity (Fig. 4A). However, the use of D-Arg or L-Arg to prevent the deleterious effects of MG and high glucose on endothelial function or possibly on other biological functions, will not be compromised by arginase or eNOS activity.

One of the deleterious effects of MG and high glucose is an increase in oxidative stress (Dhar et al., 2008; Dhar et al., 2010b; Wu et al., 2002), which can be attributed to increased activity of NADPH oxidase (Dhar et al., 2010b) and NF-κB (Wu et al., 2002). We have shown that MG and high glucose reduced ACh-induced relaxation of aortic rings partly by increasing NADPH oxidase activity and oxidative stress (Dhar et al., 2010b). In the current study D-Arg and L-Arg attenuated the increased expression of NOX4 and NF-κB (Fig. 5) and the increased production of free radicals caused by MG and high glucose (Fig. 6), which may or may not have contributed to their beneficial effect on endothelial dysfunction in aortic rings. MG is a key inducer of oxidative stress (Desai et al., 2008) and scavenging MG would prevent activation of multiple pathways of increased free radical generation.

A major deleterious effect of high glucose and reactive dicarbonyl metabolic intermediates, such as MG, glyoxal and 3-deoxyglucosone, is the formation of AGEs (Ahmed et al., 1997; Glomb et al., 2001; Shipanova et al., 1997), which are strongly implicated in the pathogenesis of conditions such as vascular complications of diabetes, atherosclerosis and aging (Vander Jagt et al., 2003). The attenuation by D-Arg and L-Arg, of the formation of the MG-specific AGE, CEL, in VSMCs (Fig. 7) is very promising in this regard. Prevention of dicarbonyls-induced AGEs, which is a major source of AGEs formation (Kilhovd et al., 2003;
Mostafa et al., 2007), by safer and specific scavengers of reactive dicarbonyls is an attractive therapeutic option.

MG has other harmful effects. Thus, acute and chronic MG cause pancreatic β cell dysfunction, reduced adipose tissue glucose uptake, reduced insulin secretion and type 2 diabetes in Sprague-Dawley rats (Dhar et al., 2010a; Dhar et al., 2011). MG has been shown to modify insulin making it dysfunctional (Jia et al., 2006), and LDL making it more atherogenic (Rabbani et al., 2011). Scavengers of MG can be very effective in preventive strategies against the deleterious effects of hyperglycemia, hyperfructoseemia (another source of MG) (Dhar et al., 2008) and elevated MG levels.

Unfortunately, specific MG scavengers are not available. The compounds currently used as MG scavengers are non-specific. Aminoguanidine, the most widely used MG scavenger and AGEs inhibitor (Brownlee et al., 1986; Edelstein et al., 1992), proved toxic in clinical trials (Bolton et al., 2004; Freedman et al., 1999). Metformin, an oral hypoglycemic drug for type 2 diabetes, and N-acetylcysteine also have MG scavenging ability but have other effects (Beisswenger et al., 2003; Jia et al., 2007; Millea, 2009; Ruggiero-Lopez et al., 1999; Vasdev et al., 1998). MG has great affinity for L-Arg and is proposed to react rapidly with the guanidino group of L-Arg (Lo et al., 1994; Takahashi, 1977). Our results from an in vitro binding assay between MG and either L-Arg, D-Arg or NAC show that after 15 min incubation almost half of the MG is bound by all three of the proposed scavengers, whereas after 3 h 80-90% of MG is bound by either D-Arg or L-Arg (Table 1). Thus, it is most likely that arginine binds MG, administered exogenously and produced from glucose and other sources in the body, and thus, attenuates the deleterious effects of MG and high glucose. While L-Arg is used as a nutritional supplement, it is a substrate for five different enzymes including eNOS and arginase (Morris,
2009). The metabolic consequences of orally administered L-Arg, which is known to enter multiple metabolic processes in the liver and the body, are difficult to predict. D-Arg, the inactive isomer of L-Arg, may prove to have more attractive pharmacokinetic and pharmacodynamic properties, and therapeutic potential than L-Arg, if its MG scavenging ability can be demonstrated to specifically inhibit the deleterious effects of MG on multiple functional and biochemical phenomena. Thus, D-Arg and L-Arg need to be evaluated more rigorously for their pharmacokinetic properties and metabolic fate after oral supplementation.

In conclusion, D-Arg and L-Arg attenuate MG- and high glucose-induced endothelial dysfunction, oxidative stress and AGE formation, in a large part by an eNOS independent mechanism, most probably by binding and inactivating MG, given exogenously or produced from high glucose. D-Arg and L-Arg attenuate the increased protein expression but not the increased activity of arginase induced by MG and high glucose, which did not restrict the attenuation of endothelial dysfunction by D-Arg and L-Arg. The effects of D-Arg and L-Arg on other deleterious effects of MG and high glucose, need to be evaluated by many more separate studies, for example on MG-induced pancreatic islet dysfunction and insulin resistance (Dhar et al., 2011), before definitive statements on their therapeutic potential can be made.
Authorship contributions

Participated in research design: Desai, Dhar I

Conducted experiments: Dhar I, Dhar A

Performed data analysis: Dhar I, Dhar A, Desai

Wrote or contributed to the writing of the manuscript: Desai, Dhar I

Took part in discussions: Desai, Dhar I, Dhar A, Wu


Footnotes

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K.D. and L.W. contributed equally to this work.
Legends for figures

Figure 1 D-arginine (D-Arg) and L-arginine (L-Arg) attenuate MG- and high glucose-induced, but not inhibition of endothelial nitric oxide synthase (eNOS)-induced reduced endothelium dependent relaxation in isolated aortic rings from Sprague-Dawley rats. Concentration-related responses were obtained to acetylcholine (Ach) in phenylephrine (PE, 1 μM) precontracted rings before (control) and 2 h after incubation with MG (100 μM) (A, B), or glucose (Glu, 25 mM) (C, D), or 15 min after incubation with the eNOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME 10 μM) (E, F). In some sets of rings L-Arg (300 μM) or D-Arg (300 μM) was preincubated for 15 min followed by co-incubation for 2 h with MG (100 μM) (A, B), or Glu (25 mM) (C, D), or for 15 min with L-NAME (10 μM) (E, F). (n = 6 rings from different rats for each treatment group). *P<0.05, **P<0.01, ***P<0.001 vs corresponding control value, †P<0.05, ††P<0.01, †††P<0.001 vs corresponding L-Arg or D-Arg co-treated value, ‡P<0.05, ‡‡P<0.01, ‡‡‡P<0.001 vs corresponding control value.

Figure 2 Exogenous methylglyoxal (MG) and high glucose increase cellular MG levels in cultured vascular smooth muscle cells and the aorta: attenuation by D-arginine (D-Arg) and L-arginine (L-Arg). Confluent rat aortic vascular smooth muscle cells (VSMC) (A, B) were incubated with normal culture medium (Control, Con) or medium containing MG (30 μM) (A), or glucose (25 mM) (B), for 24 h. L-Arg (300 μM) or D-Arg (300 μM) was incubated alone or with MG (30 μM) (A) or glucose (25 mM) (B) for 24 h. Rat isolated aorta (C, D) was incubated with normal Kreb’s solution (Control, Con) or Krebs containing MG (30 μM) (C) or (100 μM) (D), or glucose (25 mM) (C), for 3 h. L-Arg (100 μM) or D-Arg (100 μM) (C), or L-argin (300 μM), D-Arg (300 μM) or N-acetyl cysteine (NAC, 600 μM) (D) was incubated alone or with
MG for 3 h as shown. Cellular MG was measured by HPLC. \( n = 6 \) for each treatment. \( **P<0.01, \ ***P<0.001 \) vs corresponding control value, \( †††P<0.001 \) vs corresponding MG or glucose value.

**Figure 3** Attenuation by D-arginine (D-Arg) and L-arginine (L-Arg) of methylglyoxal (MG) and high glucose-induced increased protein expression, but not activity of arginase (Agn) I and II in cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs). Cultured confluent HUVECs (A, C) or VSMCs (A-10 cell line) (B, D, E) were incubated with MG (30 µM) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-Arg (300 µM) or D-Arg (300 µM) was incubated alone (E) or coincubated with MG (30 µM) or glucose (25 mM) for 24 h following which the arginase I and arginase II proteins were determined by western blotting (A-D). In (E) an arginase activity assay was performed 24 h after the treatments, based on conversion of arginine to ornithine and urea, using an activity assay kit. \( n = 6 \) for each group. \( ***P<0.001 \) vs. control, \( ††P<0.01, †††P<0.001 \) vs corresponding MG 30 µM or glucose 25 mM value.

**Figure 4** Methylglyoxal (MG) and high glucose (Glu) increase arginase activity in rat isolated aorta. The aorta was freshly isolated from Sprague-Dawley rats, cleaned and incubated with normal Kreb’s solution (Control, Con) or Kreb’s containing MG (30 µM) (A) or (100 µM) (B), or glucose (25 mM) (A), for 3 h in an incubator at 37\(^\circ\) C. L-Arg (100 µM) or D-Arg (100 µM) (A), or L_arg (300 µM), D-Arg (300 µM) or N-acetyl cysteine (NAC, 600 µM) (B) was incubated alone or with MG or glucose for 3 h as shown. An arginase activity assay was performed after the 3 h incubation, based on conversion of arginine to ornithine and urea, using an activity assay kit. \( n = 3 \) for each group. \( *P<0.05, **P<0.01, ***P<0.001 \) vs. control, \( ††P<0.01, †††P<0.001 \) vs corresponding MG 100 µM value.
Figure 5 Attenuation by D-arginine (D-Arg) and L-arginine (L-Arg) of methylglyoxal (MG) and high glucose-induced increased protein expression of nuclear factor kappa B (NF-κB) and NADPH oxidase 4 (NOX-4) in cultured human umbilical vein endothelial cells (HUVECs) and vascular smooth muscle cells (VSMCs). Cultured confluent HUVECs (A, C) or VSMCs (A-10 cell line) (B, D) were incubated with MG (30 µM) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-Arg (300 µM) or D-Arg (300 µM) was coincubated with MG (30 µM) or glucose (25 mM) for 24 h following which the NF-κB and NOX-4 proteins were determined by western blotting. \( n = 3 \) each.

Figure 6 Attenuation by D-arginine (D-Arg) and L-arginine (L-Arg) of methylglyoxal (MG) and high glucose-induced increased reactive oxygen species production in cultured human umbilical vein endothelial cells (HUVECs, A, C, E) and rat aortic vascular smooth muscle cells (VSMCs, B, D, F). Incubation of cultured HUVECs or VSMCs (A-10 cell line) with MG (30 µM) (A, B), or MG (100 µM) (C, D) or glucose (25 mM) (E, F) for 3 h or 24 h increased reactive oxygen species production, measured as oxidized dichlorofluorescein (DCF), which was attenuated by co-incubation with either L-Arg (300 µM) or D-Arg (300 µM). \( n = 8 \) for each group. **\( P < 0.01 \), ***\( P < 0.001 \) vs. corresponding control, †††\( P < 0.001 \) vs. corresponding MG (30 µM), or MG (100 µM), or glucose (25 mM) value.

Figure 7 Detection of methylglyoxal-induced advanced glycation endproduct, \( N^\varepsilon \)-carboxyethyllysine (CEL), in cultured rat aortic vascular smooth muscle cells (A-10 cells) after incubation with MG (30 µM) or glucose (25 mM) alone, or coincubated with either L-arginine (L-Arg 300 µM), D-arginine (D-Arg 300 µM) or MG (30 µM) + N-acetyl cysteine (NAC, 600 µM) for 24 h. Immunocytochemistry was performed on fixed cells with specific CEL antibody and fluorescein isothiocyanate (FITC) conjugated secondary antibody. \( n = 3 \) each. Scale bar 10 µm.
Table 1

Methylglyoxal (MG) was incubated with either L-arginine (L-Arg), D-arginine (D-Arg) or N-acetyl cysteine (NAC) in phosphate buffered saline at 37°C for different times. The solution was analyzed for free MG by HPLC after the given incubation period. The values are expressed as Mean ± S.E.M. (n = 6 each). ***P<0.001 vs respective MG alone.

<table>
<thead>
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<th>Incubation time</th>
<th>MG (30 µM) alone</th>
<th>MG (30 µM) + L-Arg (300 µM)</th>
<th>MG (30 µM) + D-Arg (300 µM)</th>
<th>MG (30 µM) + NAC (600 µM)</th>
<th>NAC (600 µM)</th>
<th>L-Arg (300 µM)</th>
<th>D-Arg (300 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>26.7 ± 0.7</td>
<td>12.6 ± 0.7***</td>
<td>12.2 ± 0.6***</td>
<td>15.2 ± 0.7***</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 min</td>
<td>26.2 ± 0.7</td>
<td>7.4 ± 0.5***</td>
<td>7.00 ± 0.3***</td>
<td>8.8 ± 0.6***</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>60 min</td>
<td>27.2 ± 0.6</td>
<td>5.7 ± 0.2***</td>
<td>4.3 ± 0.12***</td>
<td>7.00 ± 0.3***</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 h</td>
<td>27.6 ± 0.6</td>
<td>3.5 ± 0.09***</td>
<td>2.1 ± 0.05***</td>
<td>5.3 ± 0.06***</td>
<td>0</td>
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<tr>
<td>24 h</td>
<td>26.3 ± 0.6</td>
<td>1.2 ± 0.07***</td>
<td>0.8 ± 0.08***</td>
<td>2.7 ± 0.08***</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 1

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Figure 2
Figure 3
Figure 4
Figure 5
**Figure 6**

HUVECs

- **A**: 3 h vs 24 h
  - Con
  - MG 30 μM
  - +L-arg 300 μM
  - +D-arg 300 μM

VSMCs

- **B**: 3 h vs 24 h
  - Con
  - MG 30 μM
  - +L-arg 300 μM
  - +D-arg 300 μM

- **C**: 3 h vs 24 h
  - Con
  - MG 100 μM
  - +L-arg 300 μM
  - +D-arg 300 μM

- **D**: 3 h vs 24 h
  - Con
  - MG 100 μM
  - +L-arg 300 μM
  - +D-arg 300 μM

- **E**: 3 h vs 24 h
  - Con
  - Glucose 25 mM
  - +L-arg 300 μM
  - +D-arg 300 μM

- **F**: 3 h vs 24 h
  - Con
  - Glucose 25 mM
  - +L-arg 300 μM
  - +D-arg 300 μM
Figure 7