

# Arginine Attenuates Methylglyoxal- and High Glucose-Induced Endothelial Dysfunction and Oxidative Stress by an Endothelial Nitric-Oxide Synthase-Independent Mechanism

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## ABSTRACT

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 *N*<sup>ω</sup>-nitro-L-arginine methyl ester on vasorelaxation was prevented by L-argi-

nine, but not D-arginine. MG and high glucose increased protein expression of arginase, a novel finding, NADPH oxidase 4, and nuclear factor  $\kappa$ B and increased production of reactive oxygen species in HUVECs and VSMCs, which were attenuated by D-arginine and L-arginine. However, D-arginine and L-arginine did not attenuate MG- and high glucose-induced increased arginase activity in VSMCs and the aorta. D-Arginine and L-arginine also attenuated the increased formation of the MG-specific AGE *N*<sup>ε</sup>-carboxyethyl lysine, caused by MG and high glucose in VSMCs. In conclusion, arginine attenuates the increased arginase expression, oxidative stress, endothelial dysfunction, and AGE formation induced by MG and high glucose by an eNOS-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 NO and L-citrulline (Palmer et al., 1988). Endothelial NOS (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 expressed mainly 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 (Zhang et al., 2001; Berkowitz et al., 2003). 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

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**ABBREVIATIONS:** NOS, nitric-oxide synthase; eNOS, endothelial NOS; ACh, acetylcholine; AGE, advanced glycation endproduct; CEL, *N*<sup>ε</sup>-carboxyethyl lysine; Con, control; DCF, dichlorofluorescein; L-Arg, L-arginine; D-Arg, D-arginine; FBS, fetal bovine serum; Glu, glucose; HPLC, high-performance liquid chromatography; HUVEC, human umbilical vein endothelial cell; MG, methylglyoxal; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester; NAC, *N*-acetyl cysteine; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NOX4, NADPH oxidase 4; PBS, phosphate-buffered saline; PE, phenylephrine; VSMC, vascular smooth muscle cell.

to varying degrees (Thornalley, 1996), is a major precursor for the formation of advanced glycation endproducts (AGEs) (Kilhovd et al., 2003). Under physiological conditions the MG produced in the body is efficiently degraded by the glyoxalase enzymes and reduced glutathione into D-lactate (Thornalley, 1993; Vander Jagt and Hunsaker, 2003). Increased MG formation in diabetic patients increases plasma MG levels 3- to 4-fold (Thornalley, 1988; Thornalley et al., 1989; McLellan et al., 1994; 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, 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, 2011), and AGE formation (Wang et al., 2008; Dhar et al., 2011), 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 and Brownlee, 1992), alagebrium (Wolffenbuttel et al., 1998; Dhar et al., 2010a), *N*-acetyl cysteine (NAC) (Vasdev et al., 1998; Jia and Wu, 2007), and metformin (Ruggiero-Lopez et al., 1999; Beisswenger and Ruggiero-Lopez, 2003; Wang et al., 2008). MG has been reported to have high affinity for arginine (Takahashi, 1977; Lo et al., 1994). The  $K_a$  (affinity constant) of MG for arginine is  $1.9 \times 10^3 \text{ M}^{-1}$ . The  $K_m$  of L-Arg for eNOS is in the micromolar range ( $\sim 2.9 \mu\text{M}$ ) (Pollock et al., 1991). Because 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 AGE formation. We also examined the effects of MG on arginase expression and activity, which has not been reported previously.

## Materials and Methods

**Animals.** A total of 36 male 11-week-old Sprague-Dawley rats from Charles River Canada (Montreal, QC, 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* (Institute of Laboratory Animal Resources, 1996). After 1 week of acclimatization, the rats were anesthetized with an inhalation of isoflurane (Forane, Baxter Corporation, Mississauga, ON, Canada; 4% in oxygen), delivered

through a precision vaporizer and circle absorption breathing system (Ohio 30/70 Proportioner Anesthesia Machine; BOC Healthcare, Steeton, West Yorkshire, UK). When anesthesia reached sufficient depth as determined by the absence of the leg flexor response and 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 Krebs's solution and bubbled with 95%  $\text{O}_2 + 5\% \text{CO}_2$ .

**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. In brief, 3- to 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%  $\text{O}_2 + 5\% \text{CO}_2$ . After a 90-min equilibration period the rings were precontracted with phenylephrine (1  $\mu\text{M}$ ), and cumulative concentration-dependent relaxation in response to acetylcholine (ACh) was obtained before (control) and 2 h after incubation with either glucose (25 mM), MG (100  $\mu\text{M}$ ) (Dhar et al., 2010b), or the nitric-oxide synthase inhibitor *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME; 10  $\mu\text{M}$ ). In initial experiments, the responses to ACh were repeated before and 2 h after incubation with normal Krebs' solution to confirm reproducibility of responses to ACh. Some sets of rings were coincubated with either D-Arg (300  $\mu\text{M}$ ) or L-Arg (300  $\mu\text{M}$ ) (Rees et al., 1989; Desai et al., 1991; Schulz et al., 1991) for 2 h. Treatment with each compound was tested in rings from at least six different rats. Isometric tension was measured with isometric force transducers with Chart software and Powerlab equipment (ADInstruments Inc., Colorado Springs, CO).

**In Vitro Methylglyoxal Binding Assay.** MG (30  $\mu\text{M}$ ) was coincubated with either L-Arg (300  $\mu\text{M}$ ), D-Arg (300  $\mu\text{M}$ ), or NAC (600  $\mu\text{M}$ ) in phosphate-buffered saline (PBS) at 37°C for 15, 30, or 60 min or 3 and 24 h. After the prescribed incubation time the free MG in the sample was measured by HPLC (Dhar et al., 2009).

**Methylglyoxal 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 12,000 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 a Symmetry C18 column (3.9  $\times$  150 mm, and 4- $\mu\text{m}$  particle diameter; Waters, Milford, MA).

**Cell Culture.** The rat thoracic aortic smooth muscle cell line (A-10 cells) was obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  as described previously (Dhar et al., 2008). A-10 cells were seeded either in 100-mm dishes for MG measurement or 96-well plates for other assays, with an equal amount of cells ( $10^6/\text{ml}$ ) in each well, and cultured to confluence. For immunocytochemistry staining, cells were seeded on glass cover slides ( $2 \times 10^6/\text{ml}$ ). Cells were starved in FBS-free Dulbecco's modified Eagle's medium for 24 h before exposure to different metabolic precursors.

HUVECs from the American Type Culture Collection were cultured in Kaighn's F12K medium containing 10% FBS, 0.1 mg/ml heparin, and 0.03 to 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, 2011), and the protein concentration in the supernatant was determined by the BCA Protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of

cell lysates (50 µg of protein each) were separated on 7.5 to 10% SDS-polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), blocked with 5% nonfat milk in Tris-buffered saline-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 κB (NF-κB), or NADPH oxidase 4 (NOX4) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and then with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Dhar et al., 2010b, 2011).

**Arginase Activity Assay.** Arginase activity was measured by using an arginase assay kit (BioAssays Systems, Hayward, CA). Arginase catalyzes the conversion of arginine to ornithine and urea. In brief, 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 was measured at 430 nm.

**Measurement of Reactive Oxygen Species.** Confluent cells were loaded with a membrane-permeable, nonfluorescent probe 2',7'-dichlorofluorescein 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 or 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of reactive oxygen species as described previously (Dhar et al., 2008, 2010b). The protein content of the homogenate was measured by using the BCA Protein assay (Bio-Rad Laboratories).

**Immunocytochemistry.** A-10 cells were seeded on glass coverslips followed by incubation with different test compounds for 24 h and subjected to staining for the MG-induced AGE N<sup>ε</sup>-carboxyethyl lysine (CEL). As described previously (Dhar et al., 2008, 2011), the treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and washed twice with 0.01 N 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 nonspecific 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, Bagsvaerd, Denmark) overnight at room temperature. Cells were washed twice in PBS (0.01 N) for 5 min and incubated with secondary fluorescein isothiocyanate-conjugated anti-CEL antibody (Molecular Probes, Carlsbad, CA) 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 by using Metamorph image analysis software (version 7; Molecular Devices, Sunnyvale, CA). Slides from four different experiments were analyzed with five 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 ± S.E.M. and were analyzed by using one-way analysis of variance 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 coincubation (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. 1, A and B), and high glucose (25 mM; Fig. 1, C and 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. 1, A and C) or D-Arg (300 µM; Fig. 1, B and D) with MG (Fig. 1, A and B) or high glucose (Fig. 1, C and D). Incubation of the eNOS inhibitor L-NAME (10 µM; Fig. 1, E and F) prevented ACh-induced relaxation, which was restored by the coincubation 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 had shown earlier that NAC, which has been used as a positive control MG scavenger 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. 2, A and B). D-Arg or L-Arg alone did not affect basal MG levels in VSMCs (Fig. 2, A and B). Incubation of freshly isolated aorta from the rat with 30 µM (Fig. 2C) and 100 µM (Fig. 2D) MG 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).

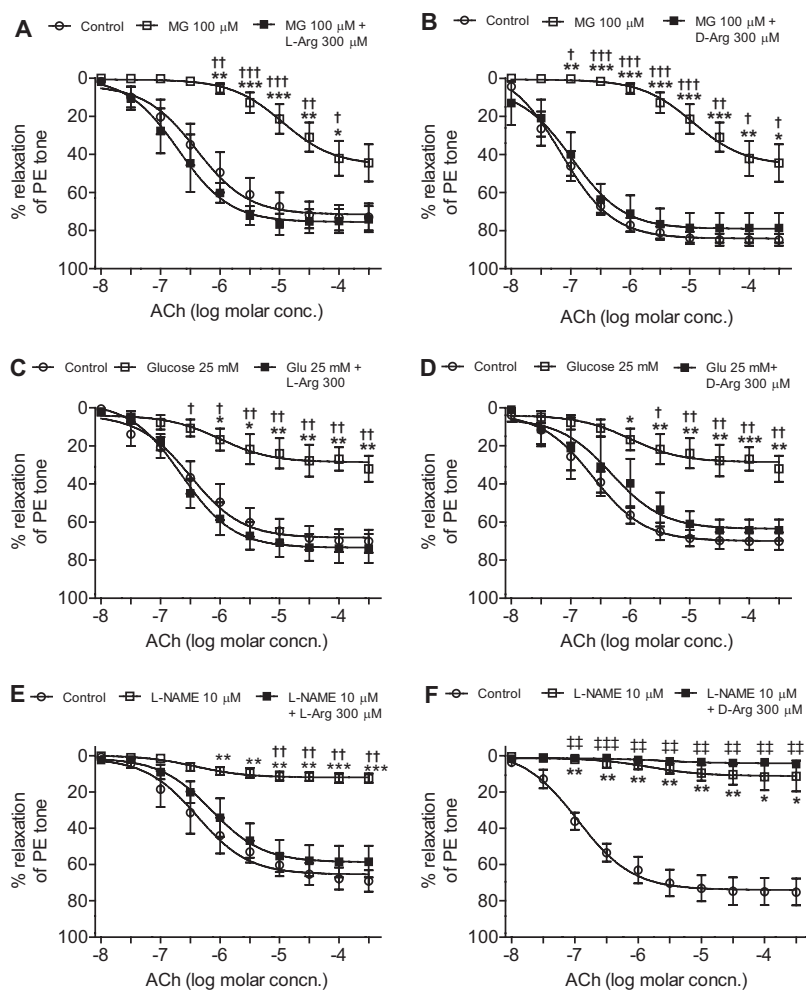
TABLE 1

MG was incubated with either L-Arg, D-Arg, or NAC in PBS at 37°C for different times

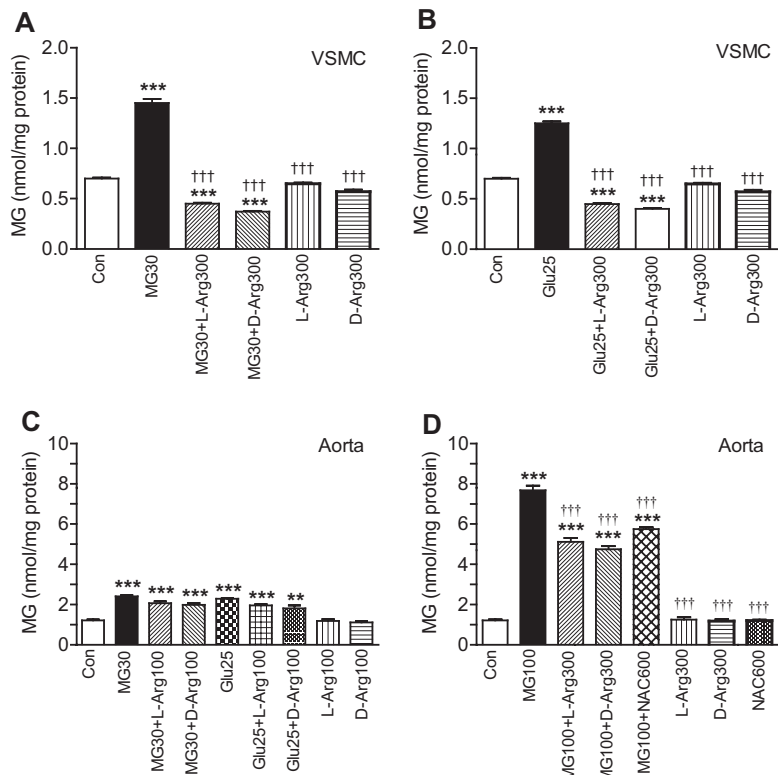
The solution was analyzed for free MG by HPLC after the given incubation period. The values are expressed as mean ± S.E.M. (*n* = 6 each).

Incubation Time	MG (30 µM) Alone	MG (30 µM) + L-Arg (300 µM)	MG (30 µM) + D-Arg (300 µM)	MG (30 µM) + NAC (600 µM)	NAC (600 µM)	L-Arg (300 µM)	D-Arg (300 µM)
15 min	26.7 ± 0.7	12.6 ± 0.7***	12.2 ± 0.6***	15.2 ± 0.7***	0	0	0
30 min	26.2 ± 0.7	7.4 ± 0.5***	7.00 ± 0.3***	8.8 ± 0.6***	0	0	0
60 min	27.2 ± 0.6	5.7 ± 0.2***	4.3 ± 0.12***	7.00 ± 0.3***	0	0	0
3 h	27.6 ± 0.6	3.5 ± 0.09***	2.1 ± 0.05***	5.3 ± 0.06***	0	0	0
24 h	26.3 ± 0.6	1.2 ± 0.07***	0.8 ± 0.08***	2.7 ± 0.08***	0	0	0

\*\*\**P* < 0.001 vs. MG alone.



**Fig. 1.** D-Arg and L-Arg attenuate MG- and high glucose-induced, but not inhibition of eNOS-induced, reduced endothelium-dependent relaxation in isolated aortic rings from Sprague-Dawley rats. Concentration-related responses were obtained to ACh in phenylephrine (PE; 1  $\mu$ M) precontracted rings before (control) and 2 h after incubation with MG (100  $\mu$ M) (A and B) or glucose (Glu; 25 mM) (C and D) or 15 min after incubation with the eNOS inhibitor L-NAME (10  $\mu$ M) (E and F). In some sets of rings L-Arg (300  $\mu$ M) or D-Arg (300  $\mu$ M) was preincubated for 15 min followed by coinubation for 2 h with MG (100  $\mu$ M) (A and B) or Glu (25 mM) (C and D) or for 15 min with L-NAME (10  $\mu$ M) (E and F).  $n = 6$  rings from different rats for each treatment group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.01$  versus corresponding control values. †,  $P < 0.05$ ; ††,  $P < 0.01$ ; †††,  $P < 0.001$  versus corresponding L-Arg- or D-Arg cotreated values. ‡,  $P < 0.01$ ; ‡‡,  $P < 0.001$  versus corresponding control values.



**Fig. 2.** Exogenous MG and high glucose increase cellular MG levels in cultured vascular smooth muscle cells and the aorta: attenuation by D-Arg and L-Arg. A and B, confluent rat aortic VSMCs were incubated with normal culture medium (control, Con) or medium containing MG (30  $\mu$ M) (A) or glucose (25 mM) (B) for 24 h. L-Arg (300  $\mu$ M) or D-Arg (300  $\mu$ M) was incubated alone or with MG (30  $\mu$ M) (A) or glucose (25 mM) (B) for 24 h. C and D, rat isolated aorta was incubated with normal Krebs' solution (control, Con) or Krebs' containing MG (30  $\mu$ M) (C) or (100  $\mu$ M) (D) or glucose (25 mM) (C) for 3 h. L-Arg (100  $\mu$ M) or D-Arg (100  $\mu$ M) (C) or L-Arg (300  $\mu$ M), D-Arg (300  $\mu$ M), or NAC (600  $\mu$ M) (D) was incubated alone or with MG for 3 h as shown. Cellular MG was measured by HPLC.  $n = 6$  for each treatment. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  versus corresponding control values. †††,  $P < 0.001$  versus corresponding MG or glucose values.

The increase of cellular MG was greater after incubation with MG (100  $\mu$ M) (Fig. 2D), which was significantly attenuated by coincubation with D-Arg or L-Arg (300  $\mu$ M) or NAC (600  $\mu$ M) (Fig. 2D). D-Arg or L-Arg alone (100 or 300  $\mu$ M) or NAC (600  $\mu$ M) did not affect basal MG levels in the isolated aorta (Fig. 2, C and D).

**Methylglyoxal and High Glucose Increase Arginase I and II Expression and Activity.** Incubation of HUVECs (Fig. 3, A and C) or VSMCs (Fig. 3, B and D) with MG (30  $\mu$ M) (Fig. 3, A and B) or high glucose (25 mM) (Fig. 3, C and D) for 24 h significantly increased the protein expression of arginase I and II in both cell types, which was attenuated by coincubation with D-Arg (300  $\mu$ M) or L-Arg (300  $\mu$ M).

Incubation of VSMCs with MG (30  $\mu$ M) or high glucose (25 mM) for 24 h significantly increased total arginase activity (Fig. 3E), which was further increased by D-Arg (300  $\mu$ M) or L-Arg (300  $\mu$ M) coincubated with MG or high glucose (Fig. 3E). However, D-Arg (300  $\mu$ M) or L-Arg (300  $\mu$ M) alone had no effect on arginase activity (Fig. 3E).

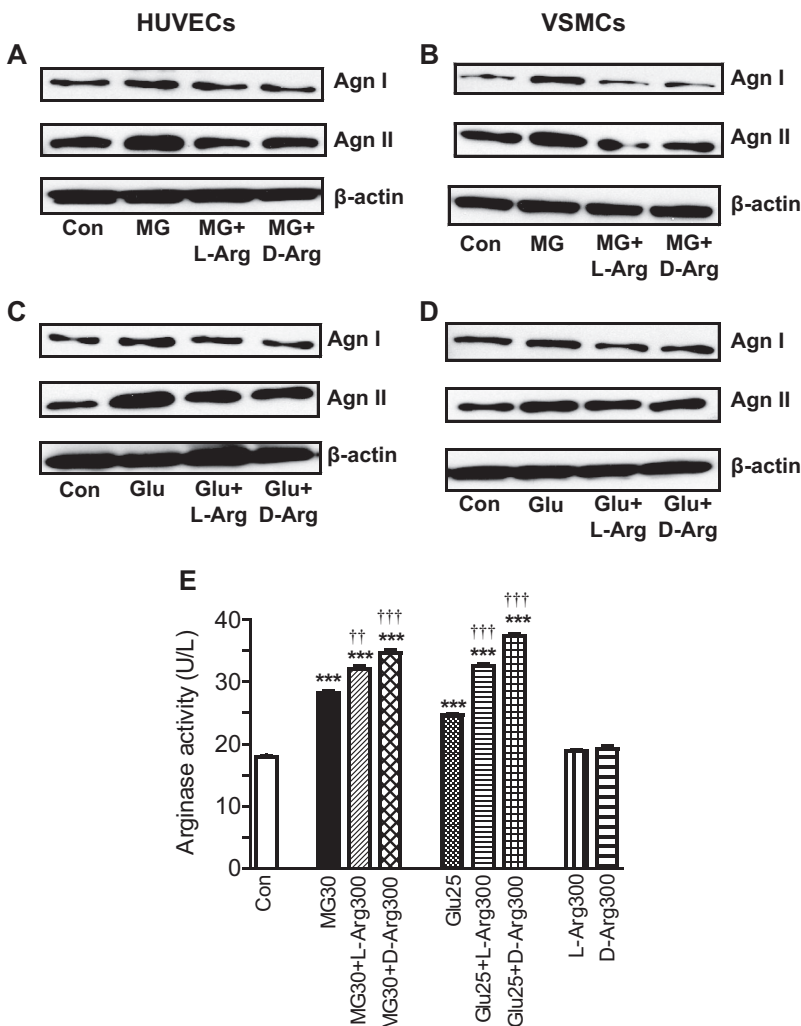
Incubation of freshly isolated aorta from the rat with 30  $\mu$ M (Fig. 4A) or 100  $\mu$ M (Fig. 4B) MG or glucose (25 mM) (Fig. 4A) for 3 h significantly increased total arginase activity, which was further increased by D-Arg (300  $\mu$ M), L-Arg (300  $\mu$ M), or NAC (600  $\mu$ M) coincubated with MG (100  $\mu$ M) (Fig. 4B), but not by D-Arg (100  $\mu$ M) or L-Arg (100  $\mu$ M) coincubated with MG (30  $\mu$ M) or glucose 25 (mM) (Fig. 4A). However,

D-Arg (100 or 300  $\mu$ M), L-Arg (100 or 300  $\mu$ M), or NAC (600  $\mu$ M) alone had no effect on arginase activity (Fig. 4).

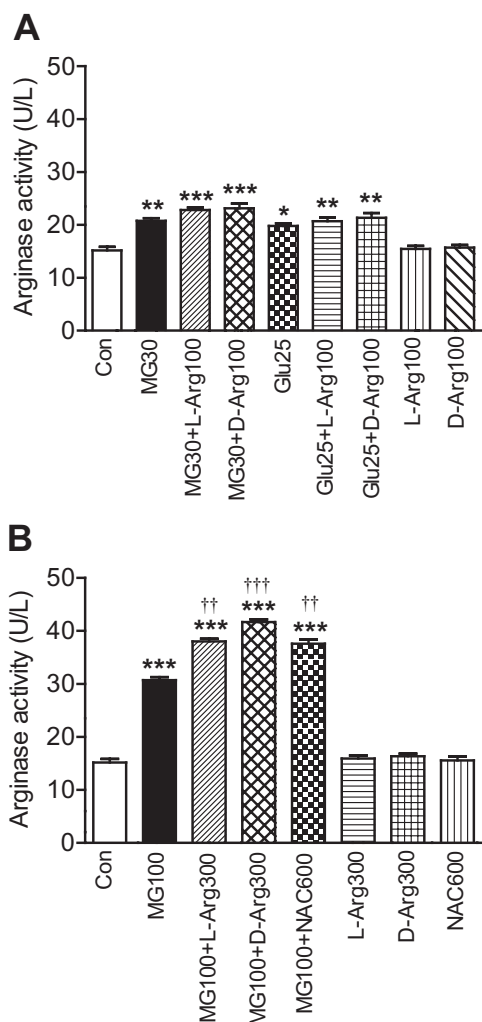
**D-Arginine and L-Arginine Prevent Increased Expression of Nuclear Factor  $\kappa$ B and NADPH Oxidase 4 Caused by Methylglyoxal and High Glucose.** Incubation of cultured HUVECs (Fig. 5, A and C) or VSMCs (Fig. 5, B and D) with MG (30  $\mu$ M) (Fig. 5, A and B) or high glucose (25 mM) (Fig. 5, C and D) for 24 h significantly increased the protein expression of NF- $\kappa$ B and NOX4 in both cell types, which was attenuated by coincubation with D-Arg (300  $\mu$ M) or L-Arg (300  $\mu$ M) (Fig. 5).

**D-Arginine and L-Arginine Prevent Methylglyoxal- and High Glucose-Induced Reactive Oxygen Species Production.** Incubation of cultured HUVECs (Fig. 6, A, C, and E) or VSMCs (Fig. 6, B, D, and F) with MG (30 or 100  $\mu$ M) (Fig. 6, A-D) or high glucose (25 mM) (Fig. 6, E and F) for 3 or 24 h significantly increased reactive oxygen species, measured as oxidized DCF, which was attenuated by D-Arg (300  $\mu$ M) and L-Arg (300  $\mu$ M) coincubated 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  $N^{\epsilon}$ -Carboxyethyl Lysine.** Incubation of cultured VSMCs with MG (30  $\mu$ M) or high glucose (25 mM) for 24 h significantly increased the formation of the



**Fig. 3.** Attenuation by D-Arg and L-Arg of MG- and high glucose-induced increased protein expression, but not activity of arginase (Agn) I and II in cultured HUVECs and VSMCs. Cultured confluent HUVECs (A and C) or VSMCs (A-10 cell line) (B, D, and E) were incubated with MG (30  $\mu$ M) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-Arg (300  $\mu$ M) or D-Arg (300  $\mu$ M) was incubated alone (E) or coincubated with MG (30  $\mu$ M) or glucose (25 mM) for 24 h after 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. \*\*\*,  $P < 0.001$  versus control. ††,  $P < 0.01$ ; †††,  $P < 0.001$  versus corresponding MG (30  $\mu$ M) or glucose (25 mM) values.



**Fig. 4.** 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 Krebs's solution (control, Con) or Krebs's containing MG (30  $\mu$ M) (A) or (100  $\mu$ M) (B) or glucose (25 mM) (A) for 3 h in an incubator at 37°C. L-Arg (100  $\mu$ M) or D-Arg (100  $\mu$ M) (A) or L-Arg (300  $\mu$ M), D-Arg (300  $\mu$ M), or NAC (600  $\mu$ M) (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. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  versus control. ††,  $P < 0.01$ ; †††,  $P < 0.001$  versus corresponding MG (100  $\mu$ M) values.

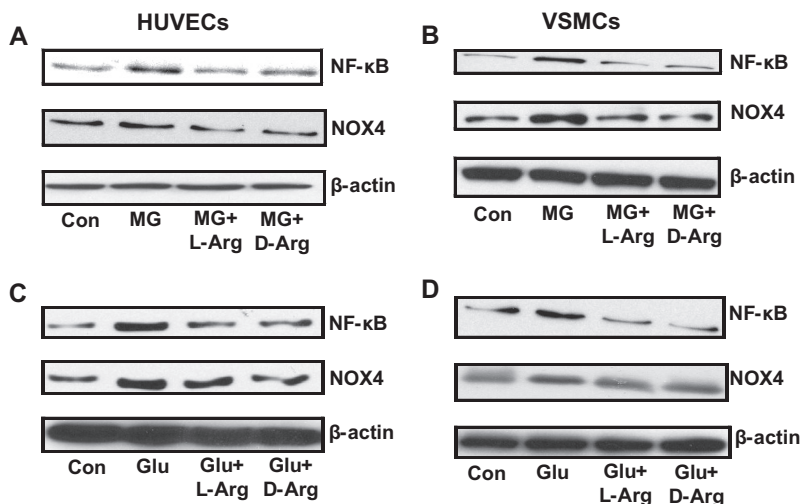
MG-specific AGE CEL (bright green stain in Fig. 7). Coincubation with D-Arg (300  $\mu$ M), L-Arg (300  $\mu$ M), or NAC (600  $\mu$ M) attenuated the formation of CEL (Fig. 7). L-Arg, D-Arg, and NAC alone did not cause any AGE (CEL) formation (data not shown).

### Discussion

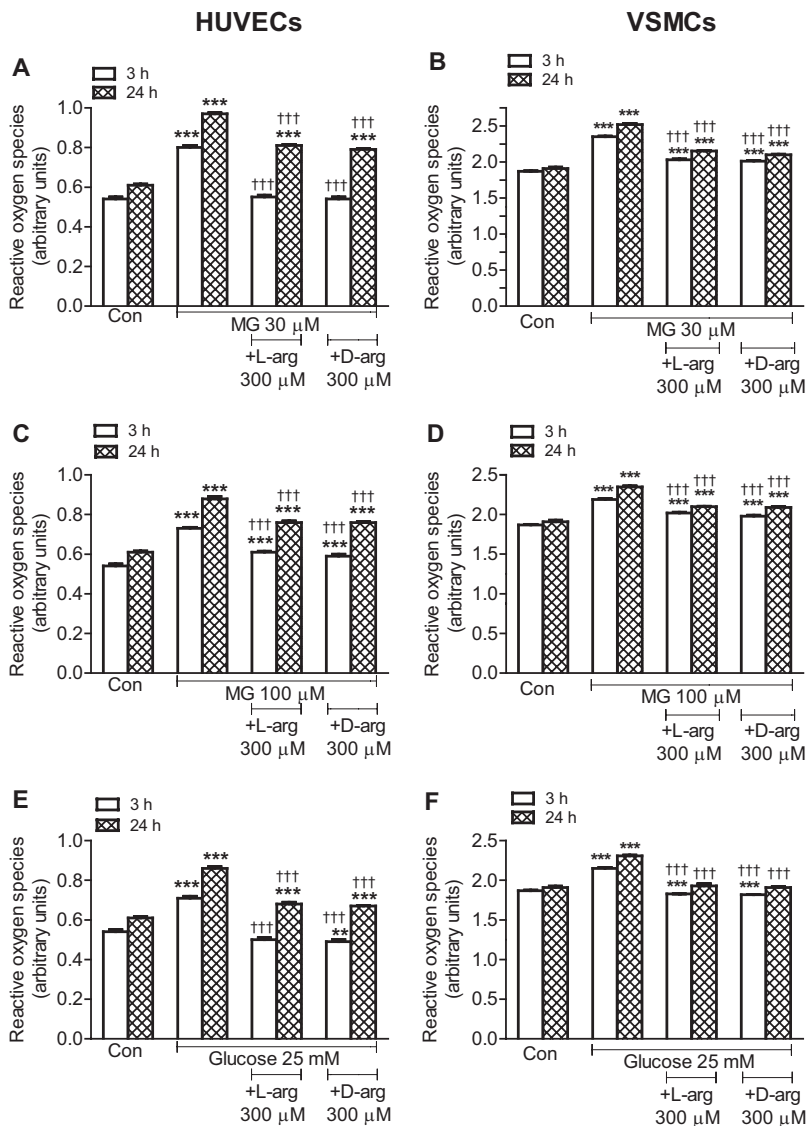
We investigated the potential of L-Arg and its inactive stereoisomer, D-Arg, as MG scavengers by focusing on endothelial dysfunction induced by MG and high glucose. We had previously reported the molecular mechanisms of eNOS dysfunction caused by MG and high glucose (Dhar et al., 2010b), so the isolated aortic ring was a good in vitro model for investigating whether D-Arg and L-Arg can prevent the endothelial dysfunction caused by MG and high glucose. 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; and 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; 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 and a precursor of cardiovascular complications of diabetes (Su et al., 2008; Potenza et al., 2009), has been widely ascribed to reduced NO availability (Potenza et al., 2009; Dhar et al., 2010b). 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 and 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-



**Fig. 5.** Attenuation by D-Arg and L-Arg of MG- and high glucose-induced increased protein expression of NF- $\kappa$ B and NOX4 in cultured HUVECs and VSMCs. Cultured confluent HUVECs (A and C) or VSMCs (A-10 cell line) (B and D) were incubated with MG (30  $\mu$ M) or glucose (Glu, 25 mM) for 24 h. In separate dishes L-Arg (300  $\mu$ M) or D-Arg (300  $\mu$ M) was coincubated with MG (30  $\mu$ M) or glucose (25 mM) for 24 h after which the NF- $\kappa$ B and NOX4 proteins were determined by Western blotting.  $n = 3$  each.



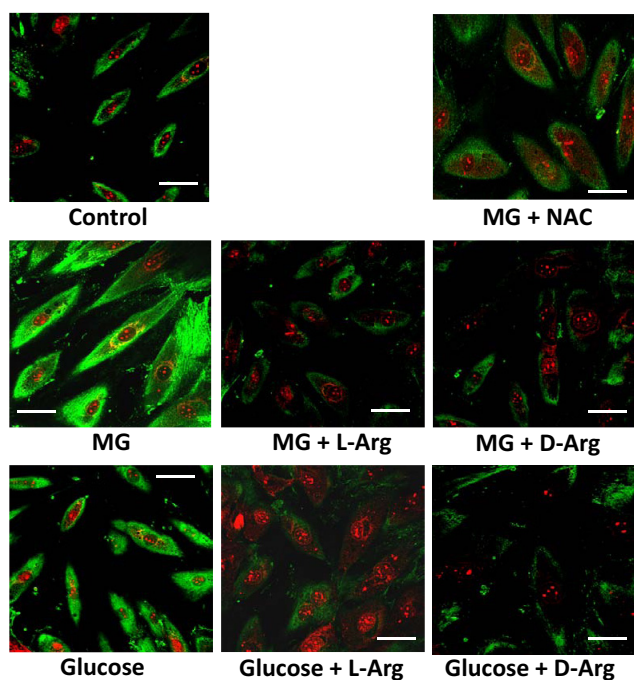
**Fig. 6.** Attenuation by D-Arg and L-Arg of MG- and high glucose-induced increased reactive oxygen species production in cultured HUVECs (A, C, and E) and rat aortic VSMCs (B, D, and F). Incubation of cultured HUVECs or VSMCs (A-10 cell line) with MG (30  $\mu$ M) (A and B), MG (100  $\mu$ M) (C and D), or glucose (25 mM) (E and F) for 3 or 24 h increased reactive oxygen species production, measured as oxidized DCF, which was attenuated by coincubation with either L-Arg (300  $\mu$ M) or D-Arg (300  $\mu$ M).  $n = 8$  for each group. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  versus corresponding control. †††,  $P < 0.001$  versus corresponding MG (30 and 100  $\mu$ M) or glucose (25 mM) value.

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 because 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 high glucose also increases arginase I and II expression in VSMCs (Fig. 3), in addition to 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 and 4), which seems to be independent of increased protein expression because even when the protein expression of arginase was normalized by cotreatment 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. Although the increased arginase activity seen with MG or high glucose plus L-Arg (300  $\mu$ M) can be explained by increased substrate availability, the increased arginase activity seen with MG or high glucose plus D-Arg (300  $\mu$ M) is surprising and needs further evaluation. Alternatively, the increased arginase activity could be an artifact of a higher concentration of 300  $\mu$ M L-Arg and D-Arg, because a lower concentration of 100  $\mu$ M L-Arg or D-Arg coincubated 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 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 (Wu and Juurlink, 2002; Dhar et



**Fig. 7.** Detection of methylglyoxal-induced advanced glycation endproduct, CEL, in cultured rat aortic vascular smooth muscle cells (A-10 cells) after incubation with MG (30  $\mu$ M) or glucose (25 mM) alone or coincubated with either L-Arg (300  $\mu$ M), D-Arg (300  $\mu$ M), or MG (30  $\mu$ M) + NAC (600  $\mu$ M) for 24 h. Immunocytochemistry was performed on fixed cells with specific CEL antibody and fluorescein isothiocyanate-conjugated secondary antibody.  $n = 3$  each. Scale bars, 10  $\mu$ m.

al., 2008, 2010b), which can be attributed to increased activity of NADPH oxidase (Dhar et al., 2010b) and NF- $\kappa$ B (Wu and Juurlink, 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- $\kappa$ B (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 and Wu, 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; Shipanova et al., 1997; Glomb et al., 2001), which are strongly implicated in the pathogenesis of conditions such as vascular complications of diabetes, atherosclerosis, and aging (Vander Jagt and Hunsaker, 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 dicarbonyl-induced AGEs, which is a major source of AGE 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. Acute and chronic MG cause pancreatic  $\beta$  cell dysfunction, reduced adipose tissue glucose uptake, reduced insulin secretion, and type 2 diabetes in Sprague-Dawley rats (Dhar et al., 2010a, 2011). MG has been shown to modify insulin, making it dysfunctional (Jia et al.,

2006), and low-density lipoprotein, making it more atherogenic (Rabbani et al., 2011). Scavengers of MG can be very effective in preventive strategies against the deleterious effects of hyperglycemia, hyperfructosemia (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 AGE inhibitor (Brownlee et al., 1986; Edelstein and Brownlee et al., 1992), proved to be toxic in clinical trials (Freedman et al., 1999; Bolton et al., 2004). Metformin, an oral hypoglycemic drug for type 2 diabetes, and *N*-acetyl cysteine also have MG-scavenging ability, but they have other effects (Vasdev et al., 1998; Ruggiero-Lopez et al., 1999; Beisswenger and Ruggiero-Lopez, 2003; Jia and Wu, 2007; Millea, 2009). MG has great affinity for L-Arg and is proposed to react rapidly with the guanidino group of L-Arg (Takahashi, 1977; Lo et al., 1994). 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 to 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. Although 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 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:* I. Dhar and Desai.

*Conducted experiments:* I. Dhar and A. Dhar.

*Performed data analysis:* I. Dhar, A. Dhar, and Desai.

*Wrote or contributed to the writing of the manuscript:* I. Dhar, Desai, and Wu.



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