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RESEARCH PAPER

Methylglyoxal scavengers attenuate endothelial dysfunction induced by methylglyoxal and high concentrations of glucose

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BACKGROUND AND PURPOSE
Endothelial dysfunction is a feature of hypertension and diabetes. Methylglyoxal (MG) is a reactive dicarbonyl metabolite of glucose and its levels are elevated in spontaneously hypertensive rats and in diabetic patients. We investigated if MG induces endothelial dysfunction and whether MG scavengers can prevent endothelial dysfunction induced by MG and high glucose concentrations.

EXPERIMENTAL APPROACH
Endothelium-dependent relaxation was studied in aortic rings from Sprague-Dawley rats. We also used cultured rat aortic and human umbilical vein endothelial cells. The MG was measured by HPLC and Western blotting and assay kits were used.

KEY RESULTS
Incubation of aortic rings with MG (30 μM) or high glucose (25 mM) attenuated endothelium-dependent, acetylcholine-induced relaxation, which was restored by two different MG scavengers, aminoguanidine (100 μM) and N-acetyl cysteine (NAC) (600 μM). Treatment of cultured endothelial cells with MG or high glucose increased cellular MG levels, effects prevented by aminoguanidine and NAC. In cultured endothelial cells, MG and high glucose reduced basal and bradykinin-stimulated nitric oxide (NO) production, cGMP levels, and serine-1177 phosphorylation and activity of endothelial NO synthase (eNOS), without affecting threonine-495 and Akt phosphorylation or total eNOS protein. These effects of MG and high glucose were attenuated by aminoguanidine or NAC.

CONCLUSIONS AND IMPLICATIONS
Our results show for the first time that MG reduced serine-1177 phosphorylation, activity of eNOS and NO production. MG caused endothelial dysfunction similar to that induced by high glucose. Specific and safe MG scavengers have potential to prevent endothelial dysfunction induced by MG and high glucose concentrations.

Abbreviations
AG, aminoguanidine; AGEs, advanced glycation endproducts; BK, bradykinin; DCF, dichlorofluorescein; eNOS, endothelial nitric oxide synthase; GSH, reduced glutathione; HUVECs, human umbilical vein endothelial cells; MG, methylglyoxal; NAC, N acetyl cysteine; RAECS, rat aortic endothelial cells; ROS, reactive oxygen species; VSMCs, vascular smooth muscle cells

Introduction

Endothelial dysfunction, commonly defined as reduced endothelium-dependent vascular relaxation, occurs as an early event in atherosclerosis, hypertension (O'Keefe *et al.*, 2009), the pre-diabetic stage of insulin resistance (Su *et al.*, 2008), and is a hallmark of type 2 and type 1 diabetes mellitus (De

Vriese *et al*., 2000; Potenza *et al*., 2009). Nitric oxide (NO) is one of the main vasodilator mediators released from the endothelium (Palmer *et al*., 1987). The NO is synthesized by endothelial nitric oxide synthase (eNOS) from L-arginine with L-citrulline as a co-product (Palmer *et al*., 1988) and has a short half-life (6–7 s). Reduced production or availability of NO is a common feature of endothelial dysfunction (De Vriese *et al*., 2000; Potenza *et al*., 2009).

Methylglyoxal (MG) is a highly reactive dicarbonyl metabolite produced during glucose metabolism (Desai and Wu, 2007). The clinical significance of MG lies in the fact that it reacts with and modifies certain proteins, lipids and DNA and alters their normal structure and/or function (Baynes and Thorpe, 1999; Desai and Wu, 2007). The MG is a major precursor of the advanced glycation endproducts (AGEs), which are involved in the pathogenesis of vascular complications of diabetes (Baynes and Thorpe, 1999; Desai and Wu, 2007). We have shown earlier that MG levels were elevated in spontaneously hypertensive rats (Wang *et al*., 2005), in fructose-fed hypertensive rats (Wang *et al*., 2008), and in diabetic patients (Wang *et al*., 2007a). We have also shown that incubation of vascular smooth muscle cells (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).

The aim of the current study was to find out if MG induced endothelial dysfunction and the mechanism(s) involved. Even though high concentrations of glucose (about 25 mm) have previously been shown to cause endothelial dysfunction (Nishikawa *et al*., 2000; Du *et al*., 2001; Triggle, 2008; Potenza *et al*., 2009) we performed parallel experiments with high glucose to see if the functional and molecular changes produced by MG were similar to those produced by high concentrations of glucose are similar. We examined whether two different MG scavengers, aminoguanidine (Lo *et al*., 1994; Wang *et al*., 2007b) and N-acetyl cysteine (NAC) (Vasdev *et al*., 1998; Jia and Wu, 2007), could prevent the deleterious effects of MG and high glucose concentrations on endothelial function.

Methods

Animals

All animal care and experimental procedures complied 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) and were approved by the Animal Care Committee at The University of Saskatchewan (Protocol no. 20070029), following guidelines of the Canadian Council on Animal Care. Male 11-week old Sprague-Dawley (SD) rats from Charles River Laboratories (St Constant, QC, Canada) were used in these studies and were acclimatized for 1 week before experimental use.

Isometric tension studies on aortic rings

A group of 24 SD rats was used. Isometric tension studies were carried out on rat aortic rings as described by Wu *et al*. (1998). Briefly, 3–4 mm thoracic aortic rings from SD rats 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% O_2 + 5% CO_2. 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 (15 or 25 mM) or MG (30 or 100 μM). 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 co-incubated with the MG scavenger aminoguanidine (AG, 100 μM) (Lo *et al*., 1994; Wang *et al*., 2007b), or another MG scavenger, NAC (600 μM) (Vasdev *et al*., 1998; Jia and Wu, 2007), or the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin (100 μM) for 2 h. Treatment with each compound was tested in rings from at least five different rats. Isometric tension was measured with isometric force transducers with the 'Chart' software and Powerlab equipment (AD Instruments Inc., Colorado Springs, CO, USA).

Cell culture

Rat aortic endothelial cells (RAECs) were isolated from male SD rats according to the method of McGuire and Orkin (1987). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.15 $mg \cdot mL^{-1}$ endothelial cell growth supplement (Biomedical Technologies Inc., MA, USA). For the initial culture Matrigel™ (Sigma-Aldrich, Oakville, ON, Canada) coated culture dishes were used. RAECs were identified by their typical cobblestone morphology and positive staining for von Willebrand factor. Immunostaining was done as described by us earlier (Dhar *et al*., 2008). Cells between passage 3 and 6 were used for the experiments. Human umbilical vein endothelial cells (HUVECs) from American Type Culture Collection were cultured in Kaighns F12K medium containing

10% FBS, 0.1 mg·mL^{-1} heparin and 0.03–0.05 mg·mL^{-1} endothelial cell growth supplement.

Assay for NO

Confluent cells were washed twice with Hanks balanced salt solution (HBSS) and incubated with MG (3, 10 or 30 μM) or glucose (15 or 25 mM) in HBSS for 3 or 24 h at 37°C in an incubator. The supernatant was analyzed for basal NO production. The cells were further incubated with bradykinin (BK, 10 μM), an endothelial cell agonist for NO production (Palmer *et al.*, 1987), for 15 min and the supernatant was collected and levels of nitrate plus nitrite were measured with the Griess assay kit (Caymen Chemicals, Ann Arbor, MI, USA) (Dhar *et al.*, 2008). Nitrate in the sample was first converted to nitrite by nitrate reductase. One set of HUVECs was also co-incubated with AG (100 μM) or NAC (600 μM), and MG (30 μM) or glucose (25 mM) for 24 h following which the basal and BK-stimulated NO production were measured.

Assay for cGMP

Briefly, control and test compounds treated cells were treated with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) (100 μM), for 30 min before agonist stimulation. Cells were harvested into the supplied lysis buffer and cGMP measured using the cGMP assay kit (R & D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. To normalize cGMP values, protein content in each dish was measured by the BCA Protein assay (Bio-Rad, Hercules, CA, USA).

Methylglyoxal assay

The MG was measured by a specific and sensitive HPLC method as described before (Dhar *et al.*, 2008; Dhar *et al.*, 2009). MG was derivatized with *o*-phenylenediamine (*o*-PD) to form the specific quinoxaline product, 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. The quinoxaline derivatives of MG (2-methylquinoxaline) and the 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, Waters, MA, USA).

eNOS activity assay

The NOS activity assay is based on the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. Briefly, control and test compounds treated cells were washed in PBS, harvested and centrifuged for 2 min to pellet the cells. The cells were resuspended in 1× homogenization buffer and sonicated briefly. The suspension was centrifuged, the supernatant was separated and the resulting protein concentration was adjusted to 5–10 mg·mL^{-1}. The eNOS activity was measured using the Cayman Chemicals NOS activity assay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Measurement of reduced glutathione

Briefly, monochlorobimane stock (100 μM) was added to endothelial cells treated with test compounds. After 30 min, the medium was collected for medium reduced glutathione (GSH) measurement. Cells were washed with PBS and harvested in 1 mL of 1% sodium dodecylsulfate (SDS) in 50 mM Tris buffer (pH 7.5), sonicated and the aliquots (100 μL) of supernatants were read in triplicate with an excitation wavelength of 380 nm and an emission wavelength of 470 nm (Kamencic *et al.*, 2000; Wu and Juurlink, 2002).

Western blotting

Cell lysates were prepared as described earlier (Wu and Juurlink, 2002; Jia and Wu, 2007) and the protein concentration in the supernatant was determined by the BCA Protein assay. 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 1.5 h at room temperature, and incubated overnight at 4°C with the primary antibody, eNOS and phospho-NOS-1177 (both from BD Transduction Laboratories, Mississauga, ON, Canada), phospho-NOS-495 and GSH-reductase (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Akt, anti-phos-Akt (Cell Signaling Technology, Inc., Danvers, MA, USA), 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 (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA) (Wu and Juurlink, 2002; Jia and Wu, 2007).

Measurement of reactive oxygen species and NADPH oxidase activity

Confluent cells were loaded with a membrane-permeable, non-fluorescent probe 2′,7′-dichlorofluorescin diacetate (CM-H_2DCFDA, 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 24 h were assayed for fluorescent oxidized dichlorofluorescein (DCF) as an indicator of production of reactive oxygen species (ROS) as described

earlier (Dhar *et al.*, 2008), and for activity of NADPH oxidase (Griendling *et al.*, 1994), which is a key endothelial cell enzyme for the production of superoxide anions (Gao and Mann, 2009). The protein content of the homogenate was measured by BCA Protein assay. The NADPH oxidase activity was measured by a luminescence assay in a 50-mmol·L^{-1} phosphate buffer, pH 7.0, containing 1 mmol·L^{-1} EGTA, 150 mmol·L^{-1} sucrose, 500 μmol·L^{-1} lucigenin as the electron acceptor, and 100 μmol·L^{-1} NADPH as the substrate (final volume, 0.9 mL) (Griendling *et al.*, 1994).

Data analysis

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.

Materials

All chemicals were of analytical grade. The MG, D-glucose, NAC, apocynin and glutathione were purchased from Sigma Aldrich, Oakville, ON, Canada. Cell culture media and reagents were purchased from Invitrogen Canada Inc., Burlington, ON, Canada.

Results

Methylglyoxal and high glucose concentrations reduce acetylcholine-induced relaxation of aortic rings: attenuation by MG scavengers

In rat aortic rings pre-contracted with phenylephrine (1 μM), incubation with MG (30 or 100 μM) for 2 h in the tissue bath, caused significant inhibition of ACh-induced endothelium-dependent relaxation, which was prevented by co-incubation of AG (100 μM) with MG (100 μM) (Figure 1A). Lower concentrations of AG (10 and 30 μM) did not prevent MG (100 μM)-induced reduction of relaxation (data not shown). Incubation in high glucose concentrations (15 and 25 mM) for 2 h also attenuated ACh-induced relaxation of rat aortic rings that was prevented by co-incubation of glucose (25 mM) with AG (100 μM) (Figure 1B). The MG (30 μM) and glucose (25 mM)-induced attenuation of relaxation was also restored by another MG scavenger, NAC (600 μM) (Figure 1C, D). However, MG and high glucose-induced attenuation of relaxation was not restored by the NADPH oxidase inhibitor apocynin (100 μM) (Figure 1E, F). In washout experiments, the reduced relaxation of aortic rings induced by MG (30 μM) and glucose (25 mM) for 2 h was restored after a further 2 h washout, with changes of Krebs solution in the bath every 15 min (data not shown). The MG and glucose did not affect endothelium-independent relaxation of aortic rings induced by sodium nitroprusside (data not shown). In preliminary experiments, incubation with lower concentrations of MG (3 and 10 μM) or with the osmotic control mannitol (25 mM) for 2 h did not affect ACh-induced relaxation (data not shown).

High glucose and exogenous methylglyoxal increase methylglyoxal levels in endothelial cells

Incubation of RAECs and HUVECs with MG (30 or 100 μM) for 24 h significantly increased the level of cellular MG that was prevented by co-incubation with AG (100 μM) or NAC (600 μM) (Figure 2A, C). Incubation of cultured RAECs and HUVECs with glucose (25 mM) for 24 h also significantly increased MG levels in these cells (Figure 2B, D), which was similarly prevented by co-incubation of HUVECs with AG (100 μM) or NAC (600 μM) and glucose (25 mM) for 24 h (Figure 2B, D). A shorter incubation (3 h) of RAECs and HUVECs with 25 mM glucose also significantly increased cellular MG levels (data not shown).

The increase in cellular MG in RAECs and HUVECs induced by glucose (25 mM) and exogenous MG (30 μM) was similar (Figure 2).

Methylglyoxal and high glucose reduce nitric oxide production in RAECs and HUVECs

Incubation of RAECs and HUVECs with 3, 10 or 30 μM MG for 3 or 24 h decreased basal and BK (10 μM)-stimulated NO production in both cell types to varying degrees depending on the concentration of MG and the incubation time (Figures 3A, C and 4A, C). The inhibition of basal and agonist-stimulated NO production was significant with 30 μM MG incubated for 24 h in both RAECs and HUVECs (Figures 3C and 4C). The attenuation of basal and BK-stimulated NO production by MG (30 μM) incubated for 24 h was restored by co-incubation with AG (100 μM) or NAC (600 μM) (Figures 3C and 4C).

Similarly, incubation of RAECs and HUVECs with glucose (15 or 25 mM for 3 or 24 h) decreased basal and BK (10 μM)-stimulated NO production to varying degrees depending on the concentration of glucose and the incubation time (Figures 3B, D and 4B, D). The inhibition of basal and agonist-stimulated NO production in both cell types was significant with 25 mM glucose incubated for 24 h (Figures 3D and 4D). The attenuation of basal and BK-stimulated NO production by glucose (25 mM) incubated for 24 h was restored by co-incubation with AG (100 μM) or NAC (600 μM) (Figures 3D and 4D). Incubation with AG (100 μM) alone for 24 h

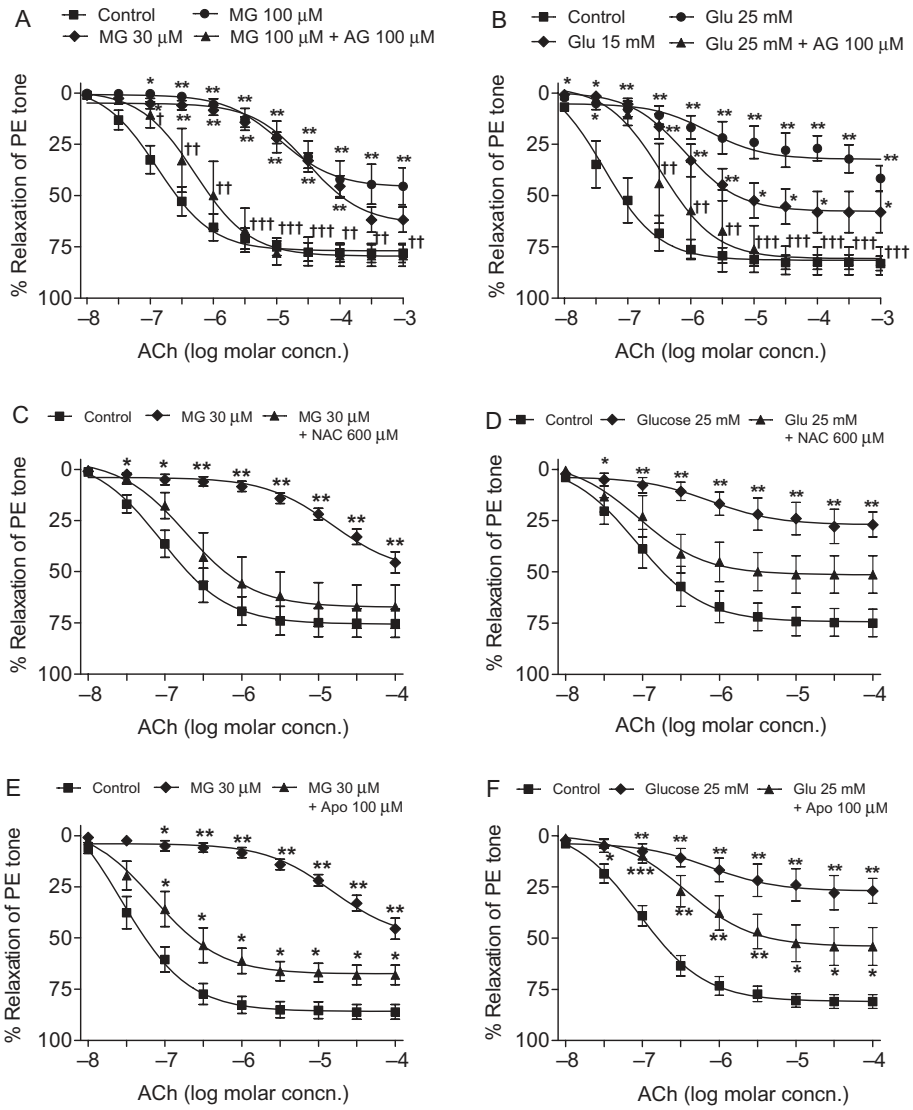

Figure 1

Methylglyoxal (MG) scavengers attenuate MG and high glucose induced endothelial dysfunction 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 (30 or 100 μM) (A, C, E), or glucose (Glu, 15 or 25 mM) (B, D, F). In some sets of rings the MG scavenger aminoguanidine (AG, 100 μM) was co-incubated with MG (100 μM) (A), or glucose (25 mM) (B); the MG scavenging antioxidant N-acetyl cysteine (NAC) (600 μM) was co-incubated with MG (30 μM) (C), or glucose (25 mM) (D); or the nicotinamide adenine dinucleotide phosphate oxidase inhibitor apocynin (Apo 100 μM) was co-incubated with MG (30 μM) (E), or glucose (25 mM) (F) for 2 h. ($n = 5$ rings from different rats for each test compound). *$P < 0.05$, **$P < 0.01$ versus corresponding control value, †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ versus corresponding values for MG 100 μM (A), or glucose (25 mM) (B).

did not affect basal or BK-stimulated NO production (data not shown).

Methylglyoxal and high glucose reduce agonist-stimulated cGMP increase in RAECs and HUVECs

The cGMP is the second messenger of NO-induced activation of soluble guanylate cyclase and is a sensitive indicator of NO production (Waldman and Murad, 1987; Papapetropoulos *et al.*, 1996). Incubation of RAECs and HUVECs with 30 μM MG or 25 mM glucose for 24 h prevented BK (10 μM)-stimulated cGMP increase in both cell types that was restored by co-incubation with AG (100 μM) or NAC (600 μM) (Figure 5A, B).

Methylglyoxal and high glucose reduce activity of the eNOS enzyme

To understand the mechanism of reduced NO production with MG and high glucose, the activity of

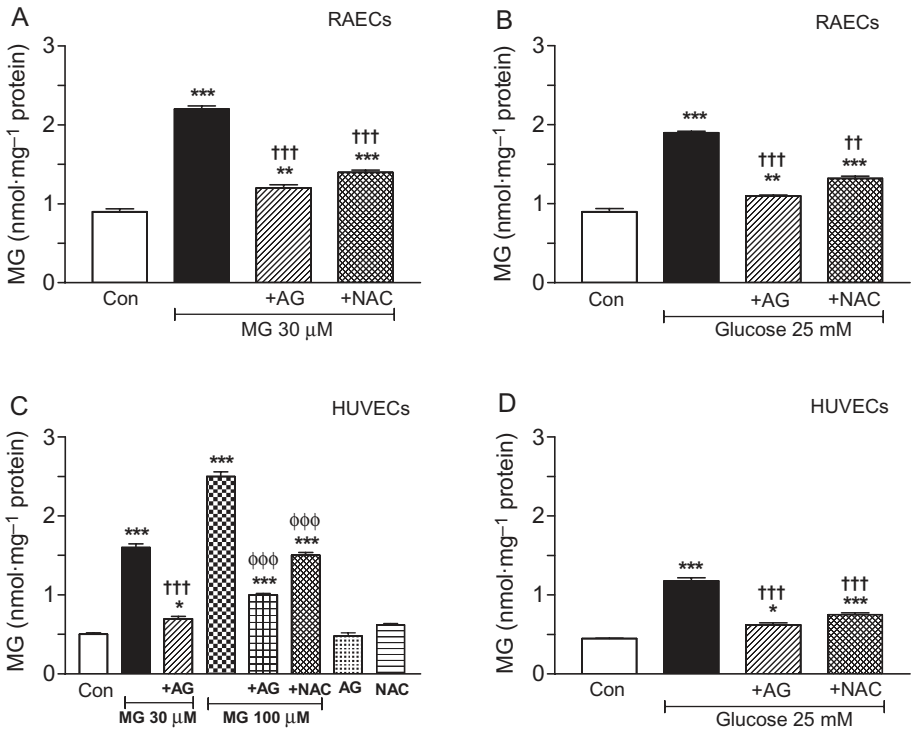

Figure 2

High glucose and exogenous methylglyoxal (MG) increase cellular MG levels in cultured endothelial cells: attenuation by MG scavengers. Confluent rat aortic endothelial cells (RAECs) and human umbilical vein endothelial cells (HUVECs) were incubated with normal culture medium (Control, Con) or medium containing MG (30 or 100 μM) (A, C), or glucose (Glu) (25 mM) (B, D), for 24 h. Aminoguanidine (AG, 100 μM) or N-acetyl cysteine (600 μM) was coincubated with MG (30 or 100 μM) (A, C), or with glucose (25 mM) (B, D) for 24 h. Cellular MG was measured by HPLC. $n = 4$ for each treatment. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus corresponding control value, ††$P < 0.01$, †††$P < 0.001$ versus corresponding values for MG (30 μM) (A, C), or glucose (25 mM) (B, D), (C) $^{\phi\phi\phi}P < 0.001$ versus MG 100 μM.

the eNOS enzyme was tested in an eNOS activity assay. Incubation of RAECs (data not shown), as well as HUVECs, with MG (30 μM) or glucose (25 mM) for 24 h reduced NO production catalyzed by eNOS in the eNOS activity assay, which was prevented by coincubation with AG (100 μM) (Figure 6A). At the same time both MG and high glucose did not affect the level of eNOS protein in HUVECs (Figure 6B) or RAECs (data not shown) under the same treatment conditions indicating that the reduced NO production from RAECs and HUVECs by MG and glucose was due to reduced activity of the eNOS enzyme and not due to reduced eNOS protein expression.

Methylglyoxal and high glucose reduce bradykinin-stimulated serine-1177 phosphorylation of the eNOS enzyme

To further elucidate the mechanism of reduced eNOS activity by MG and high glucose, we examined the serine-1177 and threonine-495 phosphorylation of eNOS, and phosphorylation of Akt, which then phosphorylates the serine-1177 of eNOS (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). In HUVECs, treatment with MG (30 μM) or glucose (25 mM) for 24 h reduced BK-stimulated serine-1177 phosphorylation of eNOS that was prevented by co-incubation with AG (100 μM) (Figure 6C). There was little basal serine-1177 phosphorylation of eNOS, which was almost abolished by MG (30 μM) or glucose (25 mM) incubated for 24 h. The antibody for serine-1177 phosphorylated eNOS did not react well with RAEC eNOS and hence that data is not shown. eNOS was phosphorylated basally at threonine-495. BK stimulation reduced the threonine-495 phosphorylation of eNOS (Figure 6C). MG (30 μM) or glucose (25 mM) incubated for 24 h did not affect basal or BK-stimulated threonine-495 phosphorylation of eNOS (Figure 6C). MG and high glucose also did not affect phosphorylation of Akt (Figure 6C).

Methylglyoxal and high glucose increase oxidative stress in HUVECs and RAECs

Incubation of cultured HUVECs and RAECs with MG (30 μM) or glucose (25 mM) for 24 h caused a significant increase in oxidized DCF, an indicator of ROS production, in both cell types which was

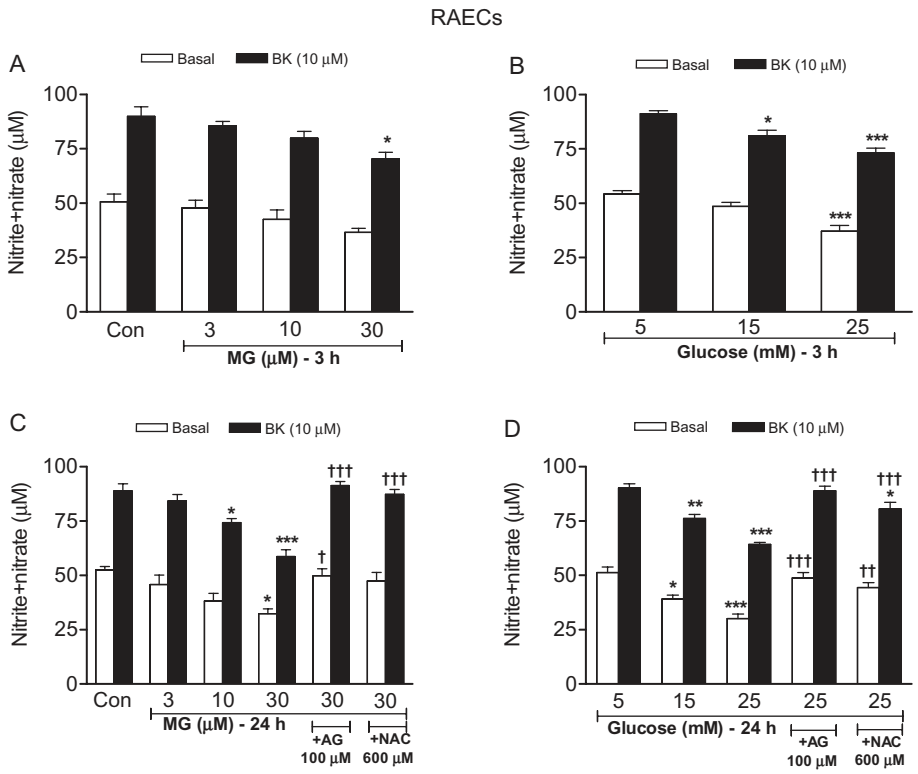

Figure 3

Methylglyoxal (MG) and high glucose reduce nitric oxide (NO) production in cultured rat aortic endothelial cells (RAECs). RAECs were incubated with MG (3, 10 or 30 μM) (A, C), or glucose (5, 15 or 25 mM) (B, D) for 3 h (A, B), or 24 h (C, D). Cells were co-incubated with MG (30 μM) (C), or glucose (25 mM) (D), and aminoguanidine (AG) (100 μM) or N-acetyl cysteine (NAC) (600 μM) for 24 h. The supernatant was collected after the 3 or 24 h incubation time (basal) and the cells were further incubated with bradykinin (BK) (10 μM) for 15 min to stimulate NO production and the supernatant was analyzed for (nitrite+nitrate) levels by the Griess assay. $n = 8$ for each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus corresponding control value (Con) (A, C), or glucose (5 mM) (B, D). †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ versus corresponding values for MG (30 μM) alone (C), or glucose (25 mM) alone (D).

attenuated by co-incubation with NAC (600 μM) (Figure 7A, B). Incubation of RAECs and HUVECs with MG (30 μM) or glucose (25 mM) for 24 h, also caused a significant increase in the activity of NADPH oxidase, as measured by superoxide anion production in an activity assay, that was prevented by the NADPH oxidase inhibitor apocynin, in both cell types (Figure 7C, D).

Methylglyoxal and high glucose decrease GSH levels and glutathione reductase protein in RAECs and HUVECs

MG is degraded by the glyoxalase enzymes that use GSH as a cofactor. Incubation of HUVECs (Figure 8A) and RAECs (data not shown) with MG (30 μM) or glucose (25 mM) for 24 h significantly reduced GSH levels and also decreased glutathione reductase protein expression, which was prevented by co-incubation with AG (100 μM) in HUVECs (Figure 8B).

Discussion

In this study, we provide evidence, for the first time, that MG, a glucose metabolite, induced endothelial dysfunction in rat aortic rings as well as in cultured RAECs and HUVECs. High glucose concentrations (25 mM) increased MG levels in both RAECs and HUVECs, and induced endothelial dysfunction in aortic rings and cultured endothelial cells, effects similar to those of MG. The effects of MG and high glucose on aortic rings and cultured endothelial cells were attenuated by two different MG scavengers, aminoguanidine (Lo *et al.*, 1994; Wang *et al.*, 2007b) and NAC (Vasdev *et al.*, 1998; Jia and Wu, 2007). Thus, our results provide a possible mechanism linking high glucose and endothelial dysfunction. The effects of MG and high glucose to reduce eNOS activity, NO production, and increase oxidative stress are seen in endothelial cells of rat and human origin and hence are not limited to one

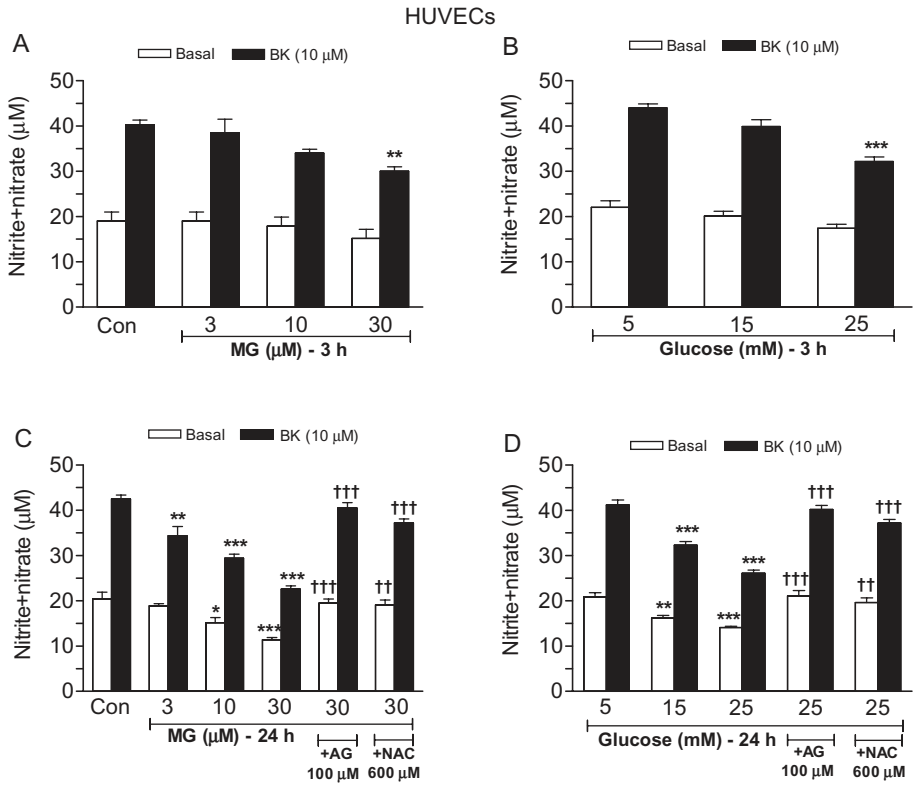

Figure 4

Methylglyoxal (MG) and high glucose reduce nitric oxide (NO) production in cultured human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with MG (3, 10 or 30 μM) (A, C), or glucose (5, 15 or 25 mM) (B, D) for 3 h (A, B), or 24 h (C, D). Aminoguanidine (AG) (100 μM) or N-acetyl cysteine (NAC) (600 μM) was co-incubated with MG (30 μM) (C), or with glucose (25 mM) (D) for 24 h in some sets of cells. The supernatant was collected after the 3 or 24 h incubation time (basal) and the cells were further incubated with bradykinin (BK, 10 μM) for 15 min to stimulate NO production and the supernatant was analyzed for (nitrite+nitrate) levels by the Griess assay. $n = 8$ for each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus corresponding control (Con) (A, C), or glucose 5 mM value (B, D). ††$P < 0.01$, †††$P < 0.001$ versus corresponding values for MG (30 μM) alone (C), or glucose (25 mM) alone (D).

species. We have recently shown that MG levels are higher in the aorta, compared to other organs such as heart, liver, lungs, kidney, and after exogenous MG administration the aortic levels increased significantly (Dhar *et al.*, 2010).

To the best of our knowledge, the effect of MG on endothelium-dependent vascular relaxation has not been reported previously. Both, MG (30 and 100 μM) and high glucose (15 and 25 mM) reduced ACh-induced relaxation to a similar extent. In fact, 25 mM glucose caused a slightly greater reduction of relaxation. It should be pointed out that exogenous MG is not fully absorbed into the cells. In one study as little as 3% of exogenous MG was absorbed by cultured L6 muscle cells incubated with 2.5 mM MG (Riboulet-Chavey *et al.*, 2006). As shown in Figure 2, 30 μM MG causes a similar increase in cellular MG levels as 25 mM glucose in endothelial cells, which justifies the use of 30 μM exogenous MG in our study. The attenuation of relaxation by both MG and high glucose was prevented by two different MG scavengers, aminoguanidine and NAC (Lo *et al.*, 1994; Vasdev *et al.*, 1998; Jia and Wu, 2007; Wang *et al.*, 2007b) (Figure 1A–D), but not by the NADPH oxidase inhibitor apocynin (Figure 1E, F), indicating MG as a possible mediator of the effects of high glucose concentrations on endothelial dysfunction.

The reduction of NO production by cultured endothelial cells treated with MG has not been reported previously. We measured the production of NO in both cell types as nitrate plus nitrite (Figures 3 and 4), as well as cGMP accumulation (Figure 5) in response to agonist stimulation. cGMP is a sensitive indicator of NO production, which activates soluble guanylate cyclase (Waldman and Murad, 1987; Papapetropoulos *et al.*, 1996). Again, both MG scavengers, aminoguanidine and NAC, prevented the reduced NO production by both MG and high glucose. It should be pointed out here that aminoguanidine is also an inhibitor of inducible NOS (Corbett *et al.*, 1992; Misko *et al.*, 1993) but inducible NOS is not normally expressed by endot-

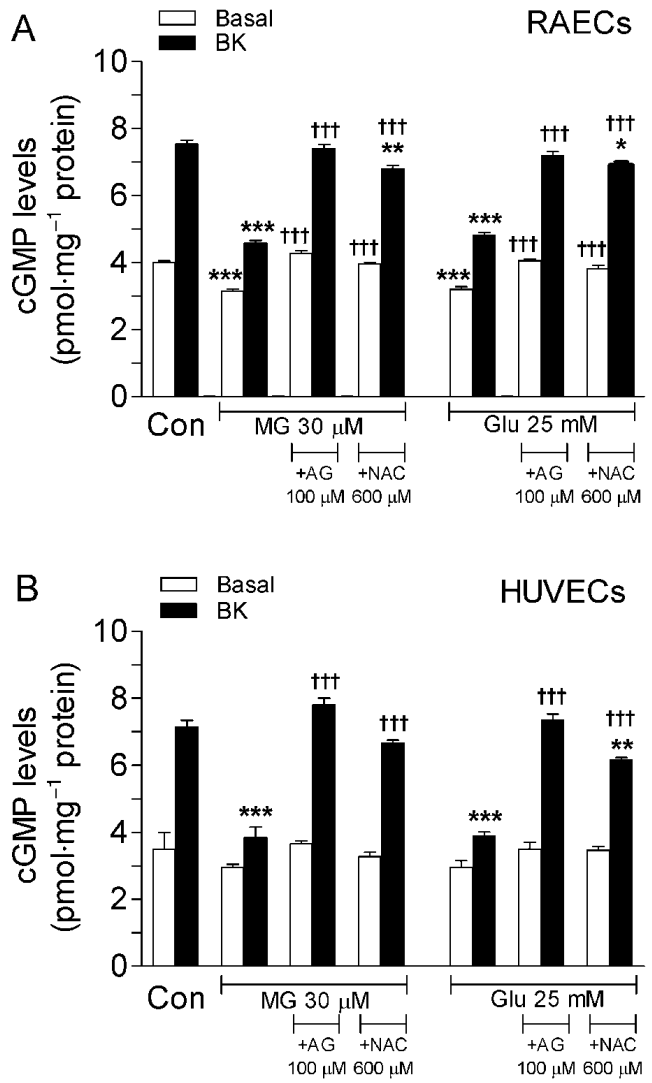

Figure 5

Methylglyoxal (MG) and high glucose concentrations decrease cGMP production in cultured rat aortic endothelial cells (RAECs) and human umbilical vein endothelial cells (HUVECs). (A) RAECs and (B) HUVECs were incubated with MG (30 μM) or glucose (25 mM) for 24 h. Aminoguanidine (AG) (100 μM) or N-acetyl cysteine (NAC) (600 μM) was co-incubated with MG (30 μM), or with glucose (25 mM) for 24 h in some sets of cells. Basal and bradykinin (BK) (10 μM)-stimulated cGMP levels were measured with an assay. $n = 8$ for each group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus corresponding control value (Con). †††$P < 0.001$ versus corresponding values for MG (30 μM) alone or glucose (Glu) (25 mM) alone.

helial cells. If aminoguanidine were to inhibit eNOS, it should further reduce the NO production along with MG and high glucose. In our study, aminoguanidine actually attenuated the reduction in NO production by MG and high glucose, suggesting a MG scavenging effect of aminoguanidine (Lo *et al.*, 1994; Wang *et al.*, 2007b). Moreover, we used a relatively lower concentration of aminoguanidine (100 μM) compared to concentrations of 1 mM and higher reported earlier (Misko *et al.*, 1993; Lo *et al.*, 1994).

The mechanism of reduced NO production can be ascribed to reduced eNOS activity induced by MG and high glucose with no change in eNOS protein level (Figure 6A, B). The reduced eNOS activity is likely due to reduced BK-stimulated serine-1177 [bovine eNOS serine-1179] phosphorylation of eNOS (Dimmeler *et al.*, 1999; Fleming *et al.*, 2001) caused by MG (30 μM) or glucose (25 mM) (Figure 6C). Serine-1177 is the most common site phosphorylated in activated eNOS (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999). Basal eNOS is phosphorylated at threonine-495 and. BK stimulation causes dephosphorylation of threonine-495 [bovine eNOS threonine-497] (Fleming *et al.*, 2001). MG (30 μM) or glucose (25 mM) did not affect threonine-495 phosphorylation of e-NOS (Figure 6C). High glucose (25 mM) has been reported to reduce serine-1179 (human serine-1177) phosphorylation of eNOS due to activation of inhibitor of κB kinase (IKKβ), a mediator of inflammation, and to reduce NO production in cultured bovine aortic endothelial cells (Kim *et al.*, 2005). As serine-1177 is the phosphorylation site in NOS for Akt (Dimmeler *et al.*, 1999; Fulton *et al.*, 1999), we looked at Akt phosphorylation itself. MG and hyperglycemia did not affect Akt phosphorylation in HUVECs (Figure 6C). Thus, reduced Akt phosphorylation is not responsible for reduced eNOS activity.

One study (Brouwers *et al.*, 2008) has reported a lack of effect of MG and MG-adducts (argpyrimidine and 5-hydro-5-methylimidazolone) (1, 10 and 100 μM of each) on eNOS activity in whole cell homogenates of HUVECs incubated with MG and its two adducts. Some possible reasons for this lack of effect of MG could be the incubation time of 60 min, and use of whole cell homogenates instead of intact cultured cells, which provides a different experimental condition. Moreover, the authors used 10 $\mu mol \cdot L^{-1}$ of total free arginine and because MG has high affinity for arginine, it is possible that the added MG bound to arginine in the reaction mix and did not affect eNOS. In contrast, Du *et al.* (2001) have reported that hyperglycemia inhibits eNOS activity by activating, the hexosamine pathway, increasing superoxide production and reducing serine-1177 phosphorylation of eNOS. We have shown that MG also reduces serine-1177 phosphorylation of eNOS and is a possible mediator of hyperglycemia-induced dysfunction.

Increased superoxide anion can quench NO to form peroxynitrite and thus reduce the bioavailability of NO (Pacher *et al.*, 2007). This is the more frequently reported mechanism of endothelial

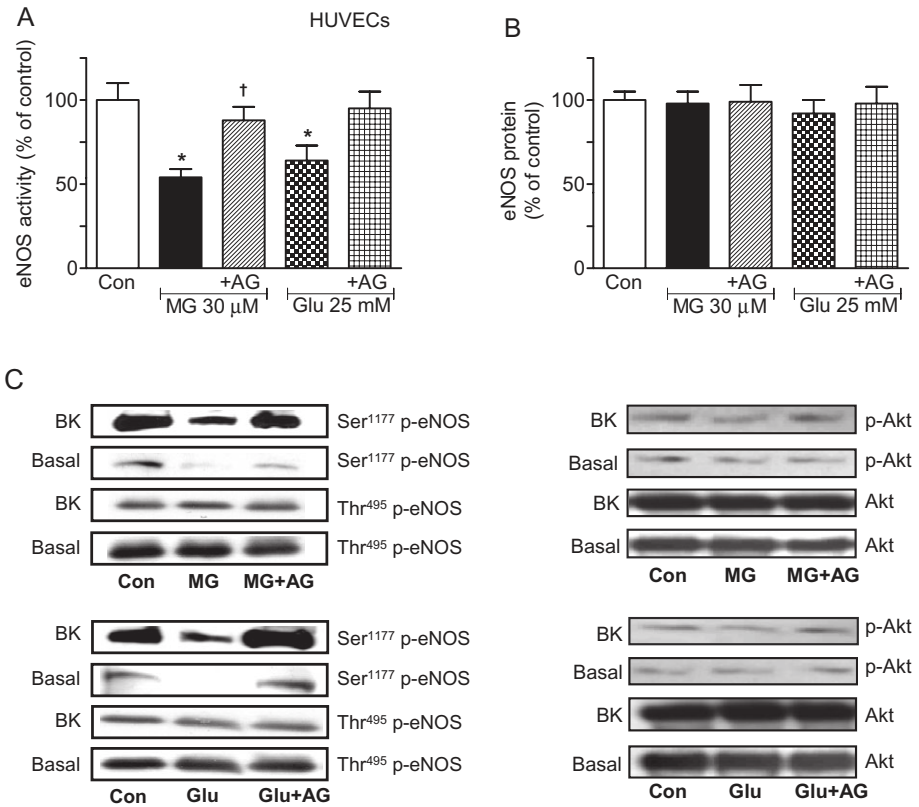

Figure 6

Methylglyoxal (MG) and high glucose reduce serine-1177 phosphorylation and activity of endothelial nitric oxide synthase (eNOS). Human umbilical vein endothelial cells (HUVECs) were incubated with MG (30 μM) alone or glucose (Glu) (25 mM) alone, or coincubated with aminoguanidine (AG) (100 μM) for 24 h following which an eNOS activity assay was performed, based on conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline, using an activity assay kit (A). (B) The total eNOS protein was determined in the same treated cells from (A) by Western blotting. (C) Basal and bradykinin (BK) (10 μM)-stimulated serine-1177, and threonine-495 phosphorylation of eNOS; and Akt and phosphorylated Akt were determined with appropriate anti-phospho-eNOS, anti-Akt and anti-phospho-Akt antibodies in cells treated as in (A) by Western blotting. $n = 5$ for each group. *$P < 0.05$ versus control (Con), †$P < 0.05$ versus MG 30 μM.

dysfunction caused by oxidative stress (Potenza *et al.*, 2009). Hyperglycemia (30 mM glucose) has been shown to induce endothelial dysfunction by increasing production of ROS, oxidative stress and activating protein kinase C and NFκB (Nishikawa *et al.*, 2000; Triggle, 2008; Potenza *et al.*, 2009). However, in these studies it was not shown if high glucose *per se* or one of its metabolites, was responsible for causing endothelial dysfunction. Our results show that MG reduces serine-1177 phosphorylation of eNOS in parallel with high glucose, an effect prevented by the MG scavenger aminoguanidine, implicating MG as a possible mediator of the effect of high glucose on reduced eNOS phosphorylation and activity.

So how does oxidative stress fit with the data presented in our study? NADPH oxidase is a key enzyme responsible for overproduction of superoxide anion and an increase in oxidative stress in endothelial dysfunction (Gao and Mann, 2009). We found that both MG and high glucose increased NADPH oxidase activity and ROS production that was prevented by the NADPH oxidase inhibitor, apocynin, and the MG-scavenging antioxidant, NAC, respectively, in RAECs and HUVECs. MG has been shown to increase NADPH oxidase activity and superoxide production in other cell types such as vascular smooth muscle cells (Chang *et al.*, 2005) and neutrophils (Ward and McLeish, 2004).

Are the effects of MG or high glucose on eNOS activity and NO production direct or through an increase in oxidative stress? Our results with eNOS activity assay (Figure 6A), apocynin and NAC indicate the effects to be partly direct on the eNOS enzyme itself. Inhibiting superoxide with apocynin did not completely restore the ACh-induced relaxation of aortic rings (Figure 1E, F) or BK-stimulated NO production in HUVECs (data not shown) that was attenuated by MG or high glucose. On the contrary NAC, which can also scavenge MG (Vasdev *et al.*, 1998), restored ACh-induced relaxation of rings (Figure 1C, D) and BK-stimulated NO produc-

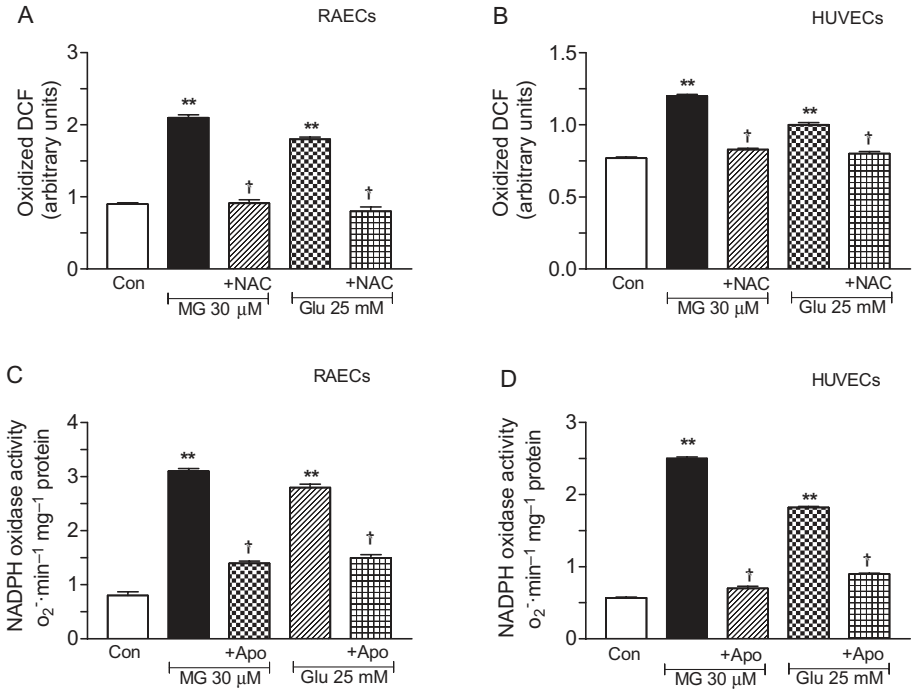

Figure 7

Methylglyoxal (MG) and high glucose increase reactive oxygen species production and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in cultured endothelial cells. Incubation of cultured rat aortic endothelial cells (RAECs) (A, C), and human umbilical vein endothelial cells (HUVECs) (B, D) with MG (30 μM) or glucose (25 mM) for 24 h increased reactive oxygen species (ROS) production (A, B), measured as oxidized dichlorofluorescein (DCF), and NADPH oxidase activity (C, D) that was prevented by co-incubation with the antioxidant N-acetylcysteine (NAC) (600 μM) (A, B), or the NADPH oxidase inhibitor, apocynin (Apo) (100 μM) (C, D) respectively. NADPH oxidase activity was measured with a luminescence assay. $n = 5$ for each group. $^{**}P < 0.01$ versus corresponding control, †$P < 0.05$ versus corresponding values for glucose (25 mM) alone or MG (30 μM) alone.

tion and cGMP increase in RAECs and HUVECs (Figures 3–5) that was attenuated by MG and high glucose.

MG and high glucose also significantly reduced GSH levels and expression of GSH-reductase, which was prevented by aminoguanidine, in HUVECs (Figure 8) and RAECs (data not shown). The GSH plays a key role in the degradation of MG by the glyoxalase enzymes (Dakin and Dudley, 1913). A reduction in GSH levels would delay the degradation of MG. Glutathione reductase replenishes GSH by reducing oxidized glutathione and is an antioxidant enzyme (Zhao *et al.*, 2009). The MG has been shown to reduce GSH and GSH reductase levels in VSMCs (Wu and Juurlink, 2002). It should be pointed out that besides MG, other reactive aldehydes, viz. glyoxal and 3-deoxyglucosone are formed from degradation of glucose (Thornalley *et al.*, 1999). According to one report, 2.7-fold more 3-deoxyglucosone and 21-fold more glyoxal was formed from glucose in phosphate buffer (Thornalley *et al.*, 1999). However, of the three aldehydes, MG is the most reactive and widely studied as an AGE precursor.

Thus, our data provides a possible link between hyperglycemia and endothelial dysfunction with MG as the mediator of the endothelial dysfunction induced by high glucose. Therefore, MG is a potential target for preventive strategies against hyperglycemia-induced endothelial dysfunction and its sequelae. The potential of NAC needs to be evaluated in this regard. NAC is already clinically used in patients for conditions such as paracetamol (acetaminophen) overdose, influenza viral infection, chronic obstructive pulmonary disease and pulmonary fibrosis (Millea, 2009). Aminoguanidine was found to be toxic in clinical trials as an AGE scavenger (Freedman *et al.*, 1999). However, for experimental studies aminoguanidine is the most effective and commonly used MG and AGEs scavenger (Lo *et al.*, 1994; Desai and Wu, 2007; Wang *et al.*, 2007b), and was a rational choice in our study as an MG scavenger.

In conclusion, hyperglycemia-induced endothelial dysfunction is receiving increasing attention as an early preventable event. Endothelial dysfunction induced by high glucose concentrations is most likely to be mediated by MG. Development of

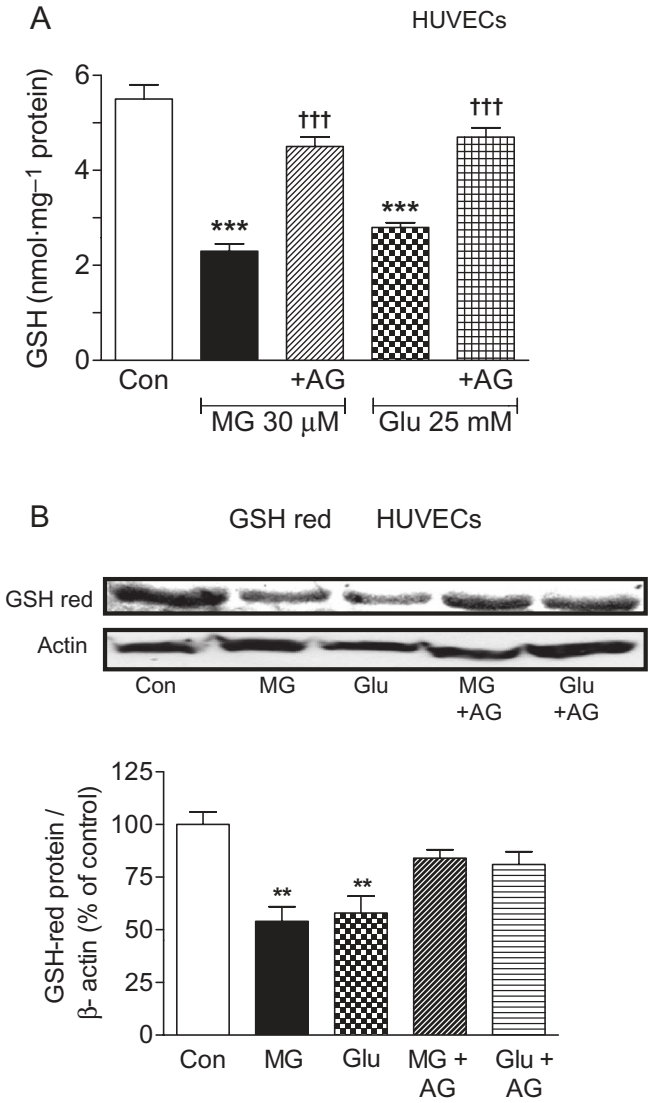

Figure 8

Methylglyoxal (MG) and high glucose decrease glutathione and glutathione reductase in cultured human umbilical vein endothelial cells (HUVECs). Incubation of HUVECs with MG (30 μM) or glucose (25 mM) for 24 h decreased cellular reduced glutathione (GSH) levels (A), and glutathione reductase (GSH red) protein levels (B) that was prevented by co-incubation with aminoguanidine (AG) (100 μM). The GSH was determined with the monochlorobimane assay. $n = 5$ for each group. $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding control (Con), †††$P < 0.001$ versus corresponding values for MG (30 μM) alone, or glucose (Glu) (25 mM) alone value.

specific and safe MG scavengers may prove very useful in blocking the many deleterious effects of hyperglycemia, including endothelial dysfunction and vascular complications of diabetes.

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Conflict of interest

None.

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