Methyglyoxal, a Reactive Glucose Metabolite, Increases Renin Angiotensin Aldosterone and Blood Pressure in Male Sprague-Dawley Rats

Indu Dhar,1 Arti Dhar,1 Lingyun Wu,2 and Kaushik M. Desai1

BACKGROUND
The majority of people with diabetes develop hypertension along with increased activity of the renin-angiotensin system. Methyglyoxal, a reactive glucose metabolite, is elevated in diabetic patients. We investigated the effects of methyglyoxal on the renin-angiotensin system and blood pressure.

METHODS
Male Sprague-Dawley rats were treated with a continuous infusion of methyglyoxal with a minipump for 4 weeks. Organs/tissues and cultured vascular smooth muscle cells (VSMCs) were used for molecular studies. High-performance liquid chromatography, Western blotting, and quantitative real-time polymerase chain reaction were used to measure methyglyoxal, proteins, and mRNA, respectively. Small interfering RNA for angiotensinogen and the receptor for advanced glycation end products (RAGE) were used to study mechanisms.

RESULTS
Methyglyoxal-treated rats developed a significant increase in blood pressure and plasma levels of aldosterone, renin, angiotensin, and catecholamines. Methyglyoxal level and protein and mRNA for angiotensin, AT1 receptor, adrenergic α1D receptor, and renin were significantly increased in the aorta and/or kidney of methyglyoxal-treated rats, a novel finding. Alagebrium attenuated the above effects of methyglyoxal. Treatment of cultured VSMCs with methyglyoxal or high glucose (25 mM) significantly increased cellular methyglyoxal and protein and mRNA for nuclear factor kappa B (NF-κB), angiotensin, AT1 receptor, and α1D receptor, which were prevented by inhibition of NF-κB, and by alagebrium. Silencing of mRNA for RAGE prevented the increase in NF-κB induced by methyglyoxal. Silencing of mRNA for angiotensinogen prevented the increase in NF-κB, angiotensin, AT1 receptor, and α1D receptor.

CONCLUSIONS
Methyglyoxal activates NF-κB through RAGE and thereby increases renin-angiotensin levels, a novel finding, and a probable mechanism of increase in blood pressure.

Keywords: blood pressure; diabetes; hyperglycemia; hypertension; methyglyoxal; receptor for advanced glycation end products; renin-angiotensin-aldosterone system.

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Type 2 diabetes and hypertension are major global health issues. Two-thirds of people with diabetes have hypertension (blood pressure (BP): systolic ≥140 mm Hg or diastolic ≥90 mm Hg).1 The mechanism of the pathogenesis of hypertension in diabetes is not well established. The renin-angiotensin-aldosterone system (RAAS) plays a major role in maintaining fluid balance, vascular tone, and BP.2 Angiotensin II also increases activity of the sympathetic nervous system.2 Increased activity of the RAAS is seen in type 2 diabetics with hypertension3 and has also been proposed to be one of the etiologic factors for type 2 diabetes mellitus.4 Glucose is one of the main precursors for the formation of methyglyoxal (MG), a reactive aldehyde.5,6 Normally, MG is rapidly degraded mainly by the glyoxalase enzymes with the help of reduced glutathione.6 In conditions of excess MG formation, such as hyperglycemia, the glyoxalase enzymes get overwhelmed and the excess MG reacts with other proteins and enzymes and disrupts their normal function.7 Plasma MG levels are elevated in people with diabetes.8,9 We have recently shown that administration of MG for 4 weeks as a continuous infusion by a subcutaneous minipump induces features of type 2 diabetes in Sprague-Dawley (SD) rats.10 MG is a major precursor for the formation of advanced glycation end products (AGEs).7 MG causes increased oxidative stress,11,12 which in turn is believed to cause the pathophysiological changes in diabetes and hypertension.13 For example, MG activates nuclear factor kappa B p65 (NF-κB p65), inactivates antioxidant enzymes such as glutathione reductase and glutathione peroxidase in rat vascular smooth muscle cells (VSMCs), and causes oxidative stress.15 On the other hand increased oxidative stress can deplete reduced glutathione and slow down degradation of MG, thereby increasing its levels and harmful effects.7,11 Elevated plasma levels of MG

Correspondence: Kaushik Desai (k.desai@usask.ca).

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have been reported in spontaneously hypertensive rats and fructose-fed rats, but it is not yet known whether MG is the cause or effect of hypertension. We hypothesized that MG upregulates the RAAS and increases BP in rats, as the basis of the work reported herein.

METHODS

Animals

Male SD rats from Charles River Laboratories (Quebec, Canada) were used according to guidelines of the Canadian Council on Animal Care. All animal protocols were approved by the University of Saskatchewan’s Animal Research Ethics Board. Twenty-eight 12-week-old male SD rats with closely matching body weights were used. A miniosmotic pump (Alzet 2ML4; Durect, Cupertino, CA) was surgically implanted subcutaneously on the back under sterile conditions. This pump holds a fixed volume (2 ml) of drug and releases a continuous small amount of MG (40% solution; Sigma-Aldrich Canada, Oakville, ON, Canada) into the body at a rate of 1 mg/hour or 24 mg/day or 672 mg/28 days. Hence each rat received a fixed dose (24 mg/day) and therefore it is not expressed as milligrams per kilogram. To control for this fixed dose, the rats were from the same batch, of the same age, and similar in weight. We have successfully used this minipump previously to deliver MG to rats for 28 days. In our previous study, subcutaneous infusion of MG with a minipump caused a significant elevation in the plasma MG compared with the control (plasma MG (µM); Control = 1.4 ± 0.1; MG group = 2.7 ± 0.1, P < 0.01 vs. control group; MG + ALA = 1.7 ± 0.05; n = 6 each). Pumps were implanted in all rats to deliver either MG or normal saline (0.9% NaCl). Anesthesia was provided by a continuously monitored inhalation of isoflurane (Forane; 2%–4%) in oxygen, with the rat placed on a heated pad to maintain a rectal temperature of 37 °C. Buprenorphine injection (0.025 mg/kg subcutaneously twice a day) was used as a preanesthetic and postsurgical recovery analgesic for 2 days after implantation.

The rats were randomly divided into the following treatment groups (n = 7 each): (i) control (0.9% saline by pump); (ii) MG (24 mg/day by pump); (iii) MG + alagebrium (ALA, an MG scavenger; 30 mg/kg/day in drinking water); (iv) ALA + 0.9% saline by pump. At the end of the study, the rats were anesthetized with sodium pentobarbital (60 mg/kg body weight, intraperitoneally). After 15 minutes of stabilization, the BP was measured by carotid artery cannulation for 30 minutes. Blood was collected from the carotid artery, and plasma was separated and stored at −80 °C. Organs/tissues cleaned in ice-cold phosphate buffer saline were immediately frozen in liquid nitrogen and stored at −80 °C until processing.

Biochemical assays

Assay kits for quantitative determination of plasma renin (AnaSpec, Fremont, CA), angiotensin II (Cayman Chemicals, Ann Arbor, MI), aldosterone (Enzo Life Sciences, Farmingdale, NY), and catecholamines (Rocky Mountain

Diagnostics, Colorado Springs, CO) were used according to the manufacturer’s instructions.

Cell culture

Rat thoracic aortic smooth muscle cells (A-10 cells, CRL-1476; American Type Culture Collection, Manassas, VA) were cultured as described previously. Cells were starved in fetal bovine serum–free Dulbecco’s modified Eagle medium medium for 24 hours before exposure to different treatments alone or in combination: MG (30 µM) (Supplementary Figure S1); glucose (5, 25 mM) (Supplementary Figure S1), ALA (100 µM), and a specific NF-κB inhibitor, CAY10512 (0.1 µM; Cayman Chemical).

MG measurement

MG was measured by a specific and sensitive high-performance liquid chromatography method, as described in the Supplementary Methods.

Western blotting

Total proteins from cultured cells, aortic tissue, and kidney were subjected to Western blot analysis as previously described. The following primary antibodies were used: AT1 receptor for angiotensin II, adrenergic α1D receptor, renin, angiotensin (the antibody for angiotensin recognizes angiotensin I, II, and III), angiotensin-converting enzyme, Erk 1/2, NFATc, sodium-dependent glucose cotransporter 2 (all from Santa Cruz Biotechnology, Santa Cruz, CA), thiazide sensitive sodium chloride cotransporter (Millipore, Billerica, MA), receptor for advanced glycation endproducts (RAGE), NF-κB (Abcam, Cambridge, MA), and anti-β-actin (diluted 1:1,000; Sigma-Aldrich Canada). Horseradish peroxidase–conjugated secondary antibodies (diluted 1:3,000; GE Healthcare, Pittsburgh, PA) were used. The proteins were visualized with enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Hercules, CA) and captured on X-ray film (GE Healthcare Life Sciences).

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction was performed as described previously and in the Supplementary Methods.

Small interfering RNA transfection

Small interfering RNA (siRNA) transfection for RAGE and angiotensinogen was performed as described in detail in the Supplementary Methods.

Materials

Glucose, MG, and other reagents were purchased from Sigma Aldrich Canada or VWR International (Mississauga, ON, Canada). Alagebrium (previously known as ALT-711; N-phenacyl-4,5-dimethyl-1,3-thiazolium) was a generous gift.
from Synvista Therapeutics (Montvale, NJ; formerly Alteon). Synvista ceased operations in 2009.\textsuperscript{20}

Statistical analysis

Data obtained from separate experiments are expressed as mean ± SEM. Statistical analysis was performed using analysis of variance with post hoc Bonferroni’s test. \( P < 0.05 \) as shown by the analysis of variance was taken as significant. \( P < 0.05 \) shown in figures indicates significant differences between different groups as revealed by the post hoc Bonferroni test to compare all of the groups.

RESULTS

Acute or chronic MG treatment increases cellular, aortic, and renal MG levels

There was no significant difference in the body weights between the different groups at the start and at the end of the treatment.\textsuperscript{19} Incubation of cultured VSMCs with different concentrations of MG or with 15 or 25 mM glucose caused a concentration-related increase in cellular MG levels (Supplementary Figure S1). Based on these results, we chose MG (30 \( \mu \)M) or high glucose (25 mM) to incubate VSMCs for 24 hours or 5 days, which caused a significant elevation of cellular MG that was attenuated by coincubation with the MG scavenger ALA (Figure 1a,b; Supplementary Table S1). Treatment of SD rats for 4 weeks with MG caused a significant increase in aortic and renal MG levels, which was attenuated by cotreatment with ALA (Figure 1c,d; Supplementary Table S1).

Chronic MG treatment increases BP and plasma catecholamines, renin, angiotensin, and aldosterone levels

Treatment of SD rats for 4 weeks with MG caused a significant increase in the BP, which was attenuated by the MG scavenger ALA (Figure 2a; Supplementary Table S1). ALA alone did not affect the BP. Chronic MG treatment also caused a significant increase in plasma norepinephrine, epinephrine, dopamine (Figure 2b; Supplementary Table S1), angiotensin (Figure 2c; Supplementary Table S1), renin (Figure 2d; Supplementary Table S1), and aldosterone (Figure 2e; Supplementary Table S1) levels, which were attenuated by the MG scavenger ALA (Figure 2; Supplementary Table S1).

Figure 1. Methylglyoxal (MG) levels in cultured vascular smooth muscle cells and in the aorta and kidney of chronic methylglyoxal treated Sprague-Dawley rats. Rat thoracic aorta smooth muscle cells (A10 cell line) were cultured and incubated with MG (30 \( \mu \)M) or high glucose (Glu, 25 mM) for (a) 24 hours or (b) 5 days. Alagebrium (30 \( \mu \)M) was used as an MG scavenger. (c and d) Groups (n = 7 each) of 12-week-old male Sprague-Dawley rats were treated for 4 weeks with a continuous infusion of MG (24 mg/day) delivering by a subcutaneous minipump. Control rats received 0.9% saline by pump. Alagebrium (ALA; 100 mg/kg/day in drinking water) was used as an MG scavenger. MG levels were determined with high-performance liquid chromatography. Abbreviation: VSMC, vascular smooth muscle cell. \(*P < 0.05, ^{**}P < 0.01\) vs. respective control (Con). \(^{†}P < 0.05, ^{‡}P < 0.01\) vs. respective MG. \(^{††}P < 0.05, ^{‡‡}P < 0.01\) vs. respective Glu group. \(P < 0.05\) indicates significant differences between different groups as revealed by the post hoc Bonferroni test.
Methylglyoxal (MG) increases the α1D receptor, AT1 receptor, angiotensin, renin, thiazide-sensitive sodium chloride cotransporter 2, and sodium-dependent glucose transporter expression in VSMCs, and/or in the aorta, and/or in the kidney.

Glucose is a major precursor of MG formation. Incubation of VSMCs with MG (30 μM) or high glucose (25 mM) for 24 hours (Figure 3a) or 5 days (Supplementary Figure S2) caused a significant increase in adrenergic α1D receptor, angiotensin AT1 receptor, and angiotensin protein and mRNA, which were attenuated by ALA (Figure 3a; Supplementary Figure S2).

Chronic treatment of SD rats with MG for 4 weeks significantly elevated aortic adrenergic α1D receptor, angiotensin AT1 receptor, and angiotensin protein and mRNA, which were attenuated by cotreatment with ALA (Figure 3b).

Chronic MG also significantly increased renal AT1 receptor, renin, and angiotensin protein and mRNA, which were attenuated by cotreatment with ALA (Figure 3c). MG also increased expression of thiazide-sensitive sodium-chloride cotransporter and sodium-dependent glucose cotransporter 2 in the kidney, which was attenuated by ALA (Supplementary Figure S3).

**Acute or chronic treatment with MG increases phosphorylated Erk 1/2 and NFATc expression**

Incubation of VSMCs with MG (30 μM) for 24 hours (Supplementary Figure S4a) caused an increase in phosphorylated Erk 1/2 (p-Erk 1/2) and NFATc protein expression, which was attenuated by ALA (Supplementary Figure S4a). Chronic MG increased aortic and renal protein expression of p-Erk 1/2 and NFATc, which was attenuated by ALA (Supplementary Figure S4b,c).

**Effects of inhibition of NF-κB or angiotensinogen siRNA and RAGE siRNA on MG-induced increase in NF-κB, angiotensin, AT1 receptor, and α1D receptor in VSMCs**

Incubation of VSMCs with MG (30 μM) for 24 hours caused a significant increase in the protein and mRNA for NF-κB, angiotensin, AT1 receptor, and adrenergic α1D receptor, which was attenuated by coincubation with the NF-κB inhibitor.
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inhibitor CAY 10512 (0.1 µM) or the MG scavenger ALA (100 µM) (Figure 4a–d). RAGE siRNA attenuated the increase in RAGE and NF-kB p65 protein expression induced by MG (30 µM) in cultured VSMCs (Figure 4e). Angiotensinogen siRNA attenuated the increase in NF-kB p65, angiotensin, AT1 receptor, and adrenergic α1D receptor protein expression induced by MG (30 µM) in cultured VSMCs (Figure 4f).

**DISCUSSION**

We report the novel finding that MG, a reactive metabolite produced from glucose and fructose, significantly increases renin-angiotensin-aldosterone levels associated with an increase in the BP in SD rats. Molecular studies in cultured VSMCs implicate RAGE and NF-κB in the signaling
pathway of MG to cause these effects. Alagebrium attenuates the pathological effects of MG on the RAAS.

The increased risk of hypertension in people with diabetes is well established.\textsuperscript{1,21} High glucose\textsuperscript{22} and high fructose diets\textsuperscript{23,24} significantly increase BP in animals and humans, but the molecular mechanisms are not very clear. In the case of high glucose diet activation of protein kinase C, increased oxidative stress and reduced bioavailability of

**Figure 4.** Nuclear factor κB (NF-κB), angiotensin, AT\(_1\) receptor (AT\(_1\)R), α\(_{1D}\) receptor (α1DR), and receptor for advanced glycation endproducts (RAGE) expression in cultured vascular smooth muscle cells treated with an NF-κB inhibitor or after RAGE small interfering RNA (siRNA) or angiotensinogen (AGT) siRNA treatment. Rat thoracic aorta smooth muscle cells (A10 cell line) were cultured and incubated with MG (30 μM) for 24 hours. (a–d) CAY 10512 (0.1 μM) was used as an NF-κB inhibitor. Alagebrium (ALA; 30 μM) was used as an MG scavenger. (e) Silencing of mRNA for RAGE or (f) silencing of mRNA for angiotensinogen was performed as described in Supplementary Methods. Protein expression was determined by Western blotting using appropriate primary antibodies, and mRNA was analyzed with quantitative real-time polymerase chain reaction. n = 4 for each group. ***P < 0.001 vs. respective control. †††P < 0.001 vs. respective MG group. P < 0.05 indicates significant differences between different groups as revealed by the post hoc Bonferroni test.
nitric oxide have been reported. In the case of high fructose diet, unregulated metabolism of fructose causes ATP depletion and an increase in uric acid, oxidative stress, and a decrease in nitric oxide production. Glucose and fructose are both precursors of MG formation, and MG is a potent inducer oxidative stress through multiple pathways and a major precursor of aldehyde conjugates formation.

To the best of our knowledge, our study is the first to report a direct effect of MG on the development of hypertension and the probable molecular mechanisms. One study had reported administration of MG in drinking water for 18 weeks and an increase in BP, but a direct link between MG and increased BP was not established. For example, plasma or aortic or renal MG levels were not measured. Instead renal levels of aldehyde conjugates were measured, which likely represent modification of several proteins/enzymes and do not identify molecular mechanisms. Moreover, in their study the authors did not address the role of the RAAS. Also, MG reacts with epithelial cells of the intestines and the colon, and its oral bioavailability is questionable. An association of elevated MG with hypertension in rats has been shown, but the cause-effect relation was not established.

Increased activity of the RAAS is seen in type 2 diabetes with hypertension. Hyperglycemia and diabetes increase renin-angiotensin activity, but the mechanism has not been clearly defined. In one study, high levels of glucose caused the release of renin through local accumulation of succinate and activation of the kidney-specific G protein–coupled receptor, GPR91, in the glomerular endothelium in rat, mouse, and rabbit. In another study 25 mM D-glucose increased the expression of the angiotensinogen gene in cultured rat proximal tubular cells through synthesis of diacylglycerol and activation of protein kinase C. We decided to examine the effect of MG on the RAAS.

In preliminary studies, oral administration of MG failed to cause any significant increase in plasma MG levels. Therefore, we administered MG by continuous infusion of very small amounts with a minipump, hoping to simulate the almost continuous production of MG in the body through metabolism. Administration of MG with a minipump results in significant elevation of plasma MG levels to values reported in pathological conditions and in our previous study. The MG levels in the kidney and the aorta also increased significantly (Figure 1), which implicates MG in the pathological effects on the RAAS. MG treatment increased plasma levels of renin, angiotensin, aldosterone, and catecholamines (Figure 2), signifying increased RAAS and adrenergic activity, which most likely increased the BP. The kidney is the main source of renin and the major regulator of BP. The kidney had increased protein expression of renin, which would explain increased plasma renin. The renin, in turn, would produce more angiotensin II and its multiple effects. Increased expression of thiazide-sensitive sodium chloride cotransporter would also cause increased sodium reabsorption.

Previously we have shown that the aorta has significantly elevated basal levels of MG compared with other organs. The increased aortic MG most likely increased aortic AT1 receptor, AT1 receptor, and angiotensin because these effects were attenuated by the MG scavenger ALA. Activation of both AT1 receptor and AT1 receptor can increase p-Erk 1/2 and NFATC, as seen here, which can contribute to increased vascular tone, inflammation, and hypertension development. Increased AT1 receptor and AT1 receptor can increase vascular tone and BP. We have previously shown that MG can reduce endothelial nitric oxide synthase activity, which can contribute to increased BP, although it was not investigated in this study.

VSMCs have been shown to produce angiotensin, including angiotensin II, which can exert an intracellular effect or a paracrine effect outside the cell. Treatment of VSMCs with MG or high glucose caused a similar significant increase in cellular MG levels, along with increased expression of AT1 receptor, AT1 receptor, and angiotensin, which were all attenuated by ALA (Figure 1), an MG scavenger and an AGES breaker. Because the attenuation by ALA of increased MG levels, AT1 receptor, AT1 receptor, and angiotensin occurred within 24 hours in cultured VSMCs (Figure 3), this indicates a direct effect of MG on AT1 receptor, AT1 receptor, and angiotensin rather than an indirect effect through MG-induced AGES, which normally take 5–7 days to form. High glucose similarly increased AT1 receptor, AT1 receptor, and angiotensin expression in VSMCs, which was attenuated by an MG scavenger, implicating MG as a mediator of the deleterious effects of hyperglycemia. Because MG is a major precursor of AGES formation, part of alagebrium's preventive effects against MG could be due to prevention of AGES formation in vivo, but not in 24-hour cell culture studies.

We investigated whether the effect of MG on angiotensin, AT1 receptor, and AT1 receptor was direct or indirect through some other mediators. RAGE is currently under intense investigation as a target to prevent diabetic complications. The activation of RAGE by AGES has been reported to increase 2 key transcription factors, NF-kB and early growth response 1, and cause oxidative stress. We report the novel finding that MG can activate RAGE and upregulate it (Figure 4e). RAGE is upregulated by its other known ligands. Thus, when we silenced RAGE, we were able to prevent the increase in NF-kB induced by MG (Figure 4e). When we inhibited NF-kB we were able to prevent the increase in angiotensin, AT1 receptor, and AT1 receptor in VSMCs (Figure 4b–d), indicating an indirect effect of MG on these parameters. When we silenced angiotensinogen mRNA, we were able to prevent the increase in angiotensin, AT1 receptor, and AT1 receptor and also the increase in NF-kB induced by MG (Figure 4f). These results indicate that MG activates RAGE, which then increases NF-kB followed by angiotensin, AT1 receptor, and AT1 receptor. The increased angiotensin II, in turn, increases NF-kB and sets up a vicious cycle. Our results establish a probable sequence of molecular events resulting from elevated MG levels, which have been reported in high carbohydrate diet–fed animals and in diabetic patients.

The main limitations of our findings are that the experiments were performed in male rats, and the results cannot be extrapolated to female rats, for which separate experiments are required to determine any effect of sex. Another limitation...
is the small number of animals in each group (n = 7 each), which can result in inadequate power for analysis. In conclusion, MG increases RAAS and BP with the probable involvement of RAGE. Because diabetic patients are known to have significantly elevated MG levels and many people with diabetes develop hypertension, MG could possibly be a mediator of hyperglycemia-induced and diabetes-associated hypertension. High carbohydrate diets are known to induce hypertension in animal models, and because glucose and fructose are both precursors for MG formation, MG could very likely be causing high dietary carbohydrate–induced hypertension, which we are currently investigating. Effective and safe MG scavengers have the potential to prevent diabetes-associated hypertension and vascular complications. Although the dietary habits of millions of people worldwide and the use of high fructose–containing processed foods is not likely to change overnight, preventive strategies will play a major role in attenuating the adverse health effects of high carbohydrate diets.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

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DISCLOSURE

The authors declared no conflict of interest.

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