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Dicarbonyl Stress in the Absence of Hyperglycemia Increases Endothelial Inflammation and Atherogenesis Similar to That Observed in Diabetes

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The deleterious effects of high glucose levels and enhanced metabolic flux on the vasculature are thought to be mediated by the generation of toxic metabolites, including reactive dicarbonyls like methylglyoxal (MG). In this article, we demonstrate that increasing plasma MG to levels observed in diabetic mice either using an exogenous source (1% in drinking water) or generated following inhibition, its primary clearance enzyme, glyoxalase-1 (with 50 mg/kg IP bromobenzyl-glutathione cyclopentyl diester every second day), was able to increase vascular adhesion and augment atherogenesis in euglycemic *apolipoprotein E* knockout mice to a similar magnitude as that observed in hyperglycemic mice with diabetes. The effects of MG appear partly mediated by activation of the receptor for advanced glycation end products (RAGE), as deletion of RAGE was able to reduce inflammation and atherogenesis associated with MG exposure. However, RAGE deletion did not completely prevent inflammation or vascular damage, possibly because the induction of mitochondrial oxidative stress by dicarbonyls also contributes to inflammation and atherogenesis. Such data would suggest that a synergistic combination of RAGE antagonism and antioxidants may offer the greatest utility for the prevention and management of diabetic vascular complications.

Diabetes leads to the increased incidence, size, and complexity of atherosclerotic plaques (1), resulting in an increased incidence and severity of cardiovascular disease in patients with diabetes (2). A number of metabolic and hemodynamic factors contribute to accelerated atherosclerosis in the setting of diabetes. One key pathway is the increased production of reactive dicarbonyls generated from triose phosphate intermediates of glycolysis, glycerol and ketone peroxidation, overactivation of the polyol pathway, and the degradation of glycated proteins (3–6). In experimental diabetes, circulating and tissue levels of methylglyoxal (MG) are three to five times higher than in the nondiabetic state (7,8). Plasma MG concentrations are also elevated in patients with diabetes (9), especially those with vascular complications (10).

Reactive α-dicarbonyls appear to be toxic to cells through their ability to produce covalent modifications of proteins, lipids, and nucleic acids via the Maillard reaction (11). These modifications, known as advanced glycation end products (AGEs), have been widely implicated in the development and progression of atherosclerosis and other vascular complications. Carbonyl-derived AGEs have been detected within atherosclerotic lesions in both extra- and intracellular locations and correlate with the size, complexity, and stability of the lesions (12,13). The potential importance of

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α-dicarbonyls in the pathogenesis of glucose-dependent atherogenesis is illustrated by the vasculoprotective effects of dicarbonyl scavengers in diabetes, in the absence of euglycemia (7,14).

AGEs are thought to act through both receptor-dependent and receptor-independent mechanisms to promote vascular damage, cellular dysfunction, and inflammation associated with diabetes (15–17). However, the relative importance of each mechanism remains to be established. Certainly, posttranslational modification of amino, guanidino, and thiol functional groups on vulnerable proteins, lipids, and DNA targets has the potential to alter their structure, stability, and/or function (18,19). For example, the modification of LDL by MG increases its arterial atherogenicity, partly by increasing its density and binding to proteoglycans in the arterial wall (20). Similarly, MG-induced modification of the platelet-derived growth factor receptor alters its mitogenic functions similar to that observed in atherosclerosis lesions of diabetic mice (21). AGEs are also able to stimulate proatherogenic pathways following activation of the receptor for AGEs (RAGE) (22). We have previously shown that genetic deletion of RAGE is able to attenuate atherosclerosis associated with diabetes in *apolipoprotein E* (*apoE*) knockout (KO) mice, although it did not completely eliminate it (23). Treatment with soluble RAGE, a dummy receptor that acts as a competitive antagonist to full-length RAGE, also significantly reduces the accumulation of atherosclerotic plaque and vascular inflammation in diabetic *apoE* KO mice (24). Interestingly, upregulation of the RAGE receptor in response to high glucose levels is partly mediated by MG and subsequent AGE modification of signaling proteins (25), while RAGE activation reduces expression of glyoxalase-1, an enzyme that metabolizes dicarbonyls, illustrating the close interconnection of receptor-independent and receptor-dependent pathways (18,19). In this article, we specifically explore these two pathways to show for the first time that increased MG levels are able to augment vascular inflammation and atherosclerosis in nondiabetic *apoE* KO mice, via both RAGE-dependent and RAGE-independent pathways, to produce similar vascular changes to those observed in hyperglycemic mice.

RESEARCH DESIGN AND METHODS

Animal Models

Male *apoE* KO mice (backcrossed 20 times to a C57BL/6 background; Animal Resource Centre, Canning Vale, Western Australia) and *RAGE/apoE* double KO (DKO) mice, generated by backcrossing *RAGE* KO mice on the C57BL/6 background into *apoE* KO mice on the same background for 10 generations (University of Heidelberg, Heidelberg, Germany), were used in these experiments (23). All animals had unrestricted access to water and standard mouse chow (Specialty Feeds, Glen Forrest, Western Australia) and were maintained on a 12-h light/12-h dark cycle at the Precinct Animal Centre at Baker IDI Heart and Diabetes Institute. All mice were maintained and studied according to National Health and Medical Research Council guidelines with local ethics approval.

Eight-week-old male *apoE* KO mice and *RAGE/apoE* DKO mice (n = 20/group) were randomized to receive standard drinking water or water into which MG had been added (50 mmol/L). This dose was chosen to achieve comparable plasma levels of MG to those observed in diabetic mice (Table 1). Male *apoE* KO mice were further randomized to receive the cell-permeable selective glyoxalase-1 inhibitor bromobenzyl-glutathione cyclopentyl diester (BBGC; 50 mg/kg IP every second day). We have previously shown this dose produces elevation in MG (and associated neuropathy) comparable to that observed in diabetic mice (8). Additional groups of *apoE* KO mice and *RAGE/apoE* DKO mice were rendered diabetic by five daily injections of streptozotocin (55 mg/kg; Sigma-Aldrich, St. Louis, MO) in citrate buffer. This results in chronic hyperglycemia (20–30 mmol/L) without requiring insulin to prevent ketosis or excessive weight loss. This model is generally associated with a two- to threefold increase in plaque accumulation in the aortic arch when compared with nondiabetic *apoE* KO mice (23).

Table 1—General parameters of mouse models of dicarbonyl stress compared with those with diabetes

	apoE KO				*RAGE/apoE* DKO		
	Control	MG treated	BBGC treated	Diabetic	Control	MG treated	Diabetic
Body weight (g)	28.2 ± 0.6	27.2 ± 0.5	23.2 ± 2.0*	22.7 ± 0.6*	27.5 ± 1.6	28.8 ± 0.6	23.5 ± 0.9*
Food intake (g/day)	3.3 ± 0.3	3.4 ± 0.2	3.6 ± 0.5	5.0 ± 0.5*	1.9 ± 0.3	2.9 ± 0.1	4.8 ± 0.5*
Water intake (mL/day)	4.2 ± 0.3	5.1 ± 0.3	3.5 ± 0.5	17.5 ± 2.7*	6.1 ± 0.2	6.7 ± 0.5	16.6 ± 2.8*
Systolic blood pressure (mmHg)	95 ± 3	90 ± 2	95 ± 2	96 ± 2	94 ± 5	88 ± 5	99 ± 2
Glycated hemoglobin (%)	4.3 ± 0.1	4.2 ± 0.2	4.1 ± 0.2	13.3 ± 1.7*	4.7 ± 0.1	4.7 ± 0.2	13.5 ± 1.7*
Plasma glucose (mmol/L)	11.8 ± 0.7	12.5 ± 0.6	11.1 ± 0.5	27.7 ± 3.1*	10.8 ± 0.8	11.3 ± 0.7	29 ± 1.6*
Total cholesterol (mmol/L)	8.8 ± 0.6	6.6 ± 0.3	7.6 ± 0.3	16.4 ± 0.3*	8.9 ± 0.3	8.4 ± 0.6	17.4 ± 0.6*
Plasma MG (μmol/L)	1.0 ± 0.2	1.9 ± 0.2*	2.1 ± 0.5*	2.3 ± 0.2*	1.0 ± 0.1	1.7 ± 0.2*	2.1 ± 0.3*

*$P < 0.05$ vs. control (nondiabetic *apoE* KO).

Disposition of Mice

All groups were then followed for 6 weeks, the earliest time point at which an increase in atherosclerosis may be detected following the induction of diabetes. At the completion of studies, mice were then humanely killed using a lethal injection of sodium pentobarbitone (100 mg/kg body weight; Euthal, Sigma-Aldrich, Castle Hill, New South Wales, Australia) followed by cardiac exsanguination. Lysates of erythrocytes were analyzed for glycated hemoglobin levels by high-performance liquid chromatography (26). Plasma levels of total cholesterol and triglycerides were measured with a standard commercial enzymatic assay using a Beckman Coulter LX20 Pro Analyzer (catalog number 467825, Beckman Coulter Diagnostics, Gladesville, New South Wales, Australia). LDL cholesterol was calculated using the Friedewald formula. Plasma MG was measured by high-performance liquid chromatography, as described previously (27). Aorta were rapidly dissected and cleaned of advential fat in ice-cold 0.9% sterile saline before being either snap frozen in liquid nitrogen and stored at −80°C or stored in buffered formalin (10%, volume/volume) for en face analysis prior to being embedded in paraffin for later immunohistochemical studies.

Plaque Area Quantitation

Plaque area was quantitated as described previously (23). In brief, aortae were removed from mice, cleaned of excess fat, and stained with Sudan IV-Herxheimer solution (0.5% weight/volume; Gurr, BDH Limited, Poole, U.K.). Aortae were then dissected longitudinally and pinned flat onto wax. Plaque accumulation across the aortic arch and total aortic surface was quantitated as the percentage area stained red.

Quantitative Real-Time PCR

Gene expression of the adhesion molecules and proinflammatory cytokines were assessed in aortic homogenates by quantitative real-time RT-PCR. This was performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, PerkinElmer Inc., PE Biosystems, Foster City, CA) as previously used by our group (23). Gene expression was normalized to 18S mRNA and reported as ratios compared with the level of expression in untreated control mice, which were given an arbitrary value of 1.

Dynamic Flow Adhesion Studies

To examine the earliest changes that contribute to dicarbonyl-dependent atherogenesis, *apoE* KO mice (6–8 weeks of age; n = 6/group) were randomized to receive MG (50 mmol/L in drinking water), BBGC (10 mg/kg/day IP), five doses of streptozotocin (55 mg/kg) for the induction of diabetes, or no treatment and were then followed for 1 week. At this time, animals were culled and aortas were isolated and mounted in a vessel chamber primed with Krebs buffer and maintained at physiological pH by infusing carbogen gas (95% O_2, 5% CO_2) through the buffer at 37°C as previously described (28). As a positive control, vessels (n = 4–5/group) were pretreated with tumor necrosis factor-α (10 ng/mL; 4 h at 37°C) before being perfused with whole blood labeled with DilC18 (1:1,000) and perfused through the aorta at 0.12 mL/min. Images and videos of vessel wall-cell interactions were observed using a fluorescence microscope (Zeiss Discovery.V20) coupled to a digital camera (Hamamatsu ORCA-ER) and analyzed with AxioVision software. Two to three frames were taken at each time point, and the number of adherent cells per frame was recorded.

In Vitro Studies in Primary Aortic Endothelial Cells

To further examine the impact of RAGE deficiency on proinflammatory pathways, primary endothelial cells were isolated and cultured from the aortae of *RAGE* KO and C57BL/6 mice. Proinflammatory responsiveness in response to exposure to 30 mmol/L D-glucose or L-glucose (a metabolically inactive osmotic control) was determined by changes in gene expression of adhesion molecules assessed by real-time RT-PCR and static adhesion assays to assess functional adhesiveness. For static adhesion assays, primary aortic endothelial cells were seeded at 50,000 cells per well into six-well plates and allowed to grow to 70% confluency before treatment with 5 mmol/L glucose and 30 mmol/L D-glucose or L-glucose for 24 h in the presence or absence of the selective inhibitor of glyoxalase-1, BBGC (1 μmol/L), the dicarbonyl scavenger alagebrium chloride (1 μmol/L), or the cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP (2.5 μmol/L). THP-1 cells were stained using CellVue Burgundy Fluorescent Cell Labeling Kit (LI-COR) as per manufacturer's instructions before seeding them onto the endothelial cell monolayers at 3×10^5 viable cells per well and incubated for 20 min at 37°C. The cells that had not adhered to the endothelial cell monolayer were removed and the wells washed with PBS before fixing with 4% formalin in PBS for 30 min. The adhesion of cells was then quantitated using the Odyssey infrared imager (LI-COR). Additionally, adhered cells were photographed at ×20 using light microscopy (Olympus CKX41).

Statistical Analysis

Data were analyzed by one-way ANOVA with comparisons of group means being performed by Fisher least-significant different method. Analyses were performed using SPSS (SPSS version 17.0). Data are shown as means ± SEM unless otherwise specified. $P < 0.05$ was considered statistically significant.

RESULTS

Dicarbonyl Stress Augments Endothelial Activation and Adhesion In Vitro

To explore the role of glucose-induced dicarbonyl stress in promoting endothelial activation, primary aortic endothelial cells were isolated from C57BL/6 mice and cultured in the presence or absence of 30 mmol/L D-glucose. As previously described, exposure to glucose was associated

with increased gene expression of adhesion molecules, including vascular cell adhesion molecule (VCAM), intracellular adhesion molecule (ICAM)-1, tetherin, and the inflammatory chemokine MCP-1 (Fig. 1*A*). This was functionally associated with increased static adhesion of labeled monocytes to a monolayer of endothelial cells previously exposed to high glucose levels (Fig. 2*A*). These increases were inhibited by the dicarbonyl scavenger alagebrium chloride (Figs. 1*A* and 2*A*). Metabolically inactive L-glucose (30 mmol/L) had no effect on the expression of adhesion molecules or in the induction of static adhesion in this model.

Exposure of endothelial cells to the cell-permeable selective glyoxalase inhibitor BBGC (1 μmol/L) had a similar effect to exposure to 30 mmol/L D-glucose, increasing the expression of adhesion molecules as well as the adhesion of labeled monocytes to a monolayer of endothelial cells previously exposed to BBGC (Figs. 1*A* and 2*A*). These increases were inhibited by the dicarbonyl scavenger alagebrium chloride, reinforcing the premise that the BBGC actions are indirectly mediated via increased generation of reactive dicarbonyls. Notably, MnTBAP, a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger, prevented BBGC-dicarbonyl–mediated induction of tetherin in aortic endothelial cells but did not affect other markers (Fig. 1). The combined treatment of high glucose and BBGC resulted in an additive increase in the expression of adhesion molecules and further increased adhesion of leukocytes to a monolayer of cells treated with both high glucose and BBGC.

Treatment of aortic endothelial cells from *RAGE* KO mice with BBGC also led to the induction of ICAM-1, VCAM, and tetherin (Fig. 1*B*) and a functional increase in adhesiveness (Fig. 2*B*), suggesting that these actions are at least partly independent of activation of RAGE by MG-derived AGEs. However, the magnitude of these changes were less than that observed in wild-type cells. Indeed, the induction of MCP-1 following treatment with BBGC was completely prevented in *RAGE* KO cells. One potential contributing factor may be that glyoxalase-1 expression activity was also three- and fivefold higher, respectively, in endothelial cells obtained from *RAGE* KO when compared with cells taken from the aortae of wild-type mice. Notably, the induction of ICAM, VCAM, and tetherin were attenuated with coadministration of MnTBAP in *RAGE* KO cells (Fig. 1*B*), similar to that observed with the dicarbonyl scavenger alagebrium.

Dicarbonyl Stress Augments the Endothelial Activation and Adhesion In Vivo

The induction of experimental diabetes in *apoE* KO mice with streptozotocin was associated with elevated plasma MG levels (Table 1), as previously described in this model (8,23), paralleling their increased plasma glucose levels.

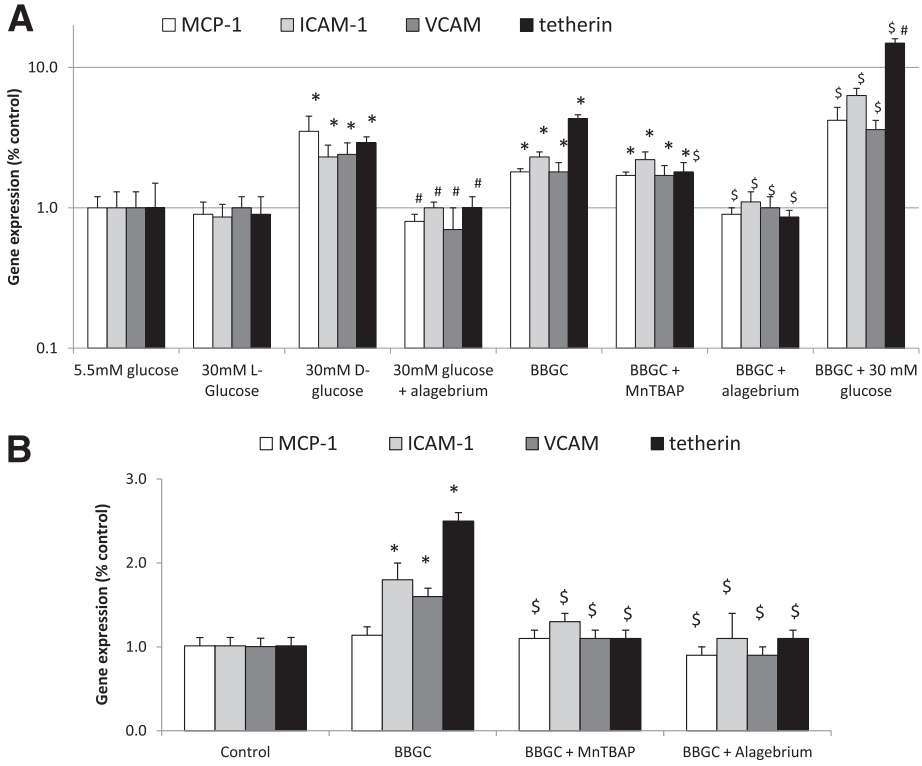

Figure 1—The induction of adhesion molecules and chemokines in aortic endothelial cells from C57BL/6 (*A*) and *RAGE* KO mice (*B*) following exposure to dicarbonyl stressors, high glucose, and the glyoxalase inhibitor, BBGC. *$P < 0.05$ vs. control; #$P < 0.05$ vs. hyperglycemia; \$$P < 0.05$ vs. BBGC.

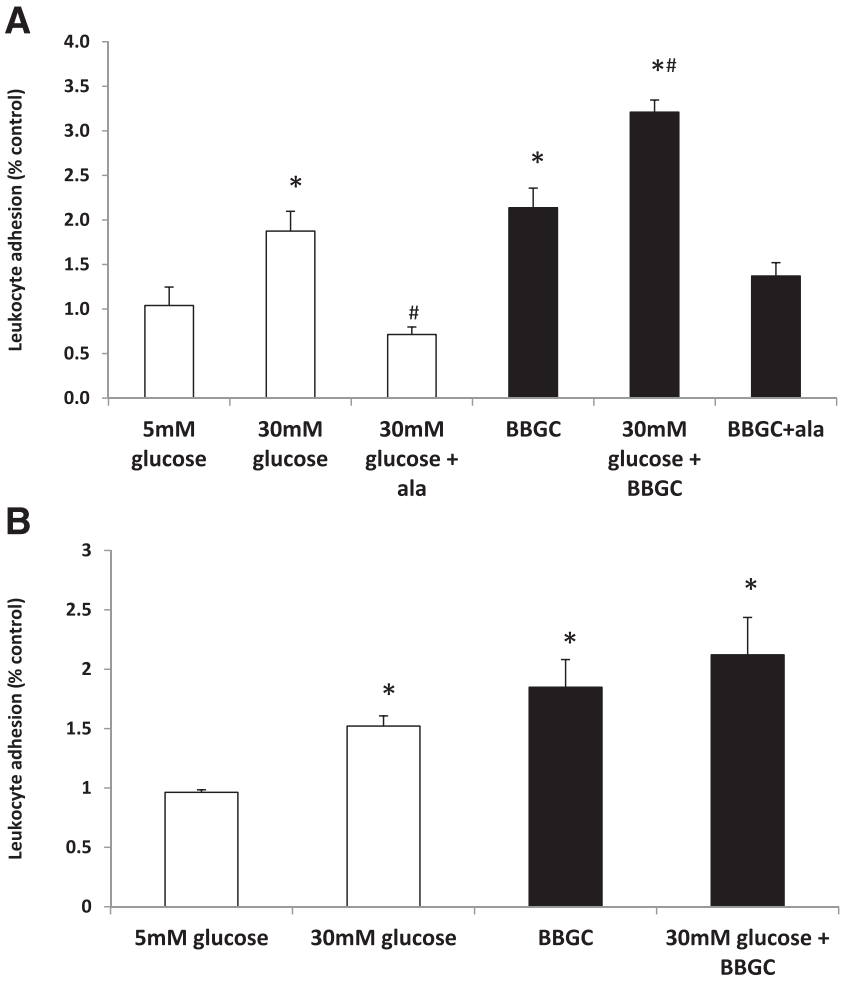

Figure 2—The induction of endothelial activation following exposure to dicarbonyl stressors, high glucose, and/or the glyoxalase inhibitor, BBGC, as denoted by increased adhesion of labeled leucocytes to an endothelial monolayer from C57BL/6 mice (*A*) and *RAGE* KO mice (*B*). Ala, alagebrium. *$P < 0.05$ vs. control; #$P < 0.05$ vs. hyperglycemia.

The induction of diabetes was also associated with increased expression of genes associated with leukocyte adhesion and vascular inflammation in the aorta, including ICAM, VCAM, tetherin, MCP-1, and macrophage markers Mac-1 and Mac-2 (Fig. 3). Soluble ICAM and MCP-1 were also increased in the circulation following the induction of diabetes. Whole aortas taken from *apoE* KO mice after 1 week of diabetes also showed increased adhesion of labeled human white cells in a dynamic flow adhesion assay when compared with nondiabetic mice (Fig. 4). After 6 weeks of diabetes, there was also a significant increase in Sudan IV–positive plaque accumulation in the aortic wall (Fig. 5), consistent with the accelerated atherogenesis previously described in this model (23).

To specifically explore the effects of MG, mice were exposed to MG in their drinking water (10 mg/kg/day). This resulted in increased circulating levels of MG, similar in magnitude to that recorded in diabetic mice, but in the absence of chronic hyperglycemia (Table 1). No weight loss or other adverse health outcomes were observed using this dose of MG. The expression of genes associated with leukocyte adhesion and vascular inflammation were increased following exposure to exogenous MG, again by a similar magnitude to that observed in diabetic mice (Fig. 3). The expression of Mac-1 and Mac-2 was also increased, denoting the accumulation of activated macrophages. Similarly, whole aortas taken from *apoE* KO mice exposed to exogenous MG showed increased adhesion of labeled human white cells in a dynamic flow adhesion assay (Fig. 4), again to a similar extent as mice exposed to diabetes. In addition, in *apoE* KO mice, exposure to MG for 6 weeks was associated with an increase in plaque accumulation in the aortic arch, as measured by en face analysis (Fig. 5). This increase in plaque area was not significantly different to that observed after 6 weeks of diabetes in *apoE* KO mice ($P = 0.09$) (Fig. 5).

Inhibition of glyoxalase-1 with BBGC also significantly increased plasma levels of MG to levels not significantly different from that observed in diabetic mice or mice receiving exogenous MG (Table 1). However, unlike with

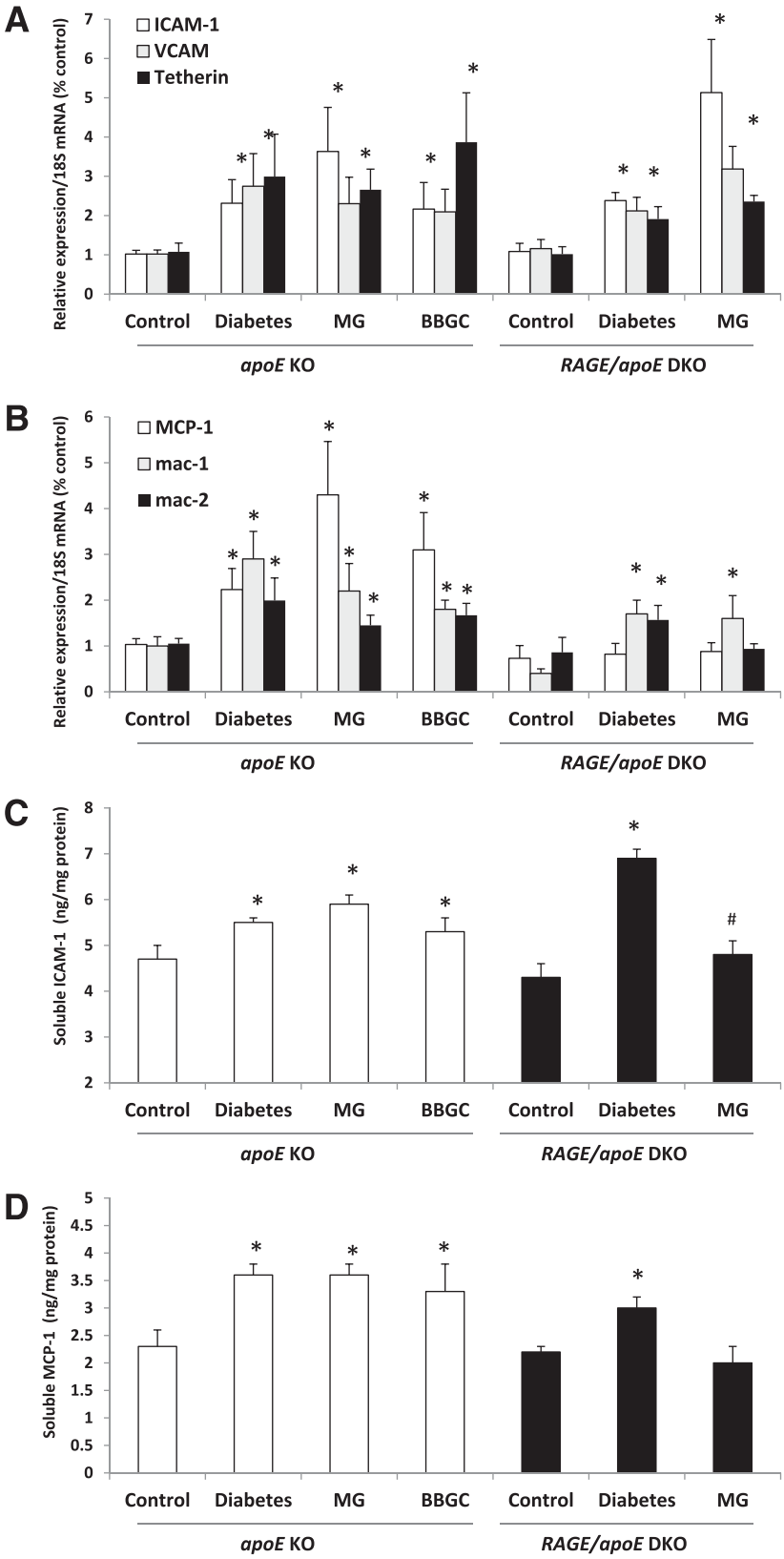

Figure 3—The gene expression of adhesion molecules ICAM-1, VCAM, and tetherin (*A*) and macrophage markers (Mac-1 and Mac-2) and the chemokine MCP-1 (*B*) in the aortic arch in *apoE* KO mice and *RAGE/apoE* double KO mice with and without 6 weeks of diabetes or exposure to MG, as assessed by real-time RT-PCR. Panels *C* and *D* show the circulating concentrations of ICAM and MCP-1 protein, respectively, in the same models. *$P < 0.05$ vs. control; #$P < 0.05$ vs. hyperglycemia.

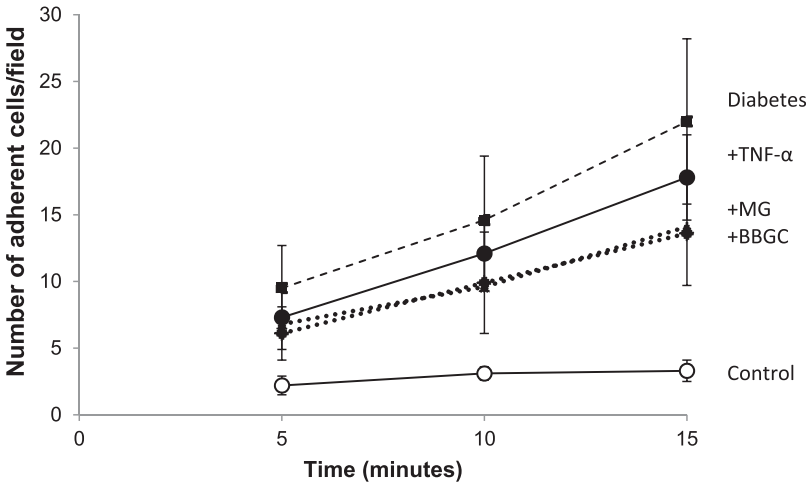

Figure 4—The induction of endothelial activation following exposure to dicarbonyl stressors, diabetes, MG, or the glyoxalase inhibitor, BBGC, as denoted by increased adhesion of labeled leukocytes to the aortic wall during dynamic flow conditions. TNF-α is presented as a positive control. TNF-α, tumor necrosis factor-α.

diabetes, inhibition of glyoxalase-1 had no significant effect on glucose levels, glycated hemoglobin, lipid or blood pressure levels, feeding/drinking behavior, or weight gain (Table 1). Whole aortas taken from *apoE* KO mice exposed to BBGC demonstrated increased vascular expression of genes associated with leukocyte adhesion and vascular inflammation in the aorta (Fig. 3). The expression of Mac-1 and Mac-2 was also increased, denoting the accumulation of activated macrophages. Whole aortas taken from *apoE* KO mice exposed to BBGC for 1 week also

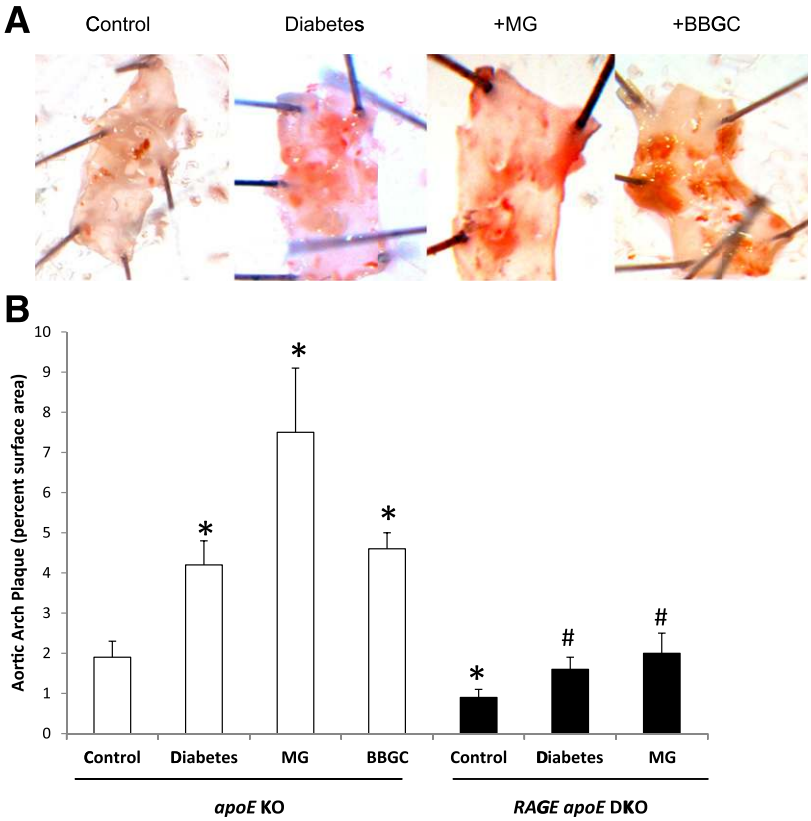

Figure 5—Plaque accumulation in the aortic arch (*A*), as assessed percentage area staining positive for Sudan IV (*B*) in *apoE* KO mice and *RAGE/apoE* double KO mice with and without 6 weeks of diabetes or exposure to MG or BBGC. *$P < 0.05$ vs. control, #$P < 0.05$ vs. *RAGE apoE* double KO mice.

showed increased adhesion of labeled human white cells in a dynamic flow adhesion assay (Fig. 4), comparable to that seen in diabetic mice. After 6 weeks of exposure to BBGC (50 mg/kg every second day), there was also significant increase in Sudan IV–positive plaque accumulation in the aortic wall (Fig. 5), again not significantly different from diabetic mice ($P = 0.13$).

Role of RAGE in Mediating Dicarbonyl-Induced Atherogenesis

Activation of the RAGE receptor by AGEs and other non-AGE ligands triggers a range of proinflammatory and proatherogenic pathways (22). We have previously demonstrated that RAGE deletion attenuates the induction of diabetes-associated atherosclerosis (23). This was also observed in the present experiments, where the induction of diabetes of *RAGE/apoE* DKO mice led to an increase in glucose, glycated hemoglobin, and plasma dicarbonyl levels comparable to that seen in diabetic *apoE* KO mice (Table 1) but was not associated with an increase in Sudan IV–positive plaque area (Fig. 5).

Although glyoxalase activity was increased in twofold in *RAGE/apoE DK*O mice when compared with wild-type mice (data not shown), as previously described (24), an infusion of MG also increased plasma MG levels in *RAGE/apoE* DKO mice to a similar extent as that observed in *apoE* KO mice (Table 1). The induction of adhesion molecules ICAM, VCAM, and tetherin were also increased in *RAGE/apoE* DKO mice following exposure to exogenous MG (Fig. 3). However, the expression of MCP-1 was not increased by either diabetes or MG in *RAGE/apoE* DKO mice, consistent with in vitro findings (Fig. 1*B*). *RAGE/apo*E KO mice have markedly reduced vascular adhesion, such that any increase induced following exposure to diabetes or BBGC, MG could not be seen (data not shown). In the 6-week model, plaque accumulation was only modestly (but significantly) increased in *RAGE/apoE* DKO mice exposed to MG, albeit off a lower baseline and far less in absolute terms than observed in *apoE* KO mice replete with the RAGE receptor (Fig. 5).

DISCUSSION

It has recently been argued that all therapeutic strategies for the prevention of complications associated with diabetes rely on the premise that the deleterious effects of high glucose levels and an enhanced metabolic flux are mediated by the generation of toxic metabolites (3,4). Of these, reactive α-dicarbonyls like MG are among the most important (6). Reactive α-dicarbonyls are the major source of intra- and extracellular AGEs (11), which contribute to the development and progression of diabetic vascular complications (5), including accelerated atherosclerosis. In this article, we demonstrate that exposure to MG, either from an exogenous source or generated following inhibition of glyoxalase-1, its primary clearance enzyme, is able to increase vascular endothelial adhesion and augment atherogenesis in *apoE* KO mice, with a similar magnitude to that observed in hyperglycemic mice with diabetes.

MG is continuously generated in the human body. It has been estimated that ~120 μmol of MG is generated every day in healthy adults (29), with intracellular concentrations of 1–5 μmol/L (30). In cells, between 1 and 5% of proteins are modified by MG (31). Even in healthy animals, 5% of aortic collagens contain MG-derived AGEs (32). However, in some circumstances, MG production is enhanced and MG-derived AGE modifications are increased. In particular, in the setting of diabetes, excessive glycolytic flux with reduced activity of GAPDH (5) and an overactive polyol pathway (4) leads to the accumulation of triose phosphate intermediates and their spontaneous fragmentation into MG (5). The proatherogenic effects of fructose feeding and the metabolic syndrome may also be partly ascribed to increased generation of MG through the polyol pathway (4). MG production may also rise in the absence of hyperglycemia via oxidation of aminoacetone (generated during protein catabolism) by semicarbazide-sensitive amine oxidase, the oxidation of ketone bodies by myeloperoxidase, and/or the cytochrome P450–mediated oxidation of acetone (generated from lipolysis). MG accumulation may also occur with impaired detoxification of α-dicarbonyls by the glyoxalase system or depletion of available glutathione due to oxidative stress (3). In our study, we used selective inhibition of glyoxalase-1 with cell-permeable inhibitor BBGC to increase plasma levels of MG to a similar extent as that observed in diabetes. This was sufficient to induce diabetes-like changes in cellular adhesion and inflammation in the absence of hyperglycemia. By contrast overexpression of glyoxalase-1, an enzyme that specifically metabolizes α-dicarbonyls, is able to prevent hyperglycemia-induced changes in vascular function in mice (33,34).

α-Dicarbonyls are also generated during fermentation and during prolonged or high-temperature cooking, meaning that certain (processed) foods and cigarette smoke (35,36) contain significant amounts of MG. Consumption of a conventional Western diet typically contains ~2 μmol of MG each day but may be up to tenfold higher in some cases. Typical mouse chow is also high in MG-derived AGEs due its thermal processing (37). In our experiments, we exposed our mice to ~45 μmol/kg (1% MG in drinking water) in order to achieve an elevation in circulating MG levels comparable to that seen with diabetes. While this exposure to exogenous MG in our experiments was potentially greater than that documented in a normal human diet, it is consistent with heavy human exposure to diets containing large amounts of fried foods. Moreover, it likely that lower exposure over much longer periods may also have pathogenic effects, especially in the setting of diabetes, where detoxification mechanisms are already saturated.

This is the first description of atherogenesis induced by dicarbonyls in the absence of hyperglycemia. Previous studies have shown that diets high in MG-derived AGEs

(and therefore, presumably, MG) are able to induce vascular inflammation and atherogenesis in diabetic *apoE* KO mice (37), but control mice were unaffected. Other studies have suggested that MG may be a pathogenic factor for the development of endothelial dysfunction (38), renal damage (39), insulin resistance (40), and macrophage activation (41), all of which may have contributed to atherogenesis in our model. It has also been reported that exposure to MG may be associated with the development of dyslipidemia (40) and/or hypertension (38). In our studies, neither MG administration nor BBGC modified lipid or blood pressure levels, although our mice had marked dyslipidemia due to genetic deletion of the *apoE* gene, a model in which diabetes itself has only modest effects on already elevated lipid levels (Table 1). However, functional changes on lipoproteins cannot be excluded. Indeed, previous studies have clearly established that the modification of LDL by MG increases its arterial atherogenicity, partly by increasing its density and binding to proteoglycans in the arterial wall (20). AGE modification of apoA-I considerably impairs its cardioprotective, antiatherogenic properties, including the ability to promote cholesterol efflux, stabilize ABCA1, and inhibit the expression of adhesion molecules (42).

Although our work has focused on changes in the vascular endothelium induced by dicarbonyl stress, it is likely that complementary changes induced in leukocytes also contribute to vascular inflammation (especially in the setting of an activated endothelium). Due to their endocytic functions, macrophages accumulate MG-modified proteins in high levels, especially within plaques (18,19). It has previously been shown that exposure of human macrophages to physiological doses of MG-modified albumin results in their activation and proliferation (41), partly by the induction of reactive oxygen species. MG has also been shown to enhance the formation of platelet-neutrophil aggregates (43) and facilitate foam cell formation (44).

RAGE is a pattern recognition receptor for AGE-modified proteins as well as several of the S-100 calgranulins, β-amyloid protein, and the neuroregulatory protein amphoterin. Activation of full-length RAGE signals a cascade of intracellular pathways, modulating overall cellular responses to various stress conditions and enhancing cell damage. These include a number of proinflammatory proatherogenic mediators, including VCAM-1, ICAM-1, interleukin-1α, interleukin-6, tumor necrosis factor-α, E-selectin, NADPH oxidase, and the expression of RAGE itself. RAGE activation also suppresses the expression of glyoxalase (25). Our data show that deletion of RAGE is able to significantly reduce atherogenesis associated with diabetes or MG exposure, through attenuation of one or many of these actions. It appears likely that MG, or the AGEs it generates, affects atherogenesis mostly by activation of RAGE-dependent pathways (6).

However, significant RAGE-independent pathways for atherogenesis also appear to exist, as RAGE deletion did not completely prevent vascular damage, and MG was still able to augment atherogenesis and vascular inflammation in *RAGE* KO mice, albeit from a much lower baseline and to a lesser extent. In addition, inhibition of glyoxalase was still proinflammatory in *RAGE* KO endothelial cells, albeit at a much lower level. These findings are consistent with our previous studies using dicarbonyl scavengers, like alagebrium chloride, aminoguanidine, and pyridoxamine to attenuate diabetic renal complications even in *RAGE* KO mice (45). The mechanism(s) by which dicarbonyls are able to induce vascular damage independent of RAGE are a matter of ongoing investigation but may result as a functional sequel of increased AGE modification of a broad range of proteins as well as lipids and DNA (46). For example, others have described how MG modification of mitochondrial proteins leads uncoupling of electrons and to oxidative stress (47). Certainly, the cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP was able to attenuate the proinflammatory effects of BBGC in *RAGE* KO cells, suggesting the proinflammatory actions of dicarbonyls in endothelial cells are partly mediated through oxidative stress. However, equivalent effects were not observed in wild-type cells. Consequently, our data suggest the presence of two key pathways for dicarbonyl-mediated dysfunction: the first (dominant) pathway mediated via RAGE signaling and the second via the (RAGE independent) induction of mitochondrial reactive oxygen species as revealed by our studies in *RAGE* KO cells and animals. These data suggest that combination approaches will be essential to attenuate the effects of dicarbonyls in the vasculature and prevent diabetic vascular complications.

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References

1. Candido R, Jandeleit-Dahm KA, Cao Z, et al. Prevention of accelerated atherosclerosis by angiotensin-converting enzyme inhibition in diabetic apolipoprotein E-deficient mice. Circulation 2002;106:246–253

2. Garcia MJ, McNamara PM, Gordon T, Kannel WB. Morbidity and mortality in diabetics in the Framingham population. Sixteen year follow-up study. Diabetes 1974;23:105–111

3. Fleming T, Cuny J, Nawroth G, et al. Is diabetes an acquired disorder of reactive glucose metabolites and their intermediates? Diabetologia 2012;55:1151–1155
4. Liu J, Wang R, Desai K, Wu L. Upregulation of aldolase B and overproduction of methylglyoxal in vascular tissues from rats with metabolic syndrome. Cardiovasc Res 2011;92:494–503
5. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813–820
6. Rabbani N, Thornalley PJ. The critical role of methylglyoxal and glyoxalase 1 in diabetic nephropathy. Diabetes 2014;63:50–52
7. Watson AM, Soro-Paavonen A, Sheehy K, et al. Delayed intervention with AGE inhibitors attenuates the progression of diabetes-accelerated atherosclerosis in diabetic apolipoprotein E knockout mice. Diabetologia 2011;54:681–689
8. Bierhaus A, Fleming T, Stoyanov S, et al. Methylglyoxal modification of Nav1.8 facilitates nociceptive neuron firing and causes hyperalgesia in diabetic neuropathy [published correction appears in Nat Med 2012;18:1445]. Nat Med 2012;18:926–933
9. Lapolla A, Flamini R, Dalla Vedova A, et al. Glyoxal and methylglyoxal levels in diabetic patients: quantitative determination by a new gc/ms method. Clin Chem Lab Med 2003;41:1166–1173
10. McLellan AC, Thornalley PJ, Benn J, Sonksen PH. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. Clin Sci (Lond) 1994;87:21–29
11. Kilhovd BK, Giardino I, Torjesen PA, et al. Increased serum levels of the specific AGE-compound methylglyoxal-derived hydroimidazolone in patients with type 2 diabetes. Metabolism 2003;52:163–167
12. Degenhardt TP, Thorpe SR, Baynes JW. Chemical modification of proteins by methylglyoxal. Cell Mol Biol (Noisy-le-grand) 1998;44:1139–1145
13. Hanssen NM, Wouters K, Huijberts MS, et al. Higher levels of advanced glycation endproducts in human carotid atherosclerotic plaques are associated with a rupture-prone phenotype. Eur Heart J 2014;35:1137–1146
14. Forbes JM, Yee LT, Thallas V, et al. Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis [published correction appears in Diabetes 2006;55:862]. Diabetes 2004;53:1813–1823
15. D'Agati V, Schmidt AM. RAGE and the pathogenesis of chronic kidney disease. Nat Rev Nephrol 2010;6:352–360
16. Sourris KC, Morley AL, Koitka A, et al. Receptor for AGEs (RAGE) blockade may exert its renoprotective effects in patients with diabetic nephropathy via induction of the angiotensin II type 2 (AT2) receptor. Diabetologia 2010;53:2442–2451
17. Watson AMD, Soro-Paavonen A, Jandeleit-Dahm KA. Age-rage signalling and endothelial dysfunction and atherosclerosis in diabetes. In *Endothelial Dysfunction and Inflammation*. Duphinee S, Karsan A, Eds. Basel, Birkhauser-Verlag/Springer, 2010, p. 161–174
18. Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetologia 2001;44:129–146
19. Thomas MC, Tikellis C, Burns WM, et al. Interactions between renin angiotensin system and advanced glycation in the kidney. J Am Soc Nephrol 2005;16:2976–2984
20. Rabbani N, Godfrey L, Xue M, et al. Glycation of LDL by methylglyoxal increases arterial atherogenicity: a possible contributor to increased risk of cardiovascular disease in diabetes. Diabetes 2011;60:1973–1980
21. Cantero AV, Portero-Otin M, Ayala V, et al. Methylglyoxal induces advanced glycation end product (ages) formation and dysfunction of pdgf receptor-beta: Implications for diabetic atherosclerosis. FASEB J 2007;21:3096–3106
22. Harja E, Bu DX, Hudson BI, et al. Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE-/- mice. J Clin Invest 2008;118:183–194
23. Soro-Paavonen A, Watson AM, Li J, et al. Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes. Diabetes 2008;57:2461–2469
24. Park L, Raman KG, Lee KJ, et al. Suppression of accelerated diabetic atherosclerosis by the soluble receptor for advanced glycation endproducts. Nat Med 1998;4:1025–1031
25. Yao D, Brownlee M. Hyperglycemia-induced reactive oxygen species increase expression of the receptor for advanced glycation end products (RAGE) and RAGE ligands. Diabetes 2010;59:249–255
26. Allen TJ, Cooper ME, O'Brien RC, Bach LA, Jackson B, Jerums G. Glomerular filtration rate in streptozocin-induced diabetic rats. Role of exchangeable sodium, vasoactive hormones, and insulin therapy. Diabetes 1990;39:1182–1190
27. Miller AG, Tan G, Binger KJ, et al. Candesartan attenuates diabetic retinal vascular pathology by restoring glyoxalase-I function. Diabetes 2010;59:3208–3215
28. Thomas MC, Pickering RJ, Tsorotes D, et al. Genetic Ace2 deficiency accentuates vascular inflammation and atherosclerosis in the ApoE knockout mouse. Circ Res 2010;107:888–897
29. Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification—a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmacol 1996;27:565–573
30. Thornalley PJ. Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems—role in ageing and disease. Drug Metabol Drug Interact 2008;23:125–150
31. Thornalley PJ, Battah S, Ahmed N, et al. Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. Biochem J 2003;375:581–592
32. Dobler D, Ahmed N, Song L, Eboigbodin KE, Thornalley PJ. Increased dicarbonyl metabolism in endothelial cells in hyperglycemia induces anoikis and impairs angiogenesis by RGD and GFOGER motif modification. Diabetes 2006;55:1961–1969
33. Berner AK, Brouwers O, Pringle R, et al. Protection against methylglyoxal-derived ages by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. Diabetologia 2012;55:845–854
34. Brouwers O, Niessen PM, Ferreira I, et al. Overexpression of glyoxalase-I reduces hyperglycemia-induced levels of advanced glycation end products and oxidative stress in diabetic rats. J Biol Chem 2011;286:1374–1380
35. Lo C-Y, Shiming L, Wang Y, et al. Reactive dicarbonyl compounds and 5-(hydroxymethyl)-2-furfural in carbonated bevarage containing high fructose corn syrup. Food Chemistry 2008;107:1099–1105
36. White JS. Misconceptions about high-fructose corn syrup: is it uniquely responsible for obesity, reactive dicarbonyl compounds, and advanced glycation endproducts? J Nutr 2009;139:1219S–1227S
37. Lin RY, Choudhury RP, Cai W, et al. Dietary glycotoxins promote diabetic atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis 2003;168:213–220
38. Vasdev S, Ford CA, Longerich L, Parai S, Gadag V, Wadhawan S. Aldehyde induced hypertension in rats: prevention by N-acetyl cysteine. Artery 1998;23:10–36
39. Golej J, Hoeger H, Radner W, Unfried G, Lubec G. Oral administration of methylglyoxal leads to kidney collagen accumulation in the mouse. Life Sci 1998;63:801–807
40. Berlanga J, Cibrian D, Guillén I, et al. Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds. Clin Sci (Lond) 2005;109:83–95
41. Fan X, Subramaniam R, Weiss MF, Monnier VM. Methylglyoxal-bovine serum albumin stimulates tumor necrosis factor alpha secretion in RAW 264.7 cells through activation of mitogen-activating protein kinase, nuclear factor kappaB and intracellular reactive oxygen species formation. Arch Biochem Biophys 2003;409:274–286
42. Hoang A, Murphy AJ, Coughlan MT, et al. Advanced glycation of apolipoprotein A-I impairs its anti-atherogenic properties. Diabetologia 2007;50:1770–1779

43. Gawlowski T, Stratmann B, Stirban AO, Negrean M, Tschoepe D. AGEs and methylglyoxal induce apoptosis and expression of Mac-1 on neutrophils resulting in platelet-neutrophil aggregation. Thromb Res 2007;121:117–126
44. Brown BE, Dean RT, Davies MJ. Glycation of low-density lipoproteins by methylglyoxal and glycolaldehyde gives rise to the in vitro formation of lipid-laden cells. Diabetologia 2005;48:361–369
45. Watson AM, Gray SP, Jiaze L, et al. Alagebrium reduces glomerular fibrogenesis and inflammation beyond preventing RAGE activation in diabetic apolipoprotein E knockout mice. Diabetes 2012;61:2105–2113
46. Thornalley PJ, Waris S, Fleming T, et al. Imidazopurinones are markers of physiological genomic damage linked to DNA instability and glyoxalase 1-associated tumour multidrug resistance. Nucleic Acids Res 2010;38:5432–5442
47. Rosca MG, Monnier VM, Szweda LI, Weiss MF. Alterations in renal mitochondrial respiration in response to the reactive oxoaldehyde methylglyoxal. Am J Physiol Renal Physiol 2002;283:F52–F59