Dicarbonyl stress in the absence of hyperglycemia increases endothelial inflammation and atherogenesis similar to that observed in diabetes.

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Abstract

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 dicarboxyls like methylglyoxal. In this paper we demonstrate that increasing plasma methylglyoxal 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 bromobenzyl-glutathione cyclopentyl diester (BBGC) 50mg/kg IP every second day) was able to increase vascular adhesion and augment atherogenesis in euglycaemic apoE knockout mice to a similar magnitude to that observed in hyperglycaemic mice with diabetes. The effects of methylglyoxal 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 as the induction of mitochondrial oxidative stress by dicarboxyls also contributes to inflammation and atherogenesis. Such data would suggest a synergistic combination of RAGE antagonism and antioxidants may offer the greatest utility for the prevention and management of diabetic vascular complications.
Introduction

Diabetes leads to the increased incidence, size and complexity of atherosclerotic plaques (1) resulting in an increased incidence and severity of cardiovascular disease (CVD) in patients with diabetes (2). A number of metabolic and haemodynamic factors contribute to accelerated atherosclerosis in the setting of diabetes. One key pathway is the increased production of reactive dicarboxyls generated from triose-phosphate intermediates of glycolysis, glycerol and ketone peroxidation, over-activation 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 non-diabetic state (7, 8). Plasma MG concentrations are also elevated in patients with diabetes (9), especially those with vascular complications(10).

Reactive α-dicarboxyls 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 intra-cellular locations, and correlate with the size, complexity and stability of the lesions (12, 13). The potential importance of α-dicarboxyls in the pathogenesis of glucose-dependent atherogenesis is illustrated by the vasculo-protective 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, post-translational 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 pro-atherogenic 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 knockout (apoE 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, up-regulation of the RAGE receptor in response to high glucose levels is partly mediated by MG and subsequent AGE-modification of signalling proteins (25), while RAGE activation reduces expression of glyoxalase-1, an enzyme that metabolises dicarbonyls, illustrating the close interconnection of receptor-independent and receptor-dependent pathways (18, 19). In this paper, 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 non-diabetic apoE KO, via both RAGE-dependent and RAGE-independent pathways, to produce similar vascular changes to those observed in hyperglycaemic mice.
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 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, WA, Australia) and were maintained on a 12 hour light–12 hour darkness 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 (NHMRC) guidelines with local ethics approval.

8-week old male apoE KO mice and RAGE/apoE DKO mice (n= 20/group) were randomised to receive standard drinking water or water into which MG had been added (50mM). 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 randomised to receive the cell-permeable selective glyoxalase 1 inhibitor bromobenzyl-glutathione cyclopentyl diester (BBGC; 50mg/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 5 daily injections of streptozotocin (55mg/kg; Sigma-Aldrich, St Louis, MO, USA) in citrate buffer. This results in chronic hyperglycaemia (20-30mM) without requiring insulin to prevent ketosis or excessive weight loss. This model is generally associated with a 2-3 fold increase in plaque accumulation in the aortic arch when compared to non-diabetic apoE KO mice(23).
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, NSW, Australia) followed by cardiac exsanguination. Lysates of erythrocytes were analysed for glycated haemoglobin levels by HPLC (Biorad, Richmond, CA, USA) (26). Plasma levels of total cholesterol and triglycerides were measured with a standard commercial enzymatic assay using a Beckman Coulter LX20PRO Analyser (Cat No. 467825 Beckman Coulter Diagnostics, Gladesville, NSW, Australia). LDL cholesterol was calculated using the Friedewald formula. Plasma MG was measured by HPLC, 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%, vol./vol.) 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 were cleaned of excess fat and stained with Sudan IV-Herxheimer’s solution (0.5% w/vol; Gurr, BDH Limited, Poole, UK). 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 pro-inflammatory 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,
Perkin-Elmer Inc, PE Biosystems, Foster City, CA, USA) as previously utilised by our group (23). Gene expression was normalized to 18S mRNA and reported as ratios compared to 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 (50mM in drinking water), BBGC (10mg/kg/day IP), five doses of streptozotocin (55mg/kg) for the induction of diabetes or no treatment, and then followed for one 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% O2; 5% CO2) through the buffer at 37°C, as previously described (28). As a positive control, vessels (n=4-5/group) were pre-treated with TNFα (10ng/ml; 4hrs at 37°C) before being perfused with whole blood labelled with DilC18 (1:1000), and then perfused through the aorta at 0.12ml/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 AxioVison software. Two to three frames were taken at each time point and the number of adherent cells per frame recorded.

*In vitro studies in primary aortic endothelial cells*

To further examine the impact of RAGE deficiency on pro-inflammatory pathways, primary endothelial cells were isolated and cultured from the aortae of RAGE KO and C57BL/6 mice. Pro-inflammatory responsiveness in response to exposure to 30mM 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 5mM glucose, 30mM D-glucose or L-Glucose for 24 hours in the presence or absence of the selective inhibitor of glyoxalase 1, BBGC (1µM), the dicarbonyl scavenger, alagebrium chloride (1µM) or the cell permeable superoxide dismutase mimetic and peroxynitrite scavenger MnTBAP (2.5µM). THP-1 cells were stained using CellVue Burgundy Fluorescent Cell labelling Kit (LICOR) as per manufacturer’s instructions before seeding them onto the endothelial cell monolayers at 3x10^5 viable cells per well and incubated for 20 minutes 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 infra-red imager (LICOR). Additionally adhered cells were photographed at x20 using light microscopy (Olympus CKX41).

Statistical analysis

Data were analysed by one-way ANOVA with comparisons of group means being performed by Fisher’s least-significant different method. Analyses were performed using SPSS (SPSS v17.0). 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 30mM D-glucose. As previously described, exposure to glucose was associated with increased gene expression of adhesion molecules including VCAM, ICAM-1 and tetherin, and the inflammatory chemokine, MCP-1 (Figure 1a). This was functionally associated with increased static adhesion of labelled monocytes to a monolayer of endothelial cells previously exposed to high glucose levels (Figure 2a). These increases were inhibited by the dicarbonyl scavenger, alagebrium chloride (Figures 1a&2a). Metabolically inactive L-glucose (30mM) 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 (1µM) had a similar effect to exposure to 30mM D-glucose, increasing the expression of adhesion molecules as well as the adhesion of labelled monocytes to a monolayer of endothelial cells previously exposed to BBGC (Figures 1a&2a). These increases were inhibited by the dicarbonyl scavenger, alagebrium chloride, reinforcing the premise that the BBGCs 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 (figure 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 leucocytes 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 (Figure 1b) and a functional increase in adhesiveness (Figure 2b), 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 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 were also 3-fold and 5-fold higher respectively in endothelial cells obtained from RAGE KO, when compared to cells taken from the aortae of wild type mice. Notably, the induction of ICAM, VCAM and tetherin were attenuated with co-administration of MnTBAP in RAGE KO cells (Figure 1b), 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. 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 the macrophage markers, Mac-1 and Mac-2 (figure 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 labelled human white cells in a dynamic flow adhesion assay when compared to non-diabetic mice (figure 4). After 6-weeks of diabetes, there was also a significant increase in Sudan IV positive plaque accumulation in the aortic wall (figure 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 (10mg/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 hyperglycaemia (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 (figure 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 labelled human white cells in a dynamic flow adhesion assay (figure 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 (figure 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, figure 5).

Inhibition of glyoxalase 1 with BBGC also significantly increased plasma levels of MG to levels not significantly different to that observed in diabetic mice or mice receiving exogenous MG (table 1). However, unlike with diabetes, inhibition of glyoxalase 1 had no significant effect on glucose levels, glycated Hb, lipid or blood pressure levels, feeding drinking behaviour 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 (figure 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 showed increased adhesion of labelled human white cells in a dynamic flow adhesion assay (figure 4), comparable to that seen in diabetic mice. After 6-weeks of exposure to BBGC (50mg/kg every second day) there was
also significant increase in Sudan IV positive plaque accumulation in the aortic wall (figure 5), again not significantly different to diabetic mice (p=0.13).

The role of RAGE in mediating dicarbonyl induced atherogenesis

Activation of the RAGE receptor by AGEs and other non-AGE ligands triggers a range of pro-inflammatory and pro-atherogenic 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 in apoE/RAGE DKO mice led to an increase in glucose, glycated haemoglobin and plasma dicarbonyl levels comparable to that seen in diabetic apoE KO mice (table 2), but was not associated with an increase in Sudan IV positive plaque area (figure 5).

Although glyoxalase activity was increased in 2 fold in RAGE/apoE DKO mice when compared to 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 observed in apoE KO mice (table 2). The induction of adhesion molecules, ICAM, VCAM and tetherin were also increased in RAGE/apoE DKO mice following exposure to exogenous MG (figure 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 (figure 1b). RAGE apoE KO mice have markedly reduced vascular adhesion, such that any increase induced following exposure to diabetes or BCCG, 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 in the RAGE receptor (figure 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 paper 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 hyperglycaemic mice with diabetes.

MG is continuously generated in the human body. It has been estimated approximately 120 µmol of MG is generated every day in healthy adults (29) with intracellular concentrations of 1-5µM (30). In cells, between 1 and 5% of proteins are modified by MG (31). Even healthy animals, 5% of aortic collagens contain methylglyoxal-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 pro-atherogenic 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 hyperglycaemia, via oxidation of aminoacetone (generated during protein catabolism) by semicarbazide-sensitive amine oxidase, the oxidation of ketone bodies by myeloperoxidase and the cytochrome P450-mediated oxidation of acetone.
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 the cell-permeable inhibitor, BBGC, to increase plasma levels of MG to a similar extent to that observed in diabetes. This was sufficient to induce diabetes-like changes in cellular adhesion and inflammation, in the absence of hyperglycaemia. By contrast over-expression of glyoxalase-1, an enzyme that specifically metabolizes α-dicarbonyls, is able to prevent hyperglycaemia-induced changes in vascular function in mice (33, 34).

α-Dicarbonyls are also generated during fermentation, 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 by Vlassara et al 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 dyslipidaemia (40) and/or hypertension (38). In our studies, neither MG administration nor BBGC modified lipid or blood pressure levels, although our mice had marked dyslipidaemia 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, Thornalley et al 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, stabilise 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 leucocytes 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 neuro-regulatory 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 pro-inflammatory pro-atherogenic mediators, including VCAM-1, ICAM-1, IL-1α, IL-6, TNF-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 also 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 pro-inflammatory 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 dicarboxyls are able to induce vascular damage independent of RAGE are a matter of ongoing investigation, but may include result as a functional sequel of increased AGE-modification of a broad range of proteins as well as lipids and DNA (46). For example, Thornalley and colleagues have described how methylglyoxal 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 pro-inflammatory effects of BBCG in RAGE KO cells suggesting the pro-inflammatory actions of dicarboxyls 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 dicarboxyl mediated dysfunction, the first (dominant) pathway mediated via RAGE signalling, and the
second via the (RAGE independent) induction of mitochondrial ROS as revealed by our studies in RAGE KO cells and animals. This data suggest that combination approaches will be essential to attenuate the effects of dicarbonyls in the vasculature and prevent diabetic vascular complications.
Author Contributions:

C.T. researched data. R.P. researched data. D.T. researched data. O.H. researched data. M.E.C. contributed to discussion, reviewed/edited manuscript. K.J.D. contributed to discussion, reviewed/edited manuscript. M.C.T designed experiments, wrote/reviewed/edited manuscript, contributed discussion).

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FIGURE LEGENDS

**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. *vs control p<0.05, # vs hyperglycaemia p<0.05

**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 labelled leucocytes to an endothelial monolayer from C57BL/6 mice (A) and RAGE KO mice (B). *vs control p<0.05, # vs hyperglycaemia p<0.05

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

**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 labelled leucocytes to the aortic wall during dynamic flow conditions.

**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. *vs control p<0.05, # vs RAGE apoE double KO mice p<0.05
Table 1. General parameters of mouse models of dicarbonyl stress compared to those with diabetes

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<thead>
<tr>
<th>Parameter</th>
<th>Control apoE KO</th>
<th>MG treated apoE KO</th>
<th>BCCG treated apoE KO</th>
<th>Diabetic apoE KO</th>
<th>Control RAGE/apoE DKO</th>
<th>MG treated RAGE/apoE DKO</th>
<th>Diabetic RAGE/apoE DKO</th>
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<td>Body weight (g/day)</td>
<td>28.2 ± 0.6</td>
<td>27.2 ± 0.5</td>
<td>23.2 ± 2.0*</td>
<td>22.7 ± 0.6*</td>
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<td>Food intake (g/day)</td>
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<td>3.4 ± 0.2</td>
<td>3.6 ± 0.5</td>
<td>5.0 ± 0.5*</td>
<td>1.9 ± 0.3</td>
<td>2.9 ± 0.1</td>
<td>4.8 ± 0.5*</td>
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<tr>
<td>Water intake (ml/day)</td>
<td>4.2 ± 0.3</td>
<td>5.1 ± 0.3</td>
<td>3.5 ± 0.5</td>
<td>17.5 ± 2.7*</td>
<td>6.1 ± 0.2</td>
<td>6.7 ± 0.5</td>
<td>16.6 ± 2.8*</td>
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<td>Systolic BP (mmHg)</td>
<td>95 ± 3</td>
<td>90 ± 2</td>
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<td>96 ± 2</td>
<td>94 ± 5</td>
<td>88 ± 5</td>
<td>99 ± 2</td>
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<td>Glycated Hb (%)</td>
<td>4.3 ±0.1</td>
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<td>4.1 ± 0.2</td>
<td>13.3 ± 1.7*</td>
<td>4.7 ± 0.1</td>
<td>4.7 ± 0.2</td>
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<td>Plasma glucose (mmol/L)</td>
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<td>11.1 ± 0.5</td>
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<td>10.8 ± 0.8</td>
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<td>Total cholesterol (mmol/L)</td>
<td>8.8 ± 0.6</td>
<td>6.6 ± 0.3</td>
<td>7.6 ± 0.3</td>
<td>16.4 ± 0.3*</td>
<td>8.9 ± 0.3</td>
<td>8.4 ± 0.6</td>
<td>17.4 ± 0.6*</td>
</tr>
<tr>
<td>Plasma MG (µmol/L)</td>
<td>1.0 ± 0.2</td>
<td>1.9 ± 0.2*</td>
<td>2.1 ± 0.5*</td>
<td>2.3 ± 0.2*</td>
<td>1.0 ± 0.1</td>
<td>1.7 ± 0.2*</td>
<td>2.1 ± 0.3*</td>
</tr>
</tbody>
</table>
References


30. Thornalley PJ. Protein and nucleotide damage by glyoxal and methylglyoxal in physiological systems--role in ageing and disease. Drug metabolism and drug interactions. 2008;23:125-150


Gene expression (% control)

- 5.5mM glucose
- 30mM D-glucose
- BBGC
- BBGC+MnTBAP
- 30mM L-Glucose
- 30mm glucose + alagebrium
- BBGC+30 mM glucose
- BBGC+alagebrium

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Gene expression (% control)

- Control
- BBGC
- BBGC + Alagebrium
- BBGC + MnTBAP

- MCP-1
- ICAM-1
- VCAM
- tetherin

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C.

![Bar chart showing soluble ICAM-1 levels in different conditions.](chart)

- Control
- Diabetes
- MG
- BBGC

**apoE KO**

**RAGE/apoE DKO**

D.

![Bar chart showing soluble MCP-1 levels in different conditions.](chart)

- Control
- Diabetes
- MG
- BBGC

**apoE KO**

**RAGE/apoE DKO**
Number of adherent cells/field

Time (minutes)

Diabetes
+TNFalpha
+MG
+BCCG
Control
Aortic arc plaque (percent surface area)

Control Diabetes MG BCCG Control Diabetes MG

ApoE KO RAGE ApoE DKO

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