Methylglyoxal administration induces diabetes-like microvascular changes and perturbs the healing process of cutaneous wounds

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ABSTRACT

Increased formation of MG (methylglyoxal) and related protein glycation in diabetes has been linked to the development of diabetic vascular complications. Diabetes is also associated with impaired wound healing. In the present study, we investigated if prolonged exposure of rats to MG (50–75 mg/kg of body weight) induced impairment of wound healing and diabetes-like vascular damage. MG treatment arrested growth, increased serum creatinine, induced hypercholesterolaemia (all \( P < 0.05 \)) and impaired vasodilation (\( P < 0.01 \)) compared with saline controls. Degenerative changes in cutaneous microvessels with loss of endothelial cells, basement membrane thickening and luminal occlusion were also detected. Acute granulation appeared immature (\( P < 0.01 \)) and was associated with an impaired infiltration of regenerative cells with reduced proliferative rates (\( P < 0.01 \)). Immunohistochemical staining indicated the presence of AGEs (advanced glycation end-products) in vascular structures, cutaneous tissue and peripheral nerve fibres. Expression of RAGE (receptor for AGEs) appeared to be increased in the cutaneous vasculature. There were also pro-inflammatory and profibrotic responses, including increased IL-1\( \beta \) (interleukin-1\( \beta \)) expression in intact epidermis, TNF-\( \alpha \) (tumour necrosis factor-\( \alpha \)) in regions of angiogenesis, CTGF (connective tissue growth factor) in medial layers of arteries, and TGF-\( \beta \) (transforming growth factor-\( \beta \)) in glomerular tufts, tubular epithelial cells and interstitial endothelial cells. We conclude that exposure to increased MG in vivo is associated with the onset of microvascular damage and other diabetes-like complications within a normoglycaemic context.

INTRODUCTION

The prevalence of diabetes is rapidly increasing worldwide and has been estimated to double within the next 25 years. Type II diabetes affects 5–10% of the population of most countries and is the most frequently diagnosed metabolic disorder [1]. The major morbidity and mortality associated with diabetes is due to the

Key words: diabetes, diabetic complication, methylglyoxal, glycation, wound healing.

Abbreviations: AGE, advanced glycation end-product; CEL, \( N^\varepsilon -(1\text{-carboxyethyl}) \)lysine; CTGF, connective tissue growth factor; H/E, haematoxylin/eosin; IL-1\( \beta \), interleukin-1\( \beta \); MG, methylglyoxal; NTG, nitroglycerine; PAS, periodate–Schiff; PCNA, proliferating cell nuclear antigen; RAGE, receptor for AGEs; TGF-\( \beta \), transforming growth factor-\( \beta \); TNF-\( \alpha \), tumour necrosis factor-\( \alpha \).

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development of both macro- and micro-vascular complications [2]. There are also cutaneous disorders linked to generalized microangiopathy, including failure of the wound healing process [3]. Prospective clinical trials in both Type I and Type II diabetic patients, the Diabetes Complications and Control Trial and the United Kingdom Prospective Diabetes Study, established hyperglycaemia as an independent risk factor for the development of microvascular complications [4,5]. Vascular complications of diabetes have been linked to dysfunction of endothelial cells, pericytes and other cells with the glucose transporter GLUT1, where hyperglycaemia activates multiple pathways of biochemical dysfunction [6]. One of these pathways is increased protein glycation by the reactive physiological dicarbonyl compound MG (methylglyoxal). MG is formed mainly by the spontaneous degradation of triosephosphates [7], the metabolism of acetone from ketone bodies, the catabolism of threonine and the degradation of glucose and glycated proteins (for review, see [8]). Increased formation of MG in diabetes occurs in vascular cells arising from an increased flux and accumulation of triosephosphates [9] following inhibition of glyceraldehyde-3,4-phosphate dehydrogenase linked to the activation of poly(ADP-ribose) polymerase [10]. Vascular tissue concentrations of MG usually appear increased 3–5-fold in clinical diabetes [11]; this may be particularly enhanced due to the pro-oxidant environment in this condition [12], which additionally impairs the detoxification process of MG by the glutathione-dependent enzyme glyoxalase I [13,14]. MG is a potent protein glycating agent and an important precursor of AGEs (advanced glycation end-products). It forms one of the most quantitatively prevalent AGEs, MG-H1 \[N^\delta-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine\], and other AGEs such as CEL \[N^\epsilon-(1-carboxyethyl)lysine\] [15]. MG-derived AGEs have been shown to accumulate at sites of vascular complications in streptozotocin-induced diabetic rats, including renal glomeruli, retina and peripheral nerves [16]. Therapeutic agents that suppress protein glycation by MG such as aminoguanidine have been shown to prevent the development of diabetic complications in experimental settings [17,18]. However, it remains to be demonstrated whether increased exposure to MG alone, as found in diabetes, can induce diabetes-like vascular complications.

In the present study, we examined the hypothesis that prolonged exposure to MG by a systemic route would reproduce characteristics of diabetic vascular disease such as microvascular damage and a torpid cutaneous wound healing process.

**MATERIALS AND METHODS**

**Reagents**

MG (lot no. 101K2507; Sigma) was diluted in sterile water for injection and stored at 4 °C protected from light. Goat polyclonal anti-CTGF (connective tissue growth factor), rabbit polyclonal anti-TGF-β (transforming growth factor-β), goat polyclonal anti-TNF-α (tumour necrosis factor-α) and rabbit polyclonal anti-IL-1β (interleukin-1β) antibodies were purchased from Santa Cruz Biotechnology. A monoclonal anti-PCNA (proliferating cell nuclear antigen) antibody was purchased from Calbiochem, and monoclonal anti-AGE and goat polyclonal anti-RAGE (receptor for AGEs) antibodies were purchased from Research Diagnostics. An anti-TNF-α antibody blocking peptide was purchased from Santa Cruz Biotechnology.

**Animals**

Male Wistar rats weighing 250–270 g (9 weeks of age) were purchased from the National Centre for Animal Breeding (CENPALAB, Havana, Cuba). Animals were housed individually in a certified room in the animal facility at the Centre for Genetic Engineering and Biotechnology, Havana, Cuba, and were maintained under controlled environmental conditions. Rats were fed with standard laboratory rodents’ chow. Access to food and water was unrestricted. Individual daily food intake was recorded during the experiment. Animals were randomly assigned to two groups, one receiving normal saline solution and the other receiving MG.

Animal care and experimentation fulfilled the criteria for standards issued by the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**Dose regimen of MG administration**

MG was administered intraperitoneally over 5 consecutive days each week for 7 consecutive weeks. The initial dose administered was 50 mg/kg of body weight for the first 2 weeks, followed by a dose of 60 mg/kg of body weight for weeks 3 and 4, and finally a dose of 75 mg/kg of body weight for the last 3 weeks. The initial dose of 50 mg/kg of body weight was selected because it has been shown previously to induce diabetic-like renal changes after 5 months of oral administration [19]. The dose of MG was increased in a stepwise manner to a maximum of 75 mg/kg of body weight when rats appeared to be adapted to the dose in use, as judged by inspection of the general well being of the rats. The doses and administration period were established from pilot experiments which indicated that longer dosing periods induced abdominal dilation, peritoneal exudation and local fibrosis. Body weight was recorded weekly throughout the study. At the beginning of the study, six rats were killed with an overdose of ketamine to collect blood samples for biochemical analysis and to record carcass weight. Baseline serum concentrations of glucose, cholesterol, triacylglycerols (triglycerides), creatinine and fructosamine were determined. The rats were treated with saline or MG as indicated above. After 4 and 6 weeks of treatment, six
animals from each group were randomly assigned and killed. Tissue fragments from skin, liver, pancreas, spleen, kidneys and eyes were harvested. Tissue samples were fixed in 10% buffered formalin and paraffin-processed to generate 5 µm sections. Specimens were stained with H/E (haematoxylin/eosin) and selected samples were stained with Congo Red and PAS (periodate–Schiff). After 6 weeks of MG treatment, full-thickness skin wounds were created and left to develop for another 8 days when rats were killed (see below).

Biochemical determinations
Serum concentrations of glucose, total cholesterol, triacylglycerols and fructosamine were determined by colorimetric assay with diagnostic kits, according to the manufacturer’s instructions (Sigma).

Wound healing
At the end of the week 6, 12 rats from each group were conditioned to receive acute controlled full-thickness skin wounds on the dorsal area under sodium pentobarbital (30 mg/kg of body weight) anaesthesia. The dorsum of the rats was surgically prepared and four symmetric ulcers were inflicted using disposable 8 mm diameter punch biotomes (Fray). Following haemostasia, the wound contours were traced upon transparent plastic sheets for planimetric analysis. This served as the original wounded area. Wound closure dynamics were studied using the standard cutaneous round ulcer model as described previously [20]. No animals were excluded due to erroneous wounding procedures. During this period, saline or MG administration was not interrupted. Half of the rats from each group (n = 6) were killed on day 4 post-wounding and the other half on day 8 post-wounding; this latter group of rats had completed approx. 7 weeks of continuous treatment with MG or saline. Ulcers and a surrounding margin of intact skin were collected, hemisectioned, fixed in 10% buffered formalin and paraffin-processed. Wound specimens were stained with H/E, van Giesson (haematoxylin/eosin) and selected samples were stained using specimens derived from saline- and MG-treated groups. The number of darkly stained nuclei was counted in 5–8 microscope fields (×20). Only nuclei of fibroblasts and endothelial cells were counted. This was repeated three times, and the number of positive cells was averaged per group.

Vasoregulatory response to NTG (nitroglycerine)
Assessment of the vasodilatory response was studied by near infra-red photoplethysmography in six rats per group after 7 weeks of treatment using an Angiodin PD 3000 plethysmographic registering device [24]. The jugular vein was dissected and 50 µg/kg of body weight NTG was administered. The maximal digital pulse of each wave was measured under basal conditions and after the NTG challenge. Vascular changes were registered and the average of wave amplitude was calculated as described previously [24].

Immunohistochemistry
Sections (5 µm) were mounted on chromalum-coated slides and exposed for 20 min at 56 °C. Afterwards, the specimens were dewaxed, rehydrated, rinsed and washed in PBS (pH 7.4) for 30 min. Once endogenous peroxidase was quenched, the specimens were washed and treated with Target Retrieval Solution (Dako) equilibrated at 99 °C. Tissue samples were then incubated for 30 min with anti-CTGF (1:300 dilution), -TGF-β (1:250 dilution), -PCNA (1:250 dilution), -AGE (1:1000 dilution), -RAGE (1:1500 dilution), -TNF-α (1:200 dilution) and -IL-1β (1:150 dilution) antibodies in background reducing solution (Dako). Controls included the use of tissue fragments of saline-treated rats, isospecies pre-immune serum and pre-absorption with antibody-blocking peptide (for TNF-α). The immunohistochemical reactions were carried out using the labelled streptavidin/biotin–HRP (horseradish peroxidase) conjugate method (Dako) according to the manufacturer’s instructions. The peroxidase reaction was developed with DAB (diaminobenzidine) and counterstained with haematoxylin or Light Green. PCNA immunostaining was conducted to determine if MG treatment negatively impacted on the proliferative capability of granulation tissue productive cells on days 4 and 8 post-wounding using specimens derived from saline- and MG-treated groups. The number of darkly stained nuclei was counted in 5–8 microscope fields (×20). Only nuclei of fibroblasts and endothelial cells were counted. This was repeated three times, and the number of positive cells was averaged per group.

In order to compare the effects of MG with the situation in human diabetes, sections stored in the Anatomical Pathology Laboratory archive of small punch samples obtained from diabetic patients enrolled as part of a controlled randomized clinical trial conducted at the National Institute of Angiology and Vascular Surgery in 2001 were used. Biopsies from this trial were collected with the approval of the local Ethics Committee and the Cuban Regulatory Agency of the Ministry of Public Health. Informed consent was obtained from the patients.
Biochemical and physiological characteristics of rats at baseline and after 7 weeks of treatment with MG

P values were determined by ANOVA. *Significant difference compared with time 0, and †significant difference compared with saline-treated rats, as determined by Duncan’s post-hoc test.

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>Saline-treated rats</th>
<th>MG-treated rats</th>
<th>P value</th>
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<tr>
<td>Body weight (g)</td>
<td>298.7 ± 48.0</td>
<td>396.5 ± 13.3*</td>
<td>322.8 ± 33.9†</td>
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<tr>
<td>Carcass weight (g)</td>
<td>222.4 ± 50.1</td>
<td>301.0 ± 8.7*</td>
<td>207.5 ± 0.5†</td>
<td>&lt; 0.001</td>
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<td>Glycaemia (mmol/l)</td>
<td>5.44 ± 2.01</td>
<td>3.37 ± 1.00*</td>
<td>2.73 ± 1.05†</td>
<td>0.029</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.66 ± 0.39</td>
<td>1.34 ± 0.23</td>
<td>2.19 ± 0.31†</td>
<td>0.06</td>
</tr>
<tr>
<td>Triacylglycerols (g/l)</td>
<td>1.44 ± 0.32</td>
<td>0.93 ± 0.11</td>
<td>1.74 ± 0.72</td>
<td>0.08</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>39.0 ± 3.9</td>
<td>52.8 ± 7.9*</td>
<td>101.3 ± 7.7†</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>153.1 ± 29.9</td>
<td>173.2 ± 110.5*</td>
<td>327.8 ± 123.0†</td>
<td>0.014</td>
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**RESULTS**

Consequences of the systemic administration of MG on body and carcasses weights and metabolism

The administration of MG to rats induced a dramatically emaciated phenotype. MG produced a significant arrest of body weight increase compared with saline-treated rats (Table 1). The mean carcass weight decreased 6 % with respect to the baseline value and 41 % compared with saline-treated controls (Table 1). Glycaemic status was similar between the MG- and saline-treated groups (P = 0.67; Table 1). MG treatment, however, increased serum total cholesterol by 63 % compared with saline-treated animals (P = 0.03, as determined by post-hoc Duncan’s test). Although mean triacylglycerol levels were increased at the end of week 7 in the MG-treated group, there was no significant difference compared with the saline-treated counterparts (Table 1). Fructosamine levels were significantly elevated in the MG-treated group (+ 114 %) compared with the saline-treated controls (Table 1).

Microvascular and other cutaneous changes

MG-treated rats exhibited an aged cutaneous phenotype characterized by thinner skin with abundant wrinkles. The qualitative analysis of full-thickness biopsies of dorsal skin showed a significant reduction in the thickness of the dermal layers, associated with a remarkable reduction in eosin affinity, collagen bundle fragmentation and hair follicle degeneration. Most importantly, MG administration introduced severe degenerative changes in cutaneous vessels, suggesting decreased peripheral perfusion by an impaired microcirculation. By serial systematic histological analysis the following sequence of changes were suggested: (i) hypertrophy of endothelial nuclei with an enhanced haematoxylin affinity, (ii) thickening of the vessel wall, (iii) detachment of the vessel from the surrounding matrix, (iv) progressive thickening and accumulation of luminal matrix, (v) luminal occlusion, (vi) endothelial pyknosis, and finally (vii) total disappearance of the vessel. Some of these degenerative changes are shown in Figure 1. An alternative infrequent feature was a regenerative response in which pericytes and endothelial nuclei appeared near the damaged vessel, suggesting a regenerative response. Hypodermal vessels were not usually affected in intact skin biopsies. Damaged cutaneous veins and capillaries were negative to Congo Red and PAS staining.

Impaired vasodilatory response to NTG

MG-treated rats exhibited a complete failure in the vasodilatory response evoked by NTG administration (Table 2). There was no change over time in the amplitude of the photoplethysmographic signal compared with basal registrations. In saline-treated animals, NTG increased the response more than 2-fold compared with the basal level. Obvious differences existed between MG- and saline-treated groups in relation to post-NTG evoked waves (Table 2).

Impairment of the wound healing process

On the first day post-injury, wounds of MG-treated rats exhibited inflamed and leaky contours. From planimetric measurements, MG treatment suppressed the wound closure process, particularly in the acute phase. Significant
Methylglyoxal administration reproduces diabetes-like tissue changes in normoglycaemic rats

Figure 1 Cutaneous vascular changes induced by MG treatment
Normal dermal vein (A) and dermal capillary (B) from a saline-treated rat. (C) Deep dermal vein from an intact skin sample and (D) a deep dermal capillary from a diabetic patient showing degenerative changes and luminal occlusion. In (C), irreversible nuclear changes are indicated by arrows. Nuclear damage is also shown in (D). (E) Section of a dermal vein from an MG-treated rat, with eosinophilic material deposition that occludes the lumen. Note also endothelial nuclear damage representative of vascular changes in MG-treated rats. (F) A representative section from an MG-treated rat showing capillary degenerative changes similar to those seen in human diabetes. All sections were stained with H/E. Magnification, × 20.

Table 2 Effect of MG administration on the vasodilatory response of peripheral vessels and in wound angiogenesis, inflammation and cellular proliferation
Values are means ± S.E.M. *P < 0.01 compared with the MG-treated group, as determined by Student’s t test and ANOVA. †P < 0.01 compared with the MG-treated group, as determined by the Mann–Whitney U test. a.u., arbitrary units.

<table>
<thead>
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<th>Parameters</th>
<th>Saline-treated rats</th>
<th>MG-treated rats</th>
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<tbody>
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<td>Vasodilatory response (a.u.)</td>
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<tr>
<td>Pre-NTG</td>
<td>4.57 ± 1.12</td>
<td>3.75 ± 1.32</td>
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<tr>
<td>Post-NTG</td>
<td>10.96 ± 2.49*</td>
<td>3.76 ± 1.24</td>
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<td>Neovessels (n)</td>
<td>40.57 ± 8.01†</td>
<td>16.14 ± 6.19</td>
</tr>
<tr>
<td>Macrophages (n)</td>
<td>9.71 ± 2.49†</td>
<td>26.29 ± 9.57</td>
</tr>
<tr>
<td>Neutrophils (n)</td>
<td>1.21 ± 1.40†</td>
<td>63.50 ± 5.95</td>
</tr>
<tr>
<td>PCNA-positive cells (n)</td>
<td>19.50 ± 8.66†</td>
<td>8.30 ± 2.77</td>
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Differences between the two groups in relation to wound area were found on days 1, 3 and 4 (P < 0.001, as determined by ANOVA; Figure 2). On day 8 post-wounding, no statistical differences were detected, although the clinical and histological appearance of the wounds was different between the two groups. The qualitative (blinded) evaluation of the slides indicated two main critical problems in the MG ulcers: (i) a severely decreased number of infiltrating cells with fibroblastic phenotype, and (ii) an apparent failure in the ability to secrete and accumulate extracellular matrix. We observed that ulcers in MG-treated rats failed to provide actively migrating cells from peripheral regions, particularly corresponding to the reticular dermis and hypodermis, which would be committed to colonize the central region of the wound and thus the onset of the granulative process. Furthermore, histological examination suggested that wounds in MG-treated rats had decreased fibrinolysis, so that a fibrin-like material was observed in most
wounds. In addition, small vessels frequently had deposits of granular eosinophilic material as an irregular ring, which may suggest a failure in vascular wall permeability. None of these changes were found in the saline-treated group. MG also significantly reduced angiogenesis and, conversely, stimulated inflammatory cell infiltration into wounds harvested on days 4 (Table 2) and 8 post-wounding (results not shown). Histochemical staining for collagen confirmed the existence of an actual failure in collagen accumulation. Collagen fibres were scarce, thin and in loose non-meshed patterns. This poor granulative response provided an image mirroring a spider web where infiltrating cells appeared suspended (Figure 3). MG treatment also produced vascular changes during the acute phase of the wound healing process. A remarkable, but not frequent, finding was the detection of arterial wall thickening of neoformed middle-sized arteries at the deepest points of the wound. Again, none of these changes were found in the wounds from the saline-treated rats. The proliferative status of granulation tissue resident cells based on PCNA immunostaining appeared significantly impaired in the MG-treated group on day 8 post-wounding compared with their saline counterparts (Table 2).

**AGE and RAGE content of skin and granulation tissue fragments**

Anti-AGE antibodies predominantly recognize the AGEs CML (Nε-carboxymethyl-lysine) and CEL [25–27]. There was no discernible staining of AGEs in intact skin dermal vessels and cells of saline-treated control rats; a faint staining was only detected in the epidermis (Figure 4). In MG-treated rats, immunostaining for AGEs showed intense staining on most of the vascular structures of intact skin fragments. AGEs were located in neoformed vessels, fibroangiogenic productive cells and nerve fibres (Figure 4). The RAGE antigen was detected in some granulation tissue cells, including infiltrating mononuclear cells of saline-treated rats (Figure 5). In MG-treated rats, RAGE expression was detected in both superficial and deep vasculature of the skin, being particularly evident in blood vessel walls; small vessels exhibited the highest intensity (Figure 5). In some cases, co-localization of both AGEs and RAGE was found on the same vessels.

**MG imposes a pro-inflammatory phenotype in granulation tissue cells**

We examined the expression of two pro-inflammatory cytokines, TNF-α and IL-1β. Granulation tissue samples corresponding to days 4 and 8 post-injury, as well as intact skin fragments from MG- and saline-treated groups, were studied. IL-1β appeared uniformly and restrictedly expressed in the epidermis in intact healthy skin fragments of saline-injected rats (Figure 6). Staining for IL-1β appeared far more intense in the epidermal layer of the MG-treated group. In the granulation tissue of saline-treated rats, IL-1β appeared particularly restricted to endothelial cells in areas of angiogenesis, which may suggest that this cytokine is involved in vascular morphogenesis. However, the intensity of IL-1β expression by endothelial cells and mature vascular structures found in the wounds from the MG-treated group was far more intense than that in the saline-treated controls (Figure 6). No TNF-α staining in the epidermis of intact skin derived from MG- or saline-treated rats was observed (Figure 6). Granulation tissue productive cells from the saline-treated controls were devoid of TNF-α immunostaining, with the exception of some infiltrating mononuclear cells. However, a widespread and intense staining was detected in the walls of neovessels, vascular precursor cells and in fibroblasts. Overall, these data suggest that MG activated and/or enhanced the expression of these two pro-inflammatory cytokines in granulation tissue cells. Small- and middle-calibre neoformed arteries at the bottom and edges of the ulcerated tissue exhibited mild arterial wall thickening in the MG-treated group. The medial layer of these arteries had a plethora of smooth muscle cells.
Methylglyoxal administration reproduces diabetes-like tissue changes in normoglycaemic rats

Figure 4  AGE expression in granulation tissue
Immunostaining of granulation tissue from (A) a saline-treated rat and (B) an MG-treated rat day 8 post-wounding with an anti-AGE antibody and counterstained with Light Green. Representative sections are shown. In (A), no AGE immunolabel was detectable. In (B), an intense immunostaining of the vascular walls (indicated by arrows) within a nerve fibre (indicated by an asterisk), as in most of the granulation tissue populating cells, is shown. (C) A granulation tissue fragment from a diabetic patient showing a similar pattern of AGE staining, particularly on the vascular structures, as found in MG-treated rats. Magnification, × 20.

with no inflammatory cell infiltration and no evidence of hyalinization. By contrast, arterial thickening was not detected in any situation in the saline-treated group. Immunohistochemistry showed a clear CTGF expression on the medial layer of arteries and in perivascular cells, possibly pericytes (Figure 7). Endothelial nuclei were not stained. Specimens incubated with anti-TGF-β antibodies did not label arterial walls in any of the samples.

Other changes associated with systemic administration of MG: effect in renal and retinal tissues
Mild glomerular mesangial expansion in rats after 7 weeks of MG treatment was confirmed after PAS staining.

Figure 5  RAGE expression in the neodermis
Immunostaining of the granulation tissue from (A) a saline-treated and (B) an MG-treated rat with an anti-RAGE antibody, followed by haematoxylin counterstaining. Representative sections are shown. Only few isolated cells appeared to be stained with the anti-RAGE antibody in controls (A), whereas RAGE was preferentially expressed by vascular walls and surrounding cells following MG treatment (B). (C) A representative granulation tissue fragment from a diabetic patient showing RAGE expression on vascular structures. Original magnification, × 20.

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Figure 6  Pro-inflammatory phenotype in granulation tissue induced by MG treatment

(A) Epidermis of a saline-treated rat showing constitutive IL-1β expression by normal epidermal cells. (B) Epidermis of an MG-treated rat showing a noticeable increase of IL-1β expression by normal epidermal cells. (C) A granulation tissue fragment from a saline-treated rat on day 8 post-injury incubated with an anti-IL-1β antibody. Cytokine expression was restricted to vascular precursor cells, but not in mature vascular structures. (D) A granulation tissue fragment from an MG-treated rat showing that IL-1β is widely expressed, including on vascular structures and granulation tissue cells. (E) A section from a saline-treated rat demonstrating that TNF-α was not expressed by normal granulation tissue components. (F) A section from an MG-treated rat showing a widespread expression of TNF-α in granulation tissue involving cells and vascular structures. Representative sections are shown. Original magnification, × 20. Sections were stained with haematoxylin and counterstained with Light Green.

(results not shown). Tubular epithelial cells exhibited ballooning, necrosis or tubular basal membrane detachment. There was accumulation of amyloid material positive for Congo Red staining the intima of small- and middle-calibre renal arteries (Figure 8). Samples from the saline-treated controls were free of renal damage and negative for both PAS and Congo Red staining. Consistent with these changes, the serum creatinine concentration increased 3-fold in MG-treated rats compared with saline controls (Table 1). Creatinine progressively accumulated from week 4 of MG treatment onwards. Renal glomeruli from the MG-treated group exhibiting mesangial expansion showed no differences in CTGF expression compared with saline samples. In contrast, there was a clear and defined increase in immunohistochemical staining for TGF-β in the glomerular tuft of MG-treated rats compared with saline-treated controls (Figure 9). There was also some focal immunostaining of tubular epithelial cells and interstitial endothelial cells to TGF-β. No TGF-β immunostaining was detected in salinetreated controls (Figure 9).

MG administration induced dilation of retinal capillaries with abundant perivascular oedema. Loss of neuronal nuclei alternating with a degenerative appearance and pyknosis were frequently observed (Figure 10). AGE epitopes were identified in most of the resident cells as in capillary walls in retinas of MG-treated animals (results not shown).

DISCUSSION

In the present study, we have shown concurrent evidence of two independent and extemporaneous experiments that converge to indicate that, when MG is systemically administered to rats, it imposes a metabolic burden which leads to systemic and local disorders involving biochemical dysfunction, histopathological damage and
Methylglyoxal administration reproduces diabetes-like tissue changes in normoglycaemic rats

Figure 7  Arterial thickening and CTGF expression induced by MG treatment
(A) The media of deep cutaneous arteries in an MG-treated rat stained with H/E showing mild thickening. (B) In a saline-treated rat, anti-CTGF immunohistochemistry demonstrated a faint expression in vascular precursor cells in areas of active angiogenesis. Vessel walls did not express the growth factor. (C) In MG-treated rats, a remarkable CTGF overexpression was observed in small arteries walls and in some cells within the angiogenesis foci. Asterisks indicate the sites of major immunostaining. In (B and C), sections were counterstained with haematoxylin. Representative sections are shown. Magnification, ×20.

Figure 8  Amyloid deposition associated with prolonged MG administration
Renal arteries in saline-treated (A) and MG-treated (B) rats stained with Congo Red are shown. No amyloid deposits were detected in (A), whereas vascular amyloid deposits were observed in (B). Representative sections are shown. Original magnification, ×20.

perturbation in organ physiology. Although caution must be exercised with regard to the wound healing data, due to differences between human and rodent skin biology, our experiments provide repeated evidence regarding the deleterious participation of MG in the consolidation of acute events within the wound resolution mechanism that somehow mirrored diabetic tissue repair disorders. Although one of the limitations of the present study is that we did not measure plasma or urinary levels of AGEs, we provide a characterization of MG-derived complications in different diabetes target organs and tissues and, in some of these, AGE deposits and RAGE overexpression are demonstrated. The effects of MG may therefore result from its direct toxicity and/or from the interaction between MG-modified proteins via a cellular receptor (i.e. RAGE). The phenotype exhibited by the rats in the present study, having attributes of human and experimental diabetes mellitus, uraemia and aging, was obtained following different dose-response pilot experiments. These pilot studies indicated that an MG dose of 50–75 mg·kg⁻¹·day⁻¹ of body weight administered using a scale-up system was required to obtain the required pathology and to prevent any lethal effects of MG on the animals. The dose of MG used in the present study, which is far lower than the reported lethal one, is approx. 6–9 times the in situ rate of MG formation under normal conditions and 3–5 times higher than the estimated flux in clinical diabetes [28,29]. Although recent studies have described the cytotoxic effects of MG on cultured pancreatic β-cells [30], the histopathological analysis of MG-treated rats in the present study indicated that the pancreas was not affected. This is supported by the finding that all the MG-induced lesions developed in a euglycaemic environment, even after 7 weeks of MG administration. Hyperglycaemia has been induced by higher doses of MG in rabbits (200 mg/kg of body weight), but this may be due to β-cell toxicity or inhibition of glucose-induced insulin secretion [30,31]. From the results of the present study, it appears that high doses of MG exert significant changes in cholesterolaemia, vasodilation and peripheral vascular and tissue function. It is likely that, under the experimental conditions used in the present study, modification of proteins and nucleosides by MG may be 6–9-fold higher than in controls, deduced from a predicted 9-fold increase in the mean MG-derived content as
Renal expression of TGF-β in glomeruli (A and B) and tubular epithelium (C and D) induced by MG treatment

Renal tissue from saline-treated (A and C) and MG-treated (B and D) rats stained with an anti-TGF-β antibody and counterstained with haematoxylin are shown. There was no evidence of TGF-β expression within the glomerular area (A) or in tubular epithelial cells (C) in saline-treated rats. MG induced a marked expression of TGF-β within the glomerular tuft (B) and in tubular epithelial cells (D). Representative sections are shown. Original magnification × 20.

reported previously [16]. This is expected to have significant negative effects on protein structure and function, with increased cellular proteolysis. The presence of increased ‘fructosamine’ levels in the serum of MG-treated rats may reflect the modification of plasma proteins by MG, since serum glucose concentrations were not increased.

Remarkably, MG administration induced a significant elevation of serum total cholesterol; however, increased triacylglycerol levels in the MG-treated group were not significant. The mechanisms underlying this effect are unknown, but modification of lipoproteins by MG may impair processing at peripheral and hepatic sites [32,33]. Hyperlipidaemia is a common feature of diabetes (particularly Type II diabetes) and uraemia. A direct or indirect pathogenic consequence of prolonged MG administration was a shift towards premature aging of the animals, as they developed an emaciated and wasted phenotype involving the whole body and, particularly, the carcass. Cachexia was associated with a loss of adipose tissue and a reduction in muscle mass, along with a decrease in skeletal compactness (as determined by radiological examination). Although MG-treated animals maintained normal food intake, the possibility that MG may have a deleterious impact on the GH (growth hormone)/IGF-1 (insulin-like growth factor-1) axis cannot be excluded. In this regard, additional studies appear justified. The fact that vascular structures of intact skin from MG-treated animals turned into an additional endogenous source of TNF-α, thus leading to the onset of a local pro-inflammatory environment, is an exciting finding, which may provide a partial explanation of the dermal damage detected in the present study. Recent evidence confirms that endothelial cells exposed to different types of AGEs in culture express and secrete TNF-α and show reduced eNOS (endothelial nitric oxide synthase) synthesis, thus contributing to the vasculopathic process common to diabetes, uraemia and aging [34]. In this context, the role played by ALEs (advanced lipoxidation end-products) in catalysing AGE formation/accumulation and thus in inducing TNF-α expression cannot be excluded [35]. The cutaneous microangiopathy described in the present study in non-wounded skin appeared particularly biased towards small dermal vessels, developing from a thickened and degenerated wall to complete luminal obliteration. Interestingly, immunohistochemical localization of AGEs, RAGE and occasionally of TNF-α appeared similar and corresponded with the severity of the degenerated vessels. Increased cellular proteolysis and activation of a pro-inflammatory axis via AGEs–RAGE interaction
Damage to retinal tissue induced by MG treatment, pyknosis and neuronal loss. Representative sections are shown. Normal architecture of the retina cell layers is seen in (A), whereas, in (B), changes induced by MG treatment include focal oedema, capillary sacular dilation (indicated by the arrow), nuclear pyknosis and neuronal loss. Representative sections are shown. Original magnification, × 10.

Figure 10 Damage to retinal tissue induced by MG treatment

Histological sections of retinal tissue from a saline-treated (A) and an MG-treated (B) rat stained with H/E (5 µm section) are shown. Normal architecture of the retina cell layers is seen in (A), whereas, in (B), changes induced by MG treatment include focal oedema, capillary sacular dilation (indicated by the arrow), nuclear pyknosis and neuronal loss. Representative sections are shown. Original magnification, × 10.

[36] may mediate the course of the vascular degenerative process. Other proteins which invoke RAGE pro-inflammatory responses, such as S100/calgranulins [37] or HMGB1 (high-mobility group box-1) [38], may be linked to MG-induced vascular cell damage.

An impaired wound healing response in diabetes is linked to the development of chronic ulcers, leading to foot and limb amputations in severe cases. Wounds derived from the MG-treated group exhibited torpid granulation, reduced neo-angiogenesis and prolonged inflammation. All of these features are common to human diabetic ulcers. Although the catabolic status of the animals may have had a negative impact on the healing process, the hypothesis of a direct consequence of MG administration is more tenable as, when wounds are topically exposed to a solution of MG, the healing events appear far more marked in the wounds from MG-treated animals than in their saline counterparts.

The insufficient granulative response described in the MG-treated group may be linked to a local pro-inflammatory and pro-degradative milieu in which TNF-α, IL-1 and MMPs (matrix metalloproteinases) may act in concert to impair the healing process [41]. TNF-α decreases the formation and tensile strength of granulation tissue possibly by enhancing the generation of activated forms of metalloproteinases; actions that are partially mediated via IL-1 [42]. The pro-inflammatory and pro-oxidative environment imposed on the wound bed by MG may also have affected the replicative capabilities of granulation tissue cells as indicated by PCNA immunolabelling. A direct cytotoxic effect exerted on such cells by MG may not be ruled out. Alternatively, glycosylation of growth factor receptors may have abrogated signalling pathways involved in cell proliferation [43] within the proliferative phase of the healing process.

Taken together, these findings suggest that MG may temporarily disrupt the pro-anabolic balance of granulation tissue, the recruitment and proliferation of fibroblasts and, thus, the organization and scaffolding of an appropriately vascularized granulation tissue.

In comparison with wounds from control animals, in which there was no evidence of medial layer thickening, a moderate arterial thickening was detected in some granulation tissue samples from MG-treated rats retrieved on day 8 post-wounding. TGF-β and CTGF are two potent pro-angiogenic and pro-matrix expansion growth factors, but only the latter appeared overexpressed in the same zones of medial layer expansion, which may validate and/or expand recent in vitro findings demonstrating that AGEs increase CTGF expression in a time- and dose-dependent manner [44]. The potential pathogenic interaction between hyperlipidaemia, arterial wall thickening and CTGF overexpression during the atherosclerotic process in the context of MG-induced disturbances merits additional and more detailed studies.

In another set of experiments, the functional effects of MG on the vasculature were investigated. A complete impairment in the vascular relaxation mechanism was observed in MG-treated rats. Decreased perfusion of subcutaneous tissue may be a further consequence of MG toxicity, possibly associated with concomitant luminal obliteration and/or dysfunctional endothelial-dependent regulation of vascular tone. Increased accumulation of AGEs has been linked to scavenging of NOS (nitric oxide synthase) [45], and MG-derived AGEs may be implicated in a decreased NTG vasodilatory response.
A previous study in which MG was given orally to mice for 5 months was the first indication that administration of exogenous MG may induce renal pathology similar to that found in diabetic nephropathy [19]. In the present study, we detected mild PAS-positive staining associated with mesangial matrix expansion and glomerular hypocellularity in the MG-treated group, which was associated with progressive creatinine retention. The glomerular TGF-β immunoreactivity found in the samples from MG-treated animals may be responsible for the mesangial extracellular matrix accumulation via PAI-1 (plasminogen activator inhibitor-1) activation [46]. Recent evidence indicates that the downstream effector for this process is CTGF [47]. It is difficult to explain, therefore, why there was no evidence of increased CTGF expression in the MG-treated group compared with the saline controls. As a corollary of renal damage, amyloid deposits were present in the vasculature of all MG-treated rats at the end of the experiment. Glycoxidative modification is associated with both aging and diabetes and appears to lead to the renal deposition of amyloid material [48]. Formation of AGEs is involved in the onset of amyloidosis and related complications [49]. In addition, amyloid is also a ligand for RAGE, and RAGE is known as a propagation factor in amyloidogenesis [50].

Retinopathy, including retinal microvascular damage, is a devastating complication of human diabetes associated with increased MG concentrations [11]. In the present study, we have demonstrated that MG administration also induced retinal damage, which appeared to correspond closely with the overexpression of AGE and RAGE in situ. As retinal damage in the rats appeared in a normoglycemic background, it is likely that this complication may be solely attributed to MG-derived effects [16].

In conclusion, our data provides further support for the notion that increased MG levels in diabetes has the potential to induce vascular damage and contribute to the development of diabetic complications. Although further studies are warranted to fully elucidate the direct or indirect participation of the exogenously administered MG on the onset of diabetic microvascular complications, therapeutic interventions leading to the inhibition of the formation of AGEs and blockade of the AGE–RAGE interaction appear once again to be a promising tool to reduce vascular and extra-vascular tissue complications in diabetes.

REFERENCES


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Methylglyoxal administration reproduces diabetes-like tissue changes in normoglycaemic rats


Received 19 January 2005/28 February 2005; accepted 9 March 2005
Published as Immediate Publication 9 March 2005, DOI 10.1042/CS20050026

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