Cardiac inflammation associated with a Western diet is mediated via activation of RAGE by AGEs

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Some of the pathogenic effects of AGEs appear to be mediated by interactions with AGE receptors (33), of which the receptor for AGEs (RAGE) is the best characterized (13). The expression of RAGE is upregulated in diabetes, aging, and other conditions associated with elevated AGE levels, where it is strongly associated with impaired cardiac function (26). Activation of RAGE is known to influence myocardial calcium homeostasis (22) and contribute to interstitial fibrogenesis in the diabetic heart (6). RAGE is also involved in the activation of inflammatory cascades, including the production of cytokines and chemokines (13). The present study demonstrates the pivotal role of the AGE-RAGE axis in cardiac disease associated with a high-fat diet and the cardioprotective actions of the AGE-reducing agent alagebrium chloride.

MATERIALS AND METHODS

Animals. RAGE knockout (KO) mice were generated on a C57Bl6 background as previously described (15). Briefly, essential elements encoding the extracellular domain of RAGE (exons 2-7) were flanked by two loxP sites in the same orientation. Mice heterozygous for Cre recombination were bred to homozygosity to generate the RAGE−/− mouse line. To confirm the abrogation of RAGE expression, different primers covering the entire RAGE molecule were used and detected no transcripts (data not shown).

Adult (8-wk-old) male C57Bl6J mice (Precinct Animal Centre, Melbourne, Australia) and RAGE KO mice were pair fed with either a standard diet (SD, AIN-93G; Specialty feeds, Perth Australia; n = 10/group). Both diets contained the same vitamin and total calorie content; however, a range of different strategies, prevents diabetes- and age-associated increases in myocardial stiffness (6, 7, 9, 16, 19).

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including carboxymethyllysine (65 nmol CML·mL⁻¹·100 mg chow⁻¹) compared with standard chow (21 nmol CML·mL⁻¹·100 mg chow⁻¹). Animals receiving a HF diet were further randomized to receive the AGE inhibitor, alagebrum chloride (Synvista, NJ) at a dose of 1 mg·kg⁻¹·day⁻¹ delivered by oral gavage. All groups were followed for 16 wk. All animal protocols were approved by the Institutional Animal Care and Use Committee and followed US National Institutes of Health guidelines.

Assessment of myocardial inflammation. The expression of inflammatory cytokines, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), along with macrophage infiltration, is augmented following cardiac stress. In our study, the gene expression of IL-6, TNF-α, the intercellular adhesion molecule ICAM-1, and the chemokine macrophage chemotactant factor (MCP-1) in left ventricular homogenates were assessed by real-time quantitative RT-PCR. The myocardial expression of IL-6 protein was further quantitated by ELISA (R&D Systems, Minneapolis, MN).

Assessment of superoxide production. The production and accumulation of superoxide and other reactive oxygen species (ROS) are key mediators of cardiac injury and cellular dysfunction associated with HF feeding (4). Superoxide production was assessed as previously described (11). Briefly, left ventricular tissues was rapidly excised and homogenized in oxygen-saturated Krebs buffer (containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 7 mM CaCl₂, 1.2 mM KH₂PO₄, 11 mM d-glucose, 0.03 mM EDTA, and 2.5 mM CaCl₂, pH 7.4). Fresh homogenates were then divided to determine superoxide production in the presence of 125 μM NADH and 30 μM of the NADPH oxidase inhibitor diphenylene iodinium (designated NADH-dependent superoxide production) and superoxide production in the presence of 125 μM NADPH and 30 μM of the respiratory chain inhibitor rotenone (designated NADPH-dependent superoxide production). This latter activity was not inhibited by 1-N⁵-nitrosoarginine or allopurinol (data not shown). Each of the samples was processed in triplicate following incubation for 60 min at 37°C. The rate of superoxide anion formation was determined by the addition of 3.8 μM lucigenin (bis-N-methylacridinium nitrate, Sigma Chemical). Chemiluminescence was monitored every 6 min for 60 min, and the integral over this period was expressed as relative light units (RLUs) normalized to 10 mg of tissue weight. The expression of the NADPH subunits, NOX2 (gp91phox), p47phox, NOX-4, and RAC-1 in left ventricular homogenates, along with the expression of heme oxygenase (HO) and glutathione peroxidases (GPX-1, GPX-3), key defence mechanisms against oxidative stress, were further assessed by real-time quantitative RT-PCR, as detailed above.

Assessment of cardiac lipid metabolism. Changes in myocardial energy metabolism, due to altered substrate handling, characterize diabetes and obesity-related cardiac disease (4). In particular, intramyocardial accumulation of triacylglycerol metabolites mediated by activation of peroxisome proliferator-activated receptor-α (PPARα) has been strongly associated with cardiac dysfunction in rodents fed a HF diet (20). To assess the interaction of AGE/RAGE with lipotoxicity pathways in this model, the ventricular expression of PPARα and the fatty acid translocase CD36 were assessed by real-time quantitative RT-PCR. Intramyocardial accumulation of neutral lipids was assessed by staining with Oil red-O in frozen cardiac sections.

Accumulation of RAGE ligands and AGE receptor expression. Cardiac levels of AGES were estimated using an indirect in-house ELISA, as previously described (11), with a monoclonal AGE antibody that recognizes the nonfluorescent AGE carboxymethyl-lysine (CML) at its primary epitope. The expression of the non-AGE RAGE ligand S100 A8/A9 (calprotectin, MRP 8/14) (12) was also assessed by commercial ELISA (R&D Systems). The expression of RAGE, together with the AGE receptors AGE-R1 and AGE-R3 were further assessed by real-time quantitative RT-PCR.

Statistical analysis. Continuous data are expressed as means ± SE except where otherwise specified. Differences in continuous variables were compared using Student’s t-tests (2 groups) or one-way ANOVA (3 or more groups). Spearman rank order correlation was used to analyze associations between continuous variables. Differences in categorical variables were compared using the Mann-Whitney rank sum test. A P value of <0.05 was considered statistically significant.
RESULTS

Physiological parameters. Feeding with the HF diet resulted in a significant increase in body weight, in both C57Bl6J and RAGE KO mice, compared with animals fed the SD diet (Table 1). Treatment with alagebrium chloride, modestly reduced weight gain associated with a Western diet but did not return it to control levels in either C57Bl6J or RAGE KO mice. This effect on weight was not due to differences in food intake, which were unaffected by the addition of alagebrium chloride (data not shown). Blood lipid levels were significantly increased in wild-type mice fed the HF diet (total cholesterol 3.0 ± 0.2 mM) compared with animals fed the SD diet (1.5 ± 0.3 mM, P < 0.01). A similar elevation was observed in RAGE KO mice (SD 1.7 ± 0.2 mM, HF 3.4 ± 0.3 mM, P < 0.01), with no significant difference observed between C57Bl6J and RAGE KO mice. Fifteen weeks of HF feeding did not influence systolic blood pressure levels in C57Bl6J or RAGE KO mice, and the systolic blood pressure was not modified in mice treated with alagebrium chloride (data not shown).

Cardiac hypertrophy in HF-fed mice. There was no significant change in the surface area adjusted total cardiac or isolated left ventricular mass in C57Bl6J or RAGE KO mice following 15 wk of feeding with a HF diet (Table 1). However, the cross-sectional area of cardiomyocytes in the left ventricular free wall was significantly increased following feeding with a HF diet in C57Bl6J mice (Table 1). In addition, the cardiac expression of β-myosin heavy chain was elevated following 15 wk of HF feeding in C57Bl6J, whereas the expression of α-myosin heavy chain was unaltered (Table 2). HF feeding RAGE KO mice failed to induce cardiomyocyte hypertrophy (Table 1) or increase the expression of β-myosin heavy chain gene (Table 2). Treatment with alagebrium chloride had no effect on hypertrophy or the expression of β-myosin heavy chain gene in HF-fed animals but reduced the expression of α-myosin heavy chain in both wild-type and RAGE KO animals.

Cardiac fibrosis in HF-fed mice. Previous studies have shown that diabetes is associated with an increase in intramyocardial fibrosis, which can be prevented by inhibition of AGE accumulation (6). In our study, feeding a HF diet also resulted in a significant increase in the gene expression of collagen I and collagen IV in C57Bl6J mice (Table 2). Untreated RAGE KO mice had a lower baseline level of collagen I and collagen IV gene expression than untreated C57 controls. However, HF feeding in these animals failed to significantly increase cardiac collagen expression beyond that seen in animals receiving a normal diet (Table 2). The differences were confirmed with significant interstitial collagen deposition in HF-fed mice (Fig. 1), which was reduced in RAGE KO animals fed the same diet (Fig. 1). However, collagen deposition was unaffected by treatment with alagebrium chloride in HF-fed animals.

Myocardial inflammation in HF-fed mice. Myocardial inflammation is significantly increased in animals fed a HF diet. In our study, 15 wk of HF feeding resulted in a significant increase in the gene expression of IL-6 and TNF-α in C57Bl6J mice (Table 2). This was associated with a significant increase in myocardial IL-6 expression at a protein level (Fig. 2). Untreated RAGE KO mice had reduced expression of inflammatory cytokines compared with untreated C57Bl6J controls (Table 2 and Fig. 2). In addition, HF feeding in RAGE KO animals failed to induce cardiac inflammation. Indeed, there was a paradoxical decline in the expression of these inflammatory cytokines following HF feeding compared with RAGE KO mice receiving a SD low in AGES. The increase in these cytokines in wild-type fed a HF diet was also reduced by alagebrium chloride, although this agent had no significant effect in RAGE KO mice.

Cardiac expressions of MCP-1 and ICAM-1 were also increased in C57Bl6J mice fed a HF diet. Following the same trend observed for inflammatory cytokines, the expressions of both ICAM-1 and MCP-1 were significantly reduced in RAGE KO mice (Table 2). Treatment with the AGE inhibitor alagebrium chloride also reduced the expression of ICAM-1 and MCP-1 in both wild-type and RAGE KO mice.

It is known that activation of RAGE triggers the activation of inflammatory pathways via NF-κB. In this study, the HF feeding significantly increased NF-κB p65 mRNA levels (Table 2). This increase was prevented in animals treated with alagebrium chloride. Expression of p65 and signaling through NF-κB-dependent pathways was downregulated in RAGE KO mice, consistent with previous reports in these animals (15).

Myocardial AGE accumulation and receptor expression in HF-fed mice. HF feeding was associated with a significant increase in the intake of AGES (4 μmol CML-lysine−1·day−1) compared with a normal chow diet (1 μmol CML-lysine−1·day−1). This resulted in a small but significant increase in cardiac AGE accumulation, as demonstrated by ELISA for CML-modified protein (Fig. 3). RAGE KO mice fed a SD diet had a similar level of cardiac CML-AGE to wild-type animals. However, HF feeding in RAGE KO mice was associated with a paradoxical reduction in the AGE content of cardiac tissues. AGE accumulation was also markedly reduced following treatment with the AGE inhibitor alagebrium chloride in both wild-type and RAGE KO mice.

HF feeding was also associated with a significant accumulation of the non-AGE RAGE ligand S100 A8/A9 (Fig. 3), correlating with changes in other inflammatory cytokines, as detailed above. This increase in S100 A8/A9 following HF feeding, was also attenuated in RAGE KO mice and in animals

<table>
<thead>
<tr>
<th>Table 1. Biophysical parameters in study groups</th>
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<tr>
<td>Wild Type</td>
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<tr>
<td>SD</td>
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<td>Weight gain, %baseline</td>
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<td>Total cardiac mass, g/m²</td>
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<tr>
<td>Total LV mass, g/m²</td>
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<td>Mean myocyte diameter, μm</td>
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Values are means ± SE. RAGE, receptor for advanced glycation end products; LV, left ventricle; KO, knockout; SD, standard diet; HF, high-fat diet; AL, alagebrium chloride. *Significant difference vs. wild-type SD; # vs. wild-type HF, P < 0.05.
receiving alagebrium chloride. Interestingly, basal levels of S100 A8/A9 were increased in the RAGE KO mice compared with wild-type animals receiving a normal diet (Fig. 3).

Paralleling changes in RAGE ligands, the gene and protein expression of RAGE was significantly increased in HF-fed C57Bl6J mice compared with mice fed a SD diet (Table 2). This increase in RAGE expression was prevented in C57Bl6J mice treated with alagebrium chloride (Table 2). There was no detectable RAGE expression in RAGE KO mice. Neither a HF diet nor alagebrium influenced the cardiac expression of the AGE receptors AGE-R1 and AGE-R3, and there was no difference in the cardiac expression of AGE-R1 or AGE-R3 between RAGE KO mice and C57Bl6J mice (data not shown).

Superoxide production in HF-fed mice. HF feeding was associated with a significant increase in NADPH-dependent (mitochondrial) superoxide production in cardiac homogenates from wild-type mice (Fig. 4). However, HF feeding in RAGE KO mice failed to increase mitochondrial superoxide production in cardiac homogenates. Treatment with the AGE inhibitor alagebrium chloride also significantly reduced mitochondrial superoxide production in both wild-type and RAGE KO mice.

With our detection methods, NADPH-dependent superoxide production was not altered by a HF diet or in RAGE KO mice (Fig. 4). However, cardiac expression of the NADPH oxidase subunit gp91phox (Nox-2) was increased in wild-type mice fed a HF diet. This increase was not observed in RAGE KO animals or in animals treated with alagebrium chloride. Expression of other NADPH subunits, such as p47phox, Nox-4, and rac-1, were not altered by HF feeding (data not shown). However, cardiac expression of the rac-1 gene was lower in RAGE KO mice (Table 2).

A HF diet also resulted in a decline in the expression of heme oxygenase (HO-1), a key protector against superoxide-dependent injury in the heart (1). Interestingly, this decline was observed in both C57Bl6J and RAGE KO mice. HO-1 expression was not influenced by treatment with alagebrium chloride (Table 2). The expression of the antioxidant enzymes GPX-1 and GPX-3 were not affected by feeding with a HF diet (data not shown).

Cardiac lipid metabolism in HF-fed mice. Diet-associated dyslipidemia following HF feeding was also associated with the intramyocardial accumulation of neutral lipid particles, as demonstrated by Oil-red O staining (Fig. 1). Lipid accumulation was similar in wild-type and RAGE KO mice and unaffected by treatment with alagebrium chloride. Similarly, the fat-dependent induction of PPARα was similar in wild-type and RAGE KO mice fed a HF diet (Table 2). However, treatment with the AGE inhibitor alagebrium chloride reduced the expression of PPARα in both wild-type and RAGE KO animals without modifying fat accumulation. Gene expression of the fatty acid translocase CD36 was not altered by HF feeding and was similar in wild-type and RAGE KO mice and following treatment with alagebrium chloride (data not shown).

DISCUSSION

The Western diet has a range of adverse effects on the heart, including enhanced inflammation, hypertrophy (2), fibrosis (2), and contractile dysfunction (21, 32). Such actions have been ascribed largely to the fat content of such diets. However, processed diets that are high in fat are also high in AGEs (29). Moreover, we have shown by two different strategies, inhibition of AGE accumulation following treatment with alagebrium chloride and prevention of RAGE activation in RAGE KO animals, are able to prevent the induction of inflammation, oxidative stress, and mitochondrial dysfunction in the heart associated with HF feeding. These data suggest an important role for the AGE/RAGE axis in cardiac disease associated with a Western diet.

Recent research on AGEs has focused largely on their pathogenic role in the development and progression of diabetic

Table 2. Expression of mediators of cardiac dysfunction associated with HF diet in C57Bl6J and RAGE KO mice, measured by real-time RT-PCR

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<th>Gene Expression (AU/18 s)</th>
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<td></td>
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<td>HF</td>
<td>HF + AL</td>
<td>SD</td>
<td>HF</td>
<td>HF + AL</td>
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<td>ND</td>
<td>ND</td>
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<td>0.8±0.1</td>
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<td>0.6±0.2#</td>
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<td>1.3±0.3#</td>
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<td>0.4±0.1*</td>
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<td>0.5±0.1*</td>
<td>0.5±0.1*</td>
<td>0.3±0.1*</td>
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<td>0.8±0.2*</td>
<td>0.5±0.1*</td>
<td>0.5±0.1*</td>
<td>0.3±0.1*</td>
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<td>0.7±0.1*</td>
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<td>0.7±0.1*</td>
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<td>gp91phox</td>
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<td>1.9±0.4*</td>
<td>1.0±0.2</td>
<td>3.0±0.6*</td>
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<td>RAC-1</td>
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<td>HO</td>
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<td>PPARα</td>
<td>1.0±0.1</td>
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Values are means ± SE. MHC, myosin heavy chain; ICAM, intercellular adhesion molecule; MCP, macrophage chemoattractant factor; HO, heme oxygenase; ND, not detected. *Significant difference vs. wild-type SD; # vs. wild-type HF, P < 0.05.
complications, as chronic hyperglycaemia leads to augmented AGE accumulation in vivo (5). However, AGEs are able to induce end-organ damage in the absence of hyperglycemia (24, 27), and indeed, inhibitors of AGE accumulation are able to prevent organ injury in diabetes without normalizing glucose levels (6). Consequently, other states in which AGEs are elevated may also see the development of “diabetes-like” AGE-induced injury, as well as being able to benefit from inhibition of the AGE/RAGE axis. For example, inhibition of cardiac AGE accumulation in the aging heart reduces myocardial damage (6, 7, 9, 16, 19). Similarly, reducing the amount of AGEs in the diet lowers markers of systemic inflammation in humans (30). Consistent with these findings, in mice pair fed a HF diet, another state associated with high exposure to AGEs, we have now shown that inhibition of AGE accumulation and/or signaling via the RAGE receptor also reduces myocardial inflammation, hypertrophy, and oxidative stress.

Fig. 1. A Western high-fat diet (HF) was associated with increased accumulation of interstitial collagen, as demonstrated by picrosirius staining (column 1); increased perivascular collagen, as demonstrated on Van-Giesen stained sections (column 2); and expression of collagen IV (column 3). Collagen accumulation associated with HF feeding was reduced in RAGE knockout (KO) mice (HF+KO, bottom row) and in mice receiving alegiberium chloride (HF+AL, 3rd row). Cardiac fat accumulation was increased in fat-fed mice, as demonstrated by staining with Oil red O (right column 4). This increase was not altered in RAGE KO mice and mice receiving alegiberium chloride.

Fig. 2. Cardiac expression of IL-6 protein, as measured by ELISA. *Significant difference vs. wild-type standard diet (SD); # vs. wild-type HF, P < 0.05.
It is known that activation of RAGE by AGEs and other non-RAGE ligands triggers the activation of secondary messenger pathways, such as NF-κB and oxidative stress, both mechanisms that have been strongly implicated in the development and progression of cardiac disease (4). In particular, in this study we show that a diet high in fat (and AGEs) produced sustained NF-κB activation and upregulation of NF-κB p65 and NF-κB-dependent signaling pathways such as the expression of MCP-1. ROS produced by the mitochondrial respiratory chain have been described as a major mediator of fat, AGE, and hyperglycemia-dependent NF-κB activation (4, 18). Indeed, in our fat-fed animals, mitochondrial superoxide production was significantly increased in the heart. This increase was prevented in animals treated with the AGE inhibitor alagebrium chloride or in RAGE KO animals. Although no detectable change in NADPH-dependent oxidase activity in any of the groups was detected, studies in endothelial (31) and smooth muscle cells (25) and in the kidney (10) have demonstrated that activation of RAGE by AGEs stimulates NADPH oxidase activity. However, cardiac expression of the NADPH oxidase subunit gp91phox (NOX-2) was increased in wild-type mice fed a HF diet, and normalized following treatment with alagebrium chloride and in RAGE KO animals.

HF feeding in wild-type animals resulted in a significant increase in the various markers of cardiac inflammation, including IL-6, TNF-α, and ICAM-1 (Table 2). This response to HF-feeding was prevented in RAGE KO animals, which, indeed, showed a paradoxical reduction in inflammation with HF feeding. One possible explanation for this phenomenon may be unopposed activation of anti-inflammatory pathways by AGEs in RAGE KO mice. For example, it is known that AGEs are able to bind and activate a number of different receptors other

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**Fig. 3.** Quantification of RAGE ligands, CML-modified protein, and S100 A8/A9 (calprotectin, MRP 8/14) by ELISA in cardiac homogenates. *Significant difference vs. wild-type SD; # vs. wild-type HF, $P < 0.05$.  

**Fig. 4.** NADH-dependent (mitochondrial) superoxide production and NADPH-dependent (cytosolic) superoxide production in cardiac homogenates. *Significant difference vs. wild-type SD; # vs. wild-type HF, $P < 0.05$.  

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than RAGE, including AGE-R1 (p60), AGE-R3 (galectin-3) (14), and lysozyme, all of which have anti-inflammatory properties. Although the cardiac expression of these AGE receptors was not altered in RAGE KO animals, their preferential activation in the setting of RAGE deficiency may have contributed to observed anti-inflammatory actions of a HF diet. Cardiac inflammation may also have been attenuated by the reduction in cardiac AGE levels observed in HF-fed RAGE KO mice (Fig. 3). The underlying mechanism of this paradoxical change remains to be established; however, it may be, in part, an epiphenomenon, as inflammation leads to the generation of AGEs, and anti-inflammatory strategies have been shown to reduce cardiac AGE accumulation (23).

In summary, AGEs represent important mediators of cardiac injury associated with a Western fast-food diet. Inhibition of AGE accumulation following treatment with alagebrium chloride and prevention of RAGE activation in knockout animals are both able to prevent the induction of inflammation, oxidative stress, and mitochondrial dysfunction in the heart associated with HF feeding. The identification of the key role of the AGE/RAGE axis in the response to HF feeding, provides a strong rationale for both dietary and pharmacological strategies directed toward this axis in the prevention and management of cardiac disease.

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DISCLOSURES

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