Research Article

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Dietary glycotoxins exacerbate progression of experimental fatty liver disease

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Background & Aims: Advanced glycation end-products (AGEs) levels are high in western diets and contribute to tissue injury via activation of RAGE (receptor for AGEs) and generation of reactive oxygen species (ROS). Here, we determined if high dietary AGE intake worsens progression of non-alcoholic fatty liver disease (NAFLD).

Methods: Male Sprague Dawley rats were fed a methionine choline deficient (MCD) diet for 6 weeks before 6 weeks of a high AGE MCD diet through baking. They were compared with animals on MCD diet or a methionine choline replete (MCR) diet alone for 12 weeks. Hepatic ROS, triglycerides, biochemistry, picro-sirius morphometry, hepatic mRNA expression and immunohistochemistry were determined. Primary hepatic stellate cells (HSCs) from both MCR and MCD animals were exposed to AGEs. ROS, proliferation and mRNA expression were determined.

Results: The high AGE MCD diet increased hepatic AGE content and elevated triglycerides, NADPH dependent superoxide production, HNE adducts, steatosis, steatohepatitis (CD43, IL-6, TNF-α) and fibrosis (α-SMA, CTGF, COL1A, picrosirius) compared to MCD alone. In HSCs, AGEs significantly increased ROS production, bromodeoxyuridine proliferation and MCP-1, IL-6, α-SMA, and RAGE expression in HSCs from MCD but not MCR animals. These effects were abrogated by RAGE or NADPH oxidase blockade.

Conclusions: In the MCD model of NAFLD, high dietary AGEs increases hepatic AGE content and exacerbates liver injury, inflammation, and liver fibrosis via oxidative stress and RAGE dependent profibrotic effects of AGEs on activated HSCs. This suggests that pharmacological and dietary strategies targeting the AGE/RAGE pathway could slow the progression of NAFLD.

Keywords: Advanced glycation end-products; Steatohepatitis; Non-alcoholic fatty liver disease; Oxidative stress; Hepatic stellate cell.

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Abbreviations: AGEs, advanced glycation end-products; RAGE, receptor for AGEs; ROS, reactive oxygen species; NAFLD, non-alcoholic fatty liver disease; MCD, methionine choline deficient; MCR, methionine choline replete; CML, N-(carboxymethyl)lysine; ALT, alanine transaminase; IL-6, interleukin-6; TNF-α, tumour necrosis factor α; α-SMA, α-smooth muscle actin; COL1A, collagen-1A; CTGF, connective tissue growth factor; HSCs, hepatic stellate cells; BrDU, bromodeoxyuridine; MCP-1, monocyte chemotactic protein-1; NADPH, nicotinamide adenine dinucleotide phosphate; NASH, non-alcoholic steatohepatitis; BSA, bovine serum albumin; HNE, 4-hydroxy-2-nonenal; HOMA-IR, homeostatic model assessment of insulin resistance.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the world, largely due to the burgeoning rates of diabetes and obesity [1]. Although most patients with NAFLD are asymptomatic and do not develop significant liver injury, many progress to non-alcoholic steatohepatitis (NASH), cirrhosis and liver cancer. However, the host and/or environmental risk factors that determine whether patients with simple hepatic steatosis go on to develop NASH and its complications remain unclear [2].

Advanced glycation end products (AGEs), also known as glycotoxins, are a complex group of compounds that are formed via the Maillard reaction, where sugar moieties become bound to proteins and lipids, causing browning and other irreversible modifications [3]. Foods that are highly processed or dry heated at high temperatures, such as broiled foods, are particularly high in AGEs [4]. AGE formation occurs at an increased rate in diabetes owing to the excess of reducing sugars available as a consequence of hyperglycaemia. The rate is also influenced by the concentration of reducing sugars in the serum, the turnover of the proteins, and the extent of the oxidative stress in the environment.

These compounds act on many receptors, including macrophage scavenger receptor type I and II, oligosaccharyl transferase-48 (AGE-R1), 80k-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3) and the receptor for advanced glycation end products (RAGE). RAGE, a member of the immunoglobulin superfamily of cell-surface molecules, is the best characterised of these receptors [5]. It is expressed in a number of cell types, including

endothelial cells, vascular smooth muscle cells, peripheral blood mononuclear cells, macrophages including Kupffer cells, and hepatic stellate cells (HSCs). There is now strong evidence that accumulation of AGEs results in changes to extracellular matrix structure and function, and they have been implicated in the pathogenesis of diabetic renal, neurological, retinal and vascular complications [6]. Cellular responses to AGEs are driven by its engagement with RAGE, which is thought to be the main way in which AGEs impart their pathogenic effects. RAGE activation increases inflammation and the generation of ROS [7].

There are a number of lines of evidence to suggest that in NAFLD, AGEs may be a factor that contributes to the progression from simple steatosis to NASH and liver fibrosis [8]. A number of studies have shown that RAGE plays a role in acute liver injury and that blockade of RAGE can ameliorate toxic, ischaemic, and cholestatic liver damage [9–11]. In chronic liver injury, hepatic expression of RAGE is significantly increased [12] and in NAFLD, AGE levels correlate with the severity of fibrosis, leading to speculation that they play a primary role in disease pathogenesis [13]. Furthermore, diabetes, which increases AGE formation and RAGE expression, worsens the progression of fibrosis in a number of human liver diseases, including NAFLD and hepatitis C [14].

In our study, we explored if excess dietary AGE consumption could exacerbate the development of NASH in experimental NAFLD in both normal and fatty livers. The MCD diet model was chosen as it does not produce the potentially confounding effect of peripheral insulin resistance, which might increase endogenous AGE production. We also performed complementary *in vitro* studies in HSCs to determine mechanistic pathways to AGE mediated effects seen in our liver disease model.

Materials and methods

Experimental design

Experiments were approved by the Austin Health Animal Ethics Committee and performed according to the National Health and Medical Research Council (NHMRC) of Australia Guidelines for animal experimentation. To explore the role of dietary AGEs in the normal liver, rats were randomised into two groups (n = 10/group) and followed for 12 weeks.

(1) A control group; fed a MCR diet (MP Biomedicals, Solon, Ohio, United States);
(2) A baked diet in normal animals; fed a MCR diet for the first 6 weeks but in the last 6 weeks this was baked (MCR baked) to increase AGE content as previously described by our group [15,16].

To explore the role of AGEs in NAFLD, steatosis was induced in 10 week old Sprague Dawley rats by depleting methionine and choline from their diet as previously described [17]. This model induces accumulation of VLDL in the liver leading to classical histopathological features of NASH without peripheral insulin resistance [18].

Rats were randomised into three groups (n = 10/group) and followed for 12 weeks.

(1) A group fed the MCR diet;
(2) A steatotic group; fed a methionine and choline deplete diet (MCD) (MP Biomedicals);
(3) A steatotic group with increased oral AGEs (MCD baked) where animals were fed an MCD diet for 12 weeks and in the last 6 weeks had this diet baked to increased dietary AGE content.

AGE production and content

A high AGE diet was produced by baking the diet at 160 °C for 1 h. AGEs for *in vitro* studies were produced as previously described (Supplementary Materials and methods) [11]. CML is the predominant AGE in food and the extent of advanced glycation was measured by ELISA [16] and this showed that baking the MCD diet increased CML levels more than 4 fold from 31 to 137 nmol/100 mg lysine, as in previous studies [19]. Liver CML content was also assessed by ELISA as previously reported [20] (Supplementary Materials and methods).

Assessment of biochemistry, superoxide production, hepatic triglyceride content and histology

Serum biochemistry, superoxide, lipids, and histology were quantified as reported [11,21]. (Supplementary Materials and methods). To ensure that there was no peripheral insulin resistance or diabetes that could confound the effects of exogenous dietary AGEs via formation of endogenous AGEs, the HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) was measured across all groups.

Quantification of gene expression and protein localisation

Gene expression and protein localisation was performed using quantitative real-time PCR and immunohistochemistry, respectively, and as previously reported [11]. The details of fluorescent labeled oligonucleotide probes and their target specific primers is given in Table 1 in Supplementary Materials and methods. Immunohistochemical staining methods are also expanded in Supplementary Materials and methods.

In vitro experiments

HSCs were isolated and cultured from livers in both MCR and MCD animals as reported [22]. Cells were seeded in 6 well plates with BSA at 100 μg/ml (vehicle) or AGE-BSA at 100 μg/ml (intervention) from 2 to 8 days post-isolation. Two additional groups were treated with AGE-BSA along with NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI) (5 μM, Sigma-Aldrich); and AGE-BSA along with goat anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA) (Supplementary Materials and methods).

Measurement of reactive oxygen species (ROS) and cell proliferation

Measurement of intracellular ROS was performed as reported [23]. Proliferative response was assessed using a BrdU assay (Roche Applied Science, Indiana, USA) as per manufacturer's instructions.

Statistical analysis

Data (mean ± SEM) were analyzed by (ANOVA) and Student's two-tailed, unpaired *t* test and log transformation where appropriate (Prism 5, GraphPad, San Diego, USA). $p < 0.05$ was considered significant.

Results

High dietary AGEs in the normal liver (MCR animals) did not result in steatohepatitis or fibrosis

Both MCR and MCR baked groups had similar food intake and no difference in weight gain, liver weight or liver to body weight ratio. Baking the MCR diet did not worsen liver biochemistry, inflammation or fibrosis (Supplementary Figs. 1 and 2). We then explored the role of increased dietary AGEs in NAFLD using the MCD model.