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Alagebrium attenuates methylglyoxal induced oxidative stress and AGE formation in H9C2 cardiac myocytes

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ABSTRACT

Aim: Diabetes mellitus associated cardiovascular complications are a leading cause of morbidity and mortality worldwide. Methylglyoxal (MG) is a reactive ketoaldehyde and a byproduct of glucose metabolism and an inducer of advanced glycation endproducts (AGEs). Alagebrium (ALA) is an AGEs crosslink breaker, however, the effects of ALA on MG levels and its consequences in cultured rat cardiomyocytes are not known. The aim of the present study was to examine the effect of high glucose and MG on cultured rat cardiomyocytes and to investigate whether ALA could prevent any deleterious effects of high glucose and MG in these cells.
Main methods: MG levels were determined by HPLC. The expression of different genes was measured by RT-PCR. Oxidative stress and AGEs formation was determined by DCF probe and immunocytochemistry respectively.
Key findings: High glucose- and MG treated- cardiomyocytes developed a significant increase in MG, and the expression for caspase-3, Bax, RAGE and NF-KB, which were all attenuated after pretreatment with ALA. A significant increase in reactive oxygen species generation and AGEs formation in high glucose- and MG treated-cultured cardiomyocytes was also observed, which was attenuated after pretreatment with ALA.
Significance: ALA may have a preventive role against the deleterious effects of high glucose and MG in the heart. Prevention of dicarbonyl-induced AGEs, by safer and specific scavengers of MG is an attractive therapeutic option.

1. Introduction

The prevalence of diabetes mellitus and the associated cardiovascular complications, including cardiomyopathy has increased drastically over the decade and is predicted to affect 300 million people worldwide by 2025 [1]. Diabetic cardiomyopathy resulting in heart failure in the absence of coronary artery disease is a serious complication of diabetes [2]. Methylglyoxal (MG), a highly reactive dicarbonyl compound reacts and modifies certain proteins to form advanced glycation end products (AGEs) such as N^{ε}-carboxyethyllysine (CEL) and argpyrimidine [3–5]. AGEs are implicated in the pathogenesis of vascular complications of diabetes and hypertension [5], however the role of MG or its end product AGEs in diabetic cardiovascular complications is not fully understood [3,4]. MG levels in healthy humans are 1 μM or less and are elevated 2–4 fold in diabetic patients with a positive correlation to the degree of hyperglycemia [6,7]. Incubation of cultured vascular smooth muscle cells with high glucose or fructose for 3 and 24 h increased MG production about 3.9–4 fold and induced oxidative stress [8]. Sprague–Dawley (SD) rats fed chronically with fructose develop insulin resistance [9,10]. It has also been reported earlier that MG modifies the structure of the insulin molecule in vitro, in a way that impairs insulin-mediated glucose uptake in adipocytes [11]. In cultured 3T3-L1 adipocytes MG decreased insulin-induced insulin-receptor substrate-1 (IRS-1) tyrosine phosphorylation and phosphatidylinositol-3-kinase (PI3K) activity [10]. Incubation of cultured L6 muscle cells with MG impaired insulin signaling [12]. We have also reported recently that chronic treatment of SD rats with MG via osmotic pump for one month induces pancreatic beta cell dysfunction, type 2 diabetes [13] and hypertension via activation of renin angiotensin aldosterone system [14]. In spite of these cellular and molecular studies on MG, the effect of high glucose-induced MG and exogenous MG, especially in pathologically relevant MG concentrations, in cultured rat cardiomyocytes is not known.

Currently, there is a lack of safe and selective MG scavengers. Safe and specific MG scavengers have the potential to prevent different high carbohydrate induced pathological conditions such as type 2 diabetes, hypertension, endothelial dysfunction and AGEs formation. Alagebrium (ALA, previously known as ALT-711) is a novel advanced glycation endproducts (AGEs) cross-link breaker [15–23]. ALA (210 $mg \cdot kg^{-1}$ twice a day for 8 weeks) given to patients with systolic hypertension reduced vascular fibrosis and markers of inflammation [17] and alagebrium at a dose of 1 $mg \cdot kg^{-1}$ daily for 1 or 3 weeks reversed diabetes-induced increase of arterial stiffness in streptozotocin-induced diabetic rats [15,16]. ALA has been shown to prevent

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ventricular dysfunction in spontaneously hypertensive rats, preserved cardiac diastolic function in aging rats and improved ventricular function in heart failure in humans [18–20]. However, the role of MG and its interaction with ALA were not examined in these studies on the heart. We have shown that ALA (100 $mg \cdot kg^{-1}$) attenuated acute methylglyoxal induced glucose intolerance in normal Sprague–Dawley rats, indicating a scavenging effect on MG [24]. ALA at a dose of 100 $mg \cdot kg^{-1}$ for 4 weeks prevented diabetes development and improved beta cell dysfunction in chronic methylglyoxal treated SD rats [13]. However, the effect of ALA on MG induced deleterious effects in cultured cardiomyocytes has not been reported so far. The aim of the present study was to examine the effect of high glucose and exogenous MG on cultured rat cardiomyocytes. More importantly, we examined whether ALA could prevent or attenuate any deleterious effects of high glucose and MG in these cells.

2. Materials and methods

2.1. Cardiomyocyte cell culture

Rat cardiac myocyte cell line (H9C2) was obtained from American Type Culture Collection (ATCC) and National Center for Cell Science, Pune, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. H9C2 cells were seeded either in 100 mm dishes for MG measurement or in 96-well plates for other assays, with an equal amount of cells (10^6/ml) in each well. For staining, cells were seeded on cover glass slides (2×10^6/ml). Cells were starved in FBS-free DMEM for 24 h before exposure to different treatments.

2.2. In vitro MG binding assay

MG (30 μM) was co-incubated with ALA (100 μM), in phosphate-buffered saline (PBS) at 37 °C for 15, 30, or 60 min or 3 and 24 h. After the prescribed incubation time the free MG in the sample was measured by HPLC [25]. The concentrations used for ALA are based on our previous studies with is in accordance with several studies from our and other laboratories [13,24,26].

2.3. MG measurement

MG was measured by an *o*-phenylene diamine (*o*-PD) method as described previously [25]. In brief, cell pellets were resuspended in ice-cold phosphate buffered saline (PBS), and lysed over ice by sonication (5 s, three times). The sample was then incubated on ice for 10 min with ¼ volume of perchloric acid (PCA) and centrifuged (12,000 rpm, 15 min) to remove the PCA-precipitated material. The supernatant was supplemented with 100 mM *o*-PD and incubated for 3 h at room temperature. The quinoxaline derivative of MG (2-methylquinoxaline) and the quinoxaline internal standard (5-methylquinoxaline) were measured using HPLC [24].

2.4. Measurement of reactive oxygen species

The formation of reactive oxygen species was determined by a dicholoro-dihydro-fluorescein diacetate (DCFH-DA) assay. Briefly, cells were loaded with a membrane-permeable, nonfluorescent probe 2′,7′-dichlorofluorescin diacetate (CM-H2DCFDA, 5 μM) for 2 h at 37 °C in FBS-free DMEM in the dark. After washing with PBS 3 times, cells were incubated with or without different treatments for 24 h, and finally subjected to detection. Once inside the cells, CM-H2DCFDA becomes membrane-impermeable DCFH2 in the presence of cytosolic esterases, and is further oxidized by peroxynitrite to form oxidized DCF which has detectable fluorescence. Oxidized DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm utilizing a Fluoroskan Ascent plate reader (Thermo Labsystems Inc., Waltham, MA, USA) and Ascent software, and expressed in arbitrary units.

2.5. Real time quantitative PCR (RT-PCR)

Total RNA from cultured cells was isolated using RNA isolation kit (Qiagen, Germantown, MD, USA). The pre-designed primers for Caspase-3, Bax, Bcl-2, NFkB and RAGE were purchased from Sigma, (Sigma-Aldrich, Bengaluru, Karnataka, India). The real-time PCR was performed in an iCycler iQ apparatus associated with the ICYCLER OPTICAL SYSTEM software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad Laboratories Ltd., Gurgaon, Haryana, India).

2.6. Immunocytochemistry

H9C2 cells were seeded on glass cover slips followed by incubation with either MG or high glucose for 24 h, and subjected to CEL staining. Similar staining procedure was followed as described previously [8]. In brief cells were first fixed in 4% paraformaldehyde followed by permeation with 0.1% Triton X-100 and pre-incubation with normal goat serum (diluted 1:30 in 0.1 N PBS) to block non-specific binding. Following overnight incubation with CEL antibody (1:100) at room temperature, cells were washed twice in PBS and incubated with FITC conjugated anti-CEL secondary antibodies (Molecular Probes) for 2 h. Cells were again washed with PBS three times and mounted in glycerol:PBS (3:7). Images were captured under fluorescence microscope. Staining intensity was quantified using the MetaMorph image analysis software (*v*. 7, Molecular Devices). Data was collected and averaged from four independent experiments with 5 fields per slide.

2.7. Statistical analysis

Data obtained from separate experiments are expressed as mean ± SEM. Statistical analysis was performed using ANOVA with post hoc Bonferroni's test. A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

The effect of ALA on MG induced deleterious effects in cultured cardiomyocytes has not been reported We studied the effect of ALA on MG levels, ROS production, RAGE expression, gene markers of apoptosis, (NFKB, Bcl2, Bax, Caspase-3) and AGEs formation in high glucose/MG treated cardiomyocytes and whether ALA could prevent any deleterious effects of high glucose and MG in these cells.

3.1. ALA scavenges MG in an in vitro assay

Incubation of MG (30 μM) with ALA (100 μM) for different times ranging from 15 min to 24 h showed progressive binding of MG.

Table 1
ALA reduces detectable MG. MG (30 μM) was incubated with ALA (100 μM) at 37 °C for different times. The solution was analyzed for MG by HPLC after the given incubation period. The values are mean ± SEM ($n = 6$ each). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. MG alone.

Incubation time	MG (30 μM) alone	MG (30 μM) + ALA (100 μM)	ALA (100 μM)
0 min	28.4 ± 1.2	26.2 ± 3.1	0
15 min	27.8 ± 1.2	19.9 ± 1.1*	0
30 min	27.5 ± 0.9	15.7 ± 0.9*	0
60 min	26.7 ± 0.5	10.2 ± 0.7**	0
3 h	26.1 ± 0.8	6.2 ± 0.3**	0
24 h	26.5 ± 0.4	1.1 ± 0.1***	0