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Advanced glycation endproduct induces ROS accumulation, apoptosis, MAP kinase activation and nuclear *O*-GlcNAcylation in human cardiac myocytes

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Abstract

Accumulation of advanced glycation endproduct (AGE) has been implicated in the pathogenesis of diabetic complications. However, the precise role and mechanism behind AGE-associated diabetic heart injury are not fully clear. This study was designed to evaluate the effect of AGE on accumulation of reactive oxygen species (ROS), apoptosis, mitogen-activated protein kinase (MAPK) activation and nuclear *O*-GlcNAcylation in fetal human cardiac myocytes. Myocytes were maintained for 24–72 h in a defined culture medium containing high glucose, the AGE carbon precursor methylglyoxal (MG), and MG-AGE derived from MG and bovine serum albumin (BSA). Generation of ROS was detected by 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate. Apoptosis was evaluated by caspase-3 activity and quantitative DNA fragmentation. Both high glucose (25.5 mM) and MG (200 μM) significantly enhanced ROS and AGE formation with greater effects elicited by MG. Both high glucose and MG-AGE significantly facilitated apoptosis with a more predominant effect from MG-AGE. In addition, phosphorylation of MAPK cascade [extracellular signal-regulated kinase-1/2 (ERK1/2) and p38] and nuclear *O*-GlcNAcylation were enhanced in MG-AGE-treated myocytes, similar to those elicited by high glucose. MG-AGE-induced phosphorylation of ERK1/2 and p38 was nullified by neutralizing AGE with specific anti-AGE antibody but not nonspecific antiserum. Collectively, these results indicated that AGE or its precursor MG may trigger ROS generation, apoptosis, MAPK activation and nuclear *O*-GlcNAcylation in human cardiac myocytes, in a manner reminiscent of high extracellular glucose.

Keywords: AGE; Cardiac myocytes; Apoptosis; MAPK; Nuclear *O*-GlcNAcylation

Introduction

Advanced glycation endproduct (AGE), a heterogeneous group of non-enzymatic glycation products of proteins, accumulates at an accelerated rate in circulation and various tissues. This process of AGE accumulation has been speculated to contribute to the pathogenesis of atherosclerosis, hypertension and complications in diabetes mellitus (Brownlee, 1995). Through interaction with its membrane receptors, AGE is believed to facilitate generation of oxygen free radicals, which consequently lead to apoptosis and compromised cardiovascular function (Brownlee, 2001). Although a large body of evidence has indicated a likely role of AGE in the pathogenesis of diabetic cardiovascular complications (Arai, 2002), the precise role and mechanism of action behind AGE-triggered early phase of diabetic heart complications are still elusive. To better understand the role of AGE in diabetes or hyperglycemia-induced cardiac injury, the present study was designed to evaluate the effect of AGE or its precursor on reactive oxygen species (ROS) generation, apoptosis and activation of mitogen-activated protein kinase (MAPK) cascade in fetal human cardiac myocytes. Nuclear *O*-GlcNAcylation, a process frequently occurring in a hyperglycemic environment (Clark et al., 2003; Li et al., 2005), was also monitored. Fetal human cardiac myocytes were chosen for our study essentially based on their ability to be optimally maintained in cell culture for a sustained period of time (Li et al., 2006; Zhang et al., 2004).

Materials and methods

Human cardiac myocyte cells and preparation of cytoplasmic or nuclear extracts

Fetal (20–24 week gestation age) human cardiac myocytes were obtained from the ScienCell Research Laboratories (San Diego, CA). Isolation and identification procedure was

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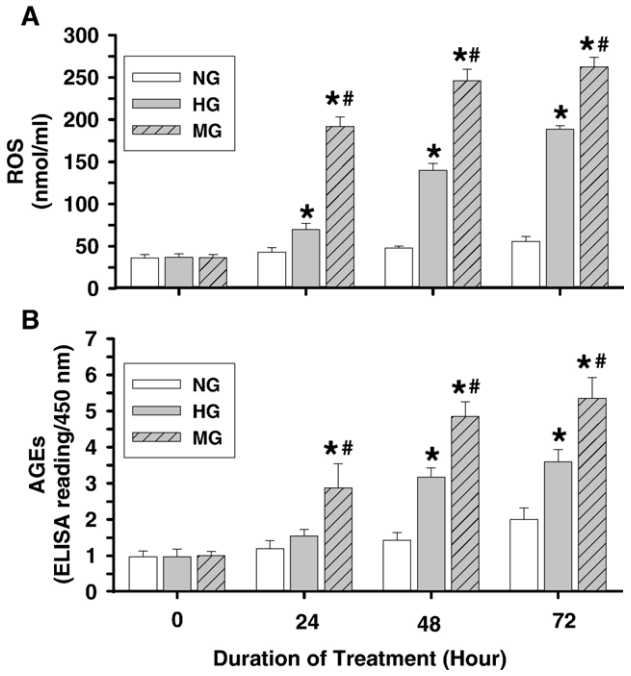

Fig. 1. Time-dependent effect of high glucose (HG, 25.5 mM) and methylglyoxal (MG, 200 μM) on ROS generation (panel A) and AGE formation (panel B). Human cardiac myocytes were cultured for up to 72 h in a medium containing normal glucose (NG, 5.5 mM), HG or NG with MG. Mean ± SEM, $n = 5$ cultures, *$p < 0.05$ *vs.* NG group, # $p < 0.05$ *vs.* HG group.

described in detail previously (Li et al., 2006). Cardiac myocytes were further purified by a differential trypsinization and immunodepletion method before being cultured at 37 °C with 5% CO_2 in a complete myocyte medium (ScienCell; Catalog# 6201), which highly supports growth of human cardiac myocytes. Cardiac myocytes, which were attached to culture plates, were incubated with high glucose (25.5 mM), methyglyoxal (MG, 200 μM) or MG-AGE derived from MG and bovine serum albumin (BSA) (200 μg/ml) for up to 72 h. An incubation time of 48 h was used in most experiments unless otherwise stated. To prepare cytosolic and nuclear extracts, cultured human cardiac myocytes were lysed in a RIPA lysis buffer (Millipore Corporation, Billerica, MA). After centrifugation at 14,000 ×*g* for 5 min, the supernatant was transferred into a pre-chilled tube (designated as cytoplasmic extracts). The pellet was resuspended in lysis buffer containing 500 mM NaCl and was gently shaken for 1 h before being centrifuged at 15,000 ×*g* for 15 min. The supernatant was designated the nuclear protein extracts. Protein concentration of the cytoplasmic and nuclear extracts was determined by the Bradford method (Bradford, 1976).

AGE assay

AGE levels in human cardiac myocytes were determined by AGE-ELISA using a specific anti-AGE monoclonal antibody 1H7G5 (1:5,000, kindly provided by Dr. Michael Brownlee, Albert Einstein College of Medicine, NY) as previously described (Li et al., 2005).

Generation of intracellular ROS

The fluorescent probe 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM–H_2DCFDA) enters cells and produces a signal following intracellular oxidation by ROS such as H_2O_2. Intracellular oxidative stress was monitored by intracellular fluorescence detection using a Spectra MaxGeminiXS device (Spectra Max, Atlanta, GA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Li et al., 2004). In brief, 200 μl cells (~10^5 cells/ml) were loaded with CM–H_2DCFDA (10 μM) for 45 min at 37 °C before intracellular fluorescence intensity was analyzed in a FluoroNunc 96-well polystyrene plate using the SOFTmax PRO 4.0 software. Intracellular ROS production was calculated from an H_2O_2 standard curve (10–200 nmol/ml).

Caspase-3 assay

Caspase-3 is an enzyme activated during induction of apoptosis. In brief, 1 ml of PBS was added to flasks containing human cardiac myocytes. Myocytes (monolayer) were scraped and collected in a microfuge tube. The cells were centrifuged at 10,000 ×*g* at 4 °C for 10 min and cell pellets were lysed in 100 μl of ice-cold cell lysis buffer (50 mM HEPES, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% NP40). Following cell lysis, 70 μl reaction buffer and 20 μl caspase-3 colorimetric substrate (Ac-DEVD-p′1NA) were added to cell lysate and incubated for 1 h at 37 °C, during which time, caspase enzyme in the sample

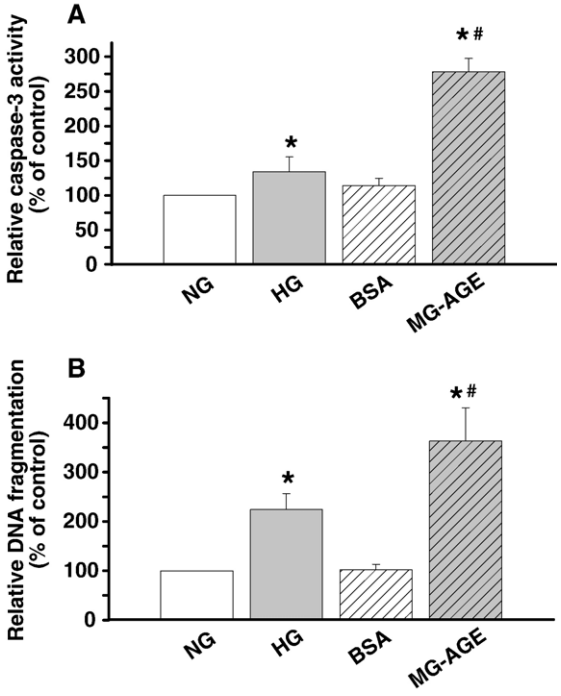

Fig. 2. Effect of high glucose (HG, 25.5 mM) and methylglyoxal-AGE (MG-AGE, 200 μg/ml) on apoptosis evaluated by caspase-3 assay (panel A) and DNA fragmentation (panel B). Human cardiac myocytes were cultured for 48 h with normal glucose (NG, 5.5 mM), HG (25.5 mM), control-BSA (200 μg/ml) and MG-AGE (200 μg/ml). Mean ± SEM, $n = 7$ cultures, *$p < 0.05$ *vs.* NG group, # $p < 0.05$ *vs.* HG group.

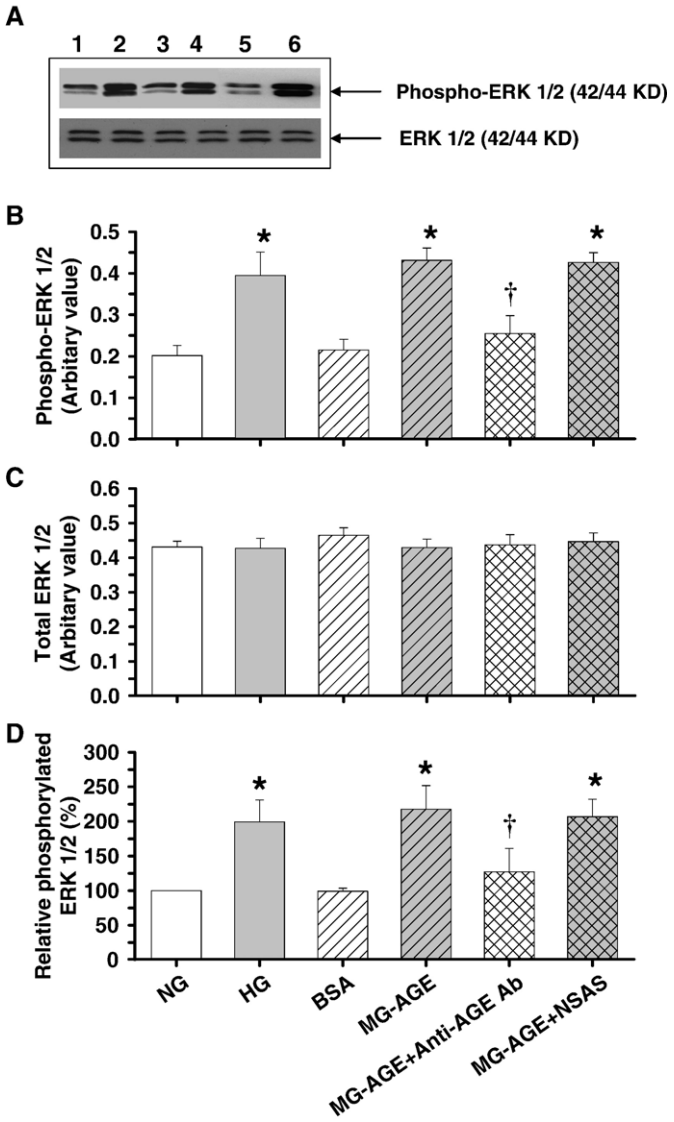

Fig. 3. Effect of 48 h-incubation of high glucose (HG, 25.5 mM) and methylglyoxal-AGE (MG-AGE, 200 μg/ml) on ERK1/2 activation in human cardiac myocytes. Panel A: Representative gels depicting total and phosphorylated ERK1/2 in myocytes incubated with normal glucose (NG 5.5 mM, lane 1), HG (25.5 mM, lane 2), BSA (200 μg/ml, control for MG-AGE, lane 3), MG-AGE (200 μg/ml, lane 4), MG-AGE (200 μg/ml)+Anti-AGE antibody 1H7G5 (1:2,000, lane 5) or MG-AGE (200 μg/ml)+non specific anti serum (NSAS, 1:1,500, lane 6); Panel B: Total ERK1/2 protein expression; Panel C: phosphorylated ERK1/2; and Panel D: phosphorylated ERK1/2 normalized to respective NG group from the same culture. Mean±SEM, $n=5$ cultures, *$p<0.05$ *vs.* NG group, † $p<0.05$ *vs.* MG-AGE group.

was allowed to cleave the chromophore pNA from its substrate molecule. The samples were then read using a microplate reader at 405 nm. Caspase-3 activity was expressed as picomoles of pNA released per microgram of protein per minute (Li et al., 2004).

Apoptosis measurement with DNA fragmentation ELISA

Apoptosis was also evaluated by determination of cytoplasmic histone-associated DNA fragmentation using an ELISA cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (Li et al., 2004).

Immunoblotting of extracellular signal-regulated kinase (ERK1/2), p38 mitogen-activated protein kinase (MAPK) and nuclear O-GlcNAcylation

Human cardiac myocytes were sonicated in a lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% SDS and protease inhibitor cocktail. Equal amount of lysates (50 μg/lane) were separated on 7%–20% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to nitrocellulose

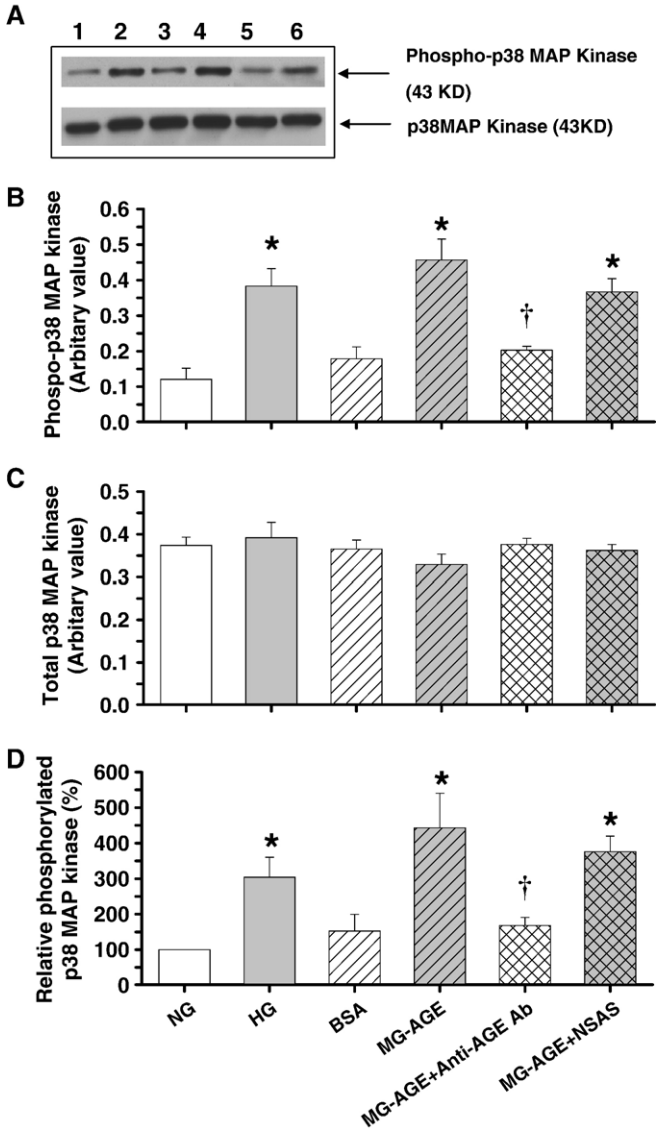

Fig. 4. Effect of 48 h-incubation of high glucose (HG, 25.5 mM) and methylglyoxal-AGE (MG-AGE) on p38 MAPK activation in human cardiac myocytes. Panel A: Representative gels depicting total and phosphorylated p38 MAPK in myocytes incubated with normal glucose (NG 5.5 mM, lane 1), HG (25.5 mM, lane 2), BSA (200 μg/ml, control for MG-AGE, lane 3), MG-AGE (200 μg/ml, lane 4), MG-AGE (200 μg/ml)+Anti-AGE antibody 1H7G5 (1:2000, lane 5) or MG-AGE (200 μg/ml)+non specific anti serum (1:2000, lane 6); Panel B: Total p38 MAPK protein expression; Panel C: phosphorylated p38 MAPK; and Panel D: phosphorylated p38 MAPK normalized to respective NG group from the same culture. Mean±SEM, $n=5$ cultures, *$p<0.05$ *vs.* NG group, † $p<0.05$ *vs.* MG-AGE group.

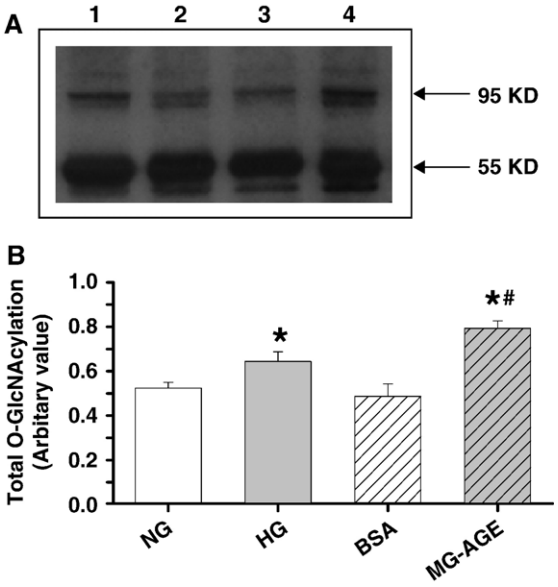

Fig. 5. Effect of 48 h-incubation of high glucose (HG, 25.5 mM) and methylglyoxal-AGE (MG-AGE, 200 μg/ml) on nuclear *O*-GlcNAcylation in human cardiac myocytes. Panel A: Representative gels depicting nuclear *O*-GlcNAcylation (especially 55 KD and 95 KD proteins). Nuclear extracts (50 μg) resolved on 4–20% gradient SDS gels were transferred to nylon membranes and reacted with an anti-*O*-GlcNAc monoclonal antibody. Lane 1 = normal glucose (NG, 5.5 mM); lane 2 = HG (25.5 mM); lane 3 = control BSA (200 μg/ml); lane 4 = MG-AGE (200 μg/ml); Panel B: pool data from 5 cultures counting all protein bands. Mean ± SEM, *$p < 0.05$ *vs.* NG group, #$p < 0.05$ *vs.* HG group.

membranes (0.2 μm). The membranes were blocked in 5% (w/v) nonfat milk in TBS-T buffer, and then incubated with anti-ERK1/2 (1:2,000), anti-phospho-ERK1/2 (1:1,000), anti-p38 MAPK (1:2,000), anti-phospho-p38 MAPK (1:1,000), anti-nuclear *O*-GlcNAc (1:1,000) and anti-β-actin (1:1,000) antibodies. Anti-ERK1/2 and anti-phospho-ERK1/2 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas anti-p38 MAPK and anti-phospho-p38 MAPK polyclonal antibodies were purchased from Cell Signaling (Beverly, MA). Anti-nuclear *O*-GlcNAc and anti-β-actin antibodies were obtained from Pierce Biotechnology (Rockford, IL) and Novus Biologicals (Littleton, CO), respectively. The antigens were detected by luminescence method (ECL Western blotting detection kit, Amersham) with peroxidase-linked IgG (1:5,000). β-actin was used as loading control (Li et al., 2006).

Data analysis

Data were presented as mean ± SEM. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) followed by a Dunnetts post hoc analysis.

Results

Effect of high glucose, MG and MG-AGE on AGE formation, ROS generation and apoptosis in human cardiac myocytes

High extracellular glucose (25.5 mM) significantly enhanced ROS generation and AGE formation in human cardiac myocytes following an incubation time of 24, 48 and 72 h with the exception of a non-significant effect on AGE formation at 24 h. The AGE carbon precursor, MG (200 μM), also stimulated AGE formation and ROS generation following 24–72 h of incubation. The amplitude of MG-induced effects on AGE and ROS was significantly greater than that elicited by high glucose. Since 72 h of incubation with high glucose or MG did not induce further increase in ROS and AGE generation compared with those elicited by 48 h of incubation, the 48-h incubation time was used for the remaining incubations in this study (Fig. 1). Consistent with their effects on intracellular ROS generation, high glucose (25.5 mM) and AGE derived from MG and bovine serum albumin (BSA)-MG-AGE (200 μg/ml), significantly enhanced apoptosis assessed by caspase-3 activity and DNA fragmentation following 48 h of incubation. The degree of MG-AGE-induced apoptosis was significantly greater than that of high glucose (Fig. 2). These data suggest that both high glucose and AGE may directly facilitate ROS generation and apoptosis.

Western blot analysis of activation of ERK1/2, p38 MAPK and nuclear O-GlcNAcylation

To elucidate potential signaling mechanism(s) involved in AGE-elicited response on ROS generation and apoptosis, phosphorylation of MAPK cascade including ERK1/2 and p38 MAPK was evaluated in human cardiac myocytes. Exposure of myocytes to MG-AGE (200 μg/ml) for 48 h significantly activated ERK1/2 and p38 MAPK (shown as phosphorylated protein expression or normalized to normal glucose group) without affecting respective total protein expression. High glucose (25.5 mM) elicited similar activation of ERK1/2 and p38 MAPK without affecting their total protein expression (Figs. 3 and 4). Interestingly, the MG-AGE-induced activation of ERK1/2 and p38 MPAK was nullified by co-incubation of specific antibody against MG-AGE but not non-specific antiserum. The anti-MG-AGE antibody and non-specific antiserum did not elicit any significant effects on activation of ERK1/2 and p38 MAPK by themselves (data not shown). In addition, levels of nuclear *O*-GlcNAcylation were significantly enhanced in both high glucose and MG-AGE-treated human cardiac myocytes, with a greater effect elicited by MG-AGE (Fig. 5). Collectively, these results revealed that AGE-induced accumulation of ROS and apoptosis may be casually associated with activation of MAPK cascade and nuclear *O*-GlcNAcylation, somewhat reminiscent of the effects elicited by high glucose.

Discussion

The salient finding of this study is that reactive dicarbonyl methylglyoxal, a major carbon precursor in AGE formation, displays stimulatory effects in ROS generation and AGE formation in human cardiac myocytes, somewhat reminiscent of high extracellular glucose. Furthermore, our in-house prepared MG-AGE enhanced apoptosis accompanied by temporal activation of ERK1/2, p38 MAPK and nuclear *O*-GlcNAcylation in human cardiac myocytes, also reminiscent of high extracellular

glucose. The MG-AGE-induced activation of ERK1/2 and p38 MPAK may be nullified by specific antibody against MG-AGE but not non-specific antiserum, suggesting a potential role of ERK1/2 and p38 MAPK in AGE-triggered ROS generation and apoptosis. These data support the notion that AGE formation may play a significant role in diabetes and hyperglycemia-induced heart complications.

Several mechanisms have been speculated for the pathogenesis of diabetic heart complications including oxidative stress, impaired glucose metabolism and intracellular Ca^{2+} mishandling (Lagadic-Gossmann et al., 1996; Schaffer et al., 1997). Nonetheless, none of these scenarios received convincing validation as the ultimate culprit responsible for diabetes or hyperglycemia-induced cellular alterations in hearts. Recent evidence has implicated a role of AGE and its receptor RAGE in the onset of diabetic complications including cardiomyopathy (Norton et al., 1996; Candido et al., 2003; Kass, 2003; Bidasee et al., 2004). As a group of heterogeneous compounds accumulated in diabetes due to increased reactive carbohydrate substrate, oxidative condition favoring glycation process and insufficient AGE detoxification (Brownlee, 2001), AGE is speculated to contribute to the development of a number of cardiovascular diseases via forming crosslink with functional macromolecules (Norton et al., 1996; Candido et al., 2003). In addition, coupling between AGE and RAGE may facilitate release of pro-inflammatory cytokines (Miyata et al., 1996; Simm et al., 2004). Although the notion of AGE theory in the pathogenesis of diabetic complications has been around for years, the critical piece of evidence supporting a direct impact of AGE in cardiomyocyte survival has not been fully established. Our current data of elevated AGE levels by high glucose environment is in line with the previous finding of enhanced methylglyoxal levels under hyperglycemia (Beisswenger et al., 2001). Methylglyoxal, a highly reactive dicarbonyl derived from either glycolytic intermediate fragmentation (triose phosphates) or acetone oxidation, is prone to AGE generation (Brownlee, 2001). Accentuated methylglyoxal metabolism has been reported in diabetes (Beisswenger et al., 2001). Therefore, our finding that MG-AGE and high glucose trigger stimulatory effects on ROS generation, apoptosis, MAPK activation and nuclear O-linked GlcNAcylation seems to consolidate a role of MG-AGE in hyperglycemia-elicited cardiac injury. ERK1/2, p38 MAPK and nuclear O-linked GlcNAcylation have all been implicated in the pathogenesis of diabetic complications although contribution from other signaling pathways should not be excluded at this time.

Experimental limitation: Although our data depicted that specific antibody against MG-AGE but not non-specific antiserum blocks MG-AGE-induced activation of ERK1/2 and p38 MAPK, use of a more unique AGE crosslink breaker such as ALT-711 would provide in-depth mechanism regarding AGE-induced activation of cardiac stress signal cascades, oxidative stress and apoptosis. However, such crosslink breaker is unavailable to us at this point. Furthermore, activation of the MAPK cascade usually occurs rapidly and our incubation time resolution of 24–72 h cannot reveal the transient response of MG-AGE on phosphorylation of ERK1/2 and p38 MAPK. Nonetheless, our earlier time course study on phosphorylation of the MAPK family using the same myocytes indicated that ERK1/2 and p38 MAPK may be turned on within 0.5–1 h and the levels of phosphorylation remains stable beyond 12 h (Li et al., 2006). Lastly, given the fact that diabetes is associated with reduced overall antioxidant capacity (Valko et al., 2007), our current study failed to provide any information whether AGE affects endogenous antioxidant capacity in the hearts. Altered redox regulation by antioxidant enzymes has been implicated in response to AGE accumulation including depressed glutathione peroxidase and unchanged superoxide dismutase (Ueda et al., 1998).

In summary, our present study provides evidence that MG-AGE facilitated cardiac myocyte injury accompanied with temporal activation of ERK1/2, p38 MAPK and nuclear *O*-GlcNAcylation, indicating their potential roles in the AGE-triggered ROS generation and apoptosis. The fact that MG-AGE and high glucose both facilitate ROS generation, apoptosis, MAPK activation and nuclear *O*-GlcNAcylation suggests a possible role of AGE in diabetes and hyperglycemia-induced heart complications. These data should help to identify new therapeutic targets for the treatment and prevention of diabetic heart complications. Future work is warranted to examine the direct impact of AGE or its precursors on cardiac excitation-contraction coupling to better understand the role of AGE in diabetic heart dysfunction.

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