Advanced glycation endproduct (AGE) accumulation and AGE receptor (RAGE) up-regulation contribute to the onset of diabetic cardiomyopathy

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Abstract

Diabetic cardiomyopathy is manifested by compromised systolic and diastolic function. This study was designed to examine the role of advanced glycation endproduct (AGE) and AGE receptor (RAGE) in diabetic cardiomyopathy. Heart function was assessed in isolated control and streptozotocin-induced diabetic hearts following in vivo RAGE gene knockdown using RNA interference. Cardiomyocyte mechanical properties were evaluated including peak shortening (PS), time-to-PS (TPS) and time-to-90% relengthening (TR90). RAGE was assayed by RT-PCR and immunoblot. Diabetes significantly enhanced cardiac MG, AGE and RAGE levels accompanied with colocalization of AGE and RAGE in cardiomyocytes. Diabetes-elicited increase in RAGE was inhibited by in vivo siRNA interference. The AGE formation inhibitor benfotiamine significantly attenuated diabetes-induced elevation in MG, AGE, RAGE and collagen cross-linking without affecting hypertriglyceridaemia and hypercholesterolaemia in diabetes. Diabetes markedly decreased LV contractility, as evidenced by reduced dP/dt and LV developed pressure (LVDP), which were protected by RAGE gene knockdown. In addition, MG-derived AGE (MG-AGE) up-regulated cardiac RAGE mRNA and triggered cardiomyocyte contractile dysfunction reminiscent of diabetic cardiomyopathy. The MG-AGE-elicited prolongation of TPS and TR90 was ablated by an anti-RAGE antibody in cardiomyocytes. Interestingly, MG-AGE-induced cardiomyocyte dysfunction was associated with mitochondrial membrane potential (MMP) depolarization and reduced GSK-3β inactivation in control cardiomyocytes, similar to those from in vivo diabetes. Treatment with siRNA-RAGE ablated diabetes-induced MMP depolarization and GSK-3β inactivation. Collectively, our result implicated a role of AGE-RAGE in the pathogenesis of diabetic cardiomyopathy.

Keywords: diabetes • advanced glycation endproduct (AGE) • RAGE • cardiac • siRNA

Introduction

The prevalence of diabetes has increased significantly due to aging, obesity and sedentary life style with diabetic cardiovascular complications being one of the leading causes of death in diabetics [1, 2]. Diabetic cardiomyopathy develops in diabetics and is characterized by systolic and diastolic dysfunction independent of coronary vascular diseases [3, 4]. Impaired diastolic function is the most prominent mechanical abnormality manifested as prolonged relaxation and reduced compliance. Several mechanisms have been proposed for diabetic cardiomyopathy including oxidative stress, impaired glucose metabolism and intracellular Ca2+-regulating protein defect [4–7]. Nevertheless, none of these cellular defects seems to be the ultimate culprit factor responsible for diabetes or hyperglycaemia-induced myopathic alterations in hearts.

Recent evidence has implicated a role of advanced glycation endproduct (AGE) in the pathogenesis of diabetic complications in various organs including hearts [8–11]. AGEs are a group of heterogeneous compounds accumulated in diabetes due to factors
including increased reactive carbohydrate substrate availability, oxidative condition favouring glycation and impaired detoxification [12]. AGE has been postulated to participate in the pathogenesis of cardiovascular diseases through a direct physiochemical alteration of tissue/cellular property by forming cross-linked macromolecules [9, 11], or an indirect AGE receptor (RAGE)-mediated release of proinflammatory cytokines and reactive oxygen species (ROS) [13, 14]. Nonetheless, whether AGE, RAGE and their interaction contribute to the pathogenesis of diabetic cardiomyopathy has not been clearly elucidated. Therefore, the goal of our present study was two-fold. First, we aimed to evaluate the causal relationship among AGE formation, RAGE expression and AGE-RAGE colocalization in diabetic hearts. Secondly, we strived to examine the impact of the reactive α-ketoaldehyde methylglyoxal-derived AGE (MG-AGE) and in vivo gene knockdown of RAGE on myocardial contractile function in control or diabetic condition.

Materials and methods

Experimental animals, benfotiamine treatment and serum AGE measurement

All animal procedures described in this study were approved by the Institutional Animal Care and Use Committee at the University of Wyoming (Laramie, WY). In brief, 10-week-old male friend virus B (FVB) albino mice were made diabetic with a single intraperitoneal injection of streptozotocin (STZ, 200 mg/kg dissolved in 0.1 mol/l citrate buffer, pH 4.5) [15]. STZ-induced experimental diabetic model is well characterized in FVB mice [16, 17]. Age- and weight-matched control mice received a similar volume of physiological saline. Three days later, fasting blood glucose levels were determined using an Accu-Chek glucose monitor (Boehringer Mannheim Diagnostics, Indianapolis, IN, USA). Mice with fasting blood glucose levels >12 mmol/l were considered diabetic. Subsets of control and diabetic mice were gavaged with the AGE formation inhibitor benfotiamine (80 mg/kg/day) for 6 weeks immediately following induction of diabetes. All animals were maintained for 6 weeks in pairs (to minimize dominance) at 22°C with a 12/12 light/dark circadian cycle and were allowed to food and water ad libitum prior to experimentation. Serum AGE level was determined by ELISA using a monoclonal anti-AGE antibody 1H7G5. Results were expressed as MG-AGE unit (1U = 10 μg/m) of our in-house prepared MG-AGE normalized to total serum protein concentration.

In vivo gene knockdown of RAGE using siRNA silence

A chemically synthesized RAGE siRNA (Santa Cruz, Santa Cruz, CA, USA; catalog sc-36375) is a pool of 3 target-specific 20–25 nt siRNA designed to knock down RAGE gene expression. A scramble siRNA duplex was also generated to serve as a non-target control (siRNA-NT). All siRNA duplexes were suspended in lipofectamine and RNAsefree 1×PBS (1:3:1) for delivery. STZ-induced diabetic (5 days following STZ injection) and FVB control mice were anaesthetized using ketamine/xylazine (3:1, 1.23 mg/kg, i.p.). A left thoracotomy through the fifth intercostal space was performed and the heart was exposed. Intramyocardial injection was performed to deliver 20 μl (0.5 μg/g, bw) RAGE siRNA or siRNA-NT at two different apical points of the hearts via a 30-gauge needle. Sham-operated mice only received a thoracotomy. Forty-eight hours following siRNA treatment, hearts were isolated for experimentation. Surgical mortality (death occurring within 4 hrs postoperatively) was similar in all cryoinjury groups [18].

Determination of methylglyoxal (MG) level

MG levels were measured using the o-phenylenediamine (o-PD)-based assay [19]. Briefly, ventricular tissue was homogenized on ice followed by sonication (3 × 5 sec.) and centrifugation (12,000 × g for 10 min.). The supernatant was derivatized with 125 nmol of o-PD (derivatizing agent) at 20°C for 4 hrs. The quinoxaline derivative of MG (2-MQ) and the quinoxaline internal standard (5-MQ) were measured using a Beckman GOLD system HPLC.

AGE detection by immunohistochemistry and ELISA

AGE level was evaluated by both immunohistochemical staining and ELISA. Presence of AGE in cardiac tissue was detected by immunohistochemical staining [20]. Briefly, formalin-fixed and paraffin-embedded mouse ventricular tissues were cut into 8 μm serial paraffin sections and then deparaffinized. Slides were incubated with 0.05% protease K in 0.01 mol/l phosphate buffered saline (PBS), pH 7.4, for 30 min. at 37°C. After washing with PBS, the slides were dipped in 0.3% H2O2-100% methanol for 30 min. to block endogenous peroxidase. 1H7G5 (1:500) was used as the primary antibody and a fluorescein isothiocyanate-conjugated (FITC) anti-mouse IgG was used as the secondary antibody. For control staining, non-immune sera and/or excessive antigen were used, and negative staining was confirmed in all preparations. The staining was visualized under an excitation wavelength of 490 nm and an emission wavelength of 517 nm using an Olympus BX51 inverted microscope (Olympus Optical, Tokyo, Japan) equipped with a digital cooled charged coupled device camera and an Image-Pro Plus image processing/analysis software (Media Cybernetics, Silver Spring, MD, USA). For ELISA assay, following coating with AGE sample and blocking, each well was washed and incubated with the 1H7G5 antibody (1:2000) at 4°C. HRP-conjugated secondary antibody (1:2000) was added and incubated at 37°C for 1 hr. After washing six times, 100 μl NeA-Blue TMB substrate (Clinical Science Products Inc., MA, USA) was added and optical intensity was determined by micro plate reader at 450 nm after 10–30 min. incubation at 37°C followed by 1 mol/l H2SO4 to terminate the reaction [21].

Analysis of myocardial collagen cross-linking

Myocardial collagen solubility was analysed using the previously described method [9]. Briefly, the left ventricular sample was minced to an approximate of 3–4 mm and mixed with 1 ml of 250 μg/ml pepsin in 0.5 mol/l acetic acid at 37°C. After 2 hrs of pepsin digestion, a condition reported to cause solubilization of unmodified heart collagen [9], 200 μl of supernatant was removed and hydroxyproline concentration was measured using the hydroxyproline assay buffer. Optical absorbance was obtained with a spectrophotometer at 557 nm. The total recoverable myocardial collagen content was determined by the hydroxyproline
RT-PCR quantification of RAGE

The total RNA was extracted from both control (non-diabetic) and diabetic heart tissues, using Trizol® Reagent according to the manufacturer’s instruction (Invitrogen, Carlsbad, CA, USA), and the RNA concentrations were determined by UV spectrophotometry. Real-time quantitative polymerase chain reaction (qPCR) was performed on cDNA generated from 2 μg of total RNA using the Platinum® SuperScript III First Strand Synthesis System (Invitrogen). The RAGE primers (5′-GGA CTG CAT GCC GGA GTG TCT ATT 3′ (forward) and 5′-GCA TAG AAC CCG TCG GTG AGG 3′ (reverse)) were validated against GAPDH primers ([house keeping gene]: 5′-CTG gaa gtc gtt atg-3′ (forward) and 5′-GCC AGT TAG CTT CCG GTT CAG 3′ (reverse)) across a 10,000-fold dilution series to ensure that amplification efficiencies were not affected by transcript concentrations. For PCR, one-tenth of the cDNA reaction was used in the standard Platinum® SYBR-Green® qPCR SuperMix UDG (Invitrogen) and amplified in an iCycler (Bio-Rad). Melt curves were performed at the end of each amplification process to demonstrate the presence of a single amplification product. Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. The RAGE PCR Ct value was normalized by subtracting the GAPDH value, which gives the ΔCt value. From this value, the relative expression level to GAPDH for RAGE can be calculated using the ΔΔCt method [22].

Immunofluorescence detection of MG-AGE and RAGE in isolated cardiomyocytes

To localize MG-AGE and RAGE in the same cells, cover slips were incubated in 0.1% BSA in PBS for 30 min. at 37°C to minimize nonspecific binding of antibodies. Cells were stained with mouse anti-Aging (1:200) followed by fluorescein-conjugated sheep anti-mouse IgG. The cover slips were then incubated with rabbit anti-RAGE antiserum (1:100) and rhodamine-conjugated goat anti-rabbit IgG [23].

Preparation of methylglyoxal-modified bovine serum albumin (MG-AGE)

MG-AGE was prepared by incubating 7.2 mg/ml BSA with 100 mM methylglyoxal in 100 mM NaHCO3, pH 7.4, for 48 hrs at 37°C in sterile conditions as previously reported [24]. Control BSA was obtained following incubation in the same condition without methylglyoxal. After incubation, unreacted carbonyls were removed by extensive dialysis against ammonium bicarbonate buffer (30 mM, pH 7.9, 4°C). MG-AGE and control-BSA preparations were further filter-sterilized before assay and stored in a freezer at −20°C until used. MG-AGE (or control-BSA) concentration was expressed as the BSA protein concentration added to culture medium.

Mouse heart perfusions

Isolated mouse hearts were retrogradely perfused with a Krebs-Henseleit buffer containing 7 mM glucose, 0.4 mM oleate, 1% BSA and a low fasting concentration of insulin (10 μU/ml). Hearts were perfused at a constant flow of 4 ml/min. (equal to an aortic pressure of 80 mmHg) at baseline for 60 min. A fluid-filled latex balloon connected to a solid-state pressure transducer was inserted into the left ventricle through a left atriotomy to measure pressure. LVDP, the first derivative of LVDP (±dP/dt) and heart rate were recorded using a digital acquisition system at a balloon volume, which resulted in a baseline LV end-diastolic pressure of 5 mmHg [25].

Cardiomyocyte isolation and cultivation with MG-AGE

Mouse hearts were removed and perfused with Krebs-Henseleit bicarbonate buffer containing (in mM/l): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES and 11.1 glucose, with 5% CO2-95% O2. Hearts were subsequently digested with 223 U/ml collagenase D (Boehringer Mannheim, Indianapolis, IN, USA) for 20 min. at 37°C. After perfusion, left ventricles were removed and minced before being filtered. Extracellular Ca2+ was slowly added back to 1.25 mM/l. Isolated myocytes were cultured in minimum essential medium (MEM) containing 100 U/ml penicillin-streptomycin, 2 mM/l L-Glutamine, 0.1 mg/ml BSA supplemented with MG-AGE or control-BSA (0–5 μM/l) at 37°C for 2 hrs. Myocytes with obvious sarcoplastic blebs or spontaneous contractions were not used [26].

Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using a SoftEdge MyoCam® system (IonOptix Corporation, Milton, MA, USA) [28]. In brief, myocytes were placed in a Warner chamber mounted on the stage of an inverted microscope (Olympus, IX-70) and were field stimulated with suprathreshold voltage at a frequency of 0.5 Hz. The myocyte being studied was displayed on a computer monitor using an IonOptix MyoCam camera. An IonOptix SoftEdge software was used to capture changes cell shortening and relengthening and the indices assessed included peak shortening (PS) amplitude, time-to-peak...
Table 1  Biometric and myocardial contractile features of control and STZ-induced diabetic mice

| Mouse group                      | Control      | Diabetic     | Diabetic + siRNA-RAGE | Diabetic + siRNA-NT |
|----------------------------------|--------------|--------------|------------------------|---------------------|
| Body weight (g, pre-STZ)         | 23.6 ± 0.2   | 23.3 ± 0.4   | 23.2 ± 0.5             | 22.9 ± 1.1          |
| Body weight (g, at killing)      | 27.1 ± 0.3   | 22.6 ± 0.3*  | 23.1 ± 0.6             | 22.7 ± 1.0          |
| Heart weight (mg)                | 194 ± 5      | 153 ± 5*     | 151 ± 7*               | 150 ± 6*            |
| Heart/weight (mg/g)              | 7.17 ± 0.16  | 6.81 ± 0.22  | 6.52 ± 0.15            | 6.64 ± 0.10         |
| Liver weight (mg)                | 1466 ± 61    | 1404 ± 48    | 1397 ± 63              | 1389 ± 71           |
| Liver/body weight (mg/g)         | 54.2 ± 2.3   | 62.4 ± 2.4*  | 60.3 ± 1.4*            | 61.3 ± 1.9*         |
| Kidney weight (mg)               | 409 ± 7      | 420 ± 8      | 428 ± 5                | 411 ± 10            |
| Kidney/body weight (mg/g)        | 15.1 ± 0.3   | 18.7 ± 0.5*  | 18.5 ± 0.3*            | 18.2 ± 0.4*         |
| Fasting blood glucose (mmol/l)   | 5.95 ± 0.14  | 21.0 ± 0.83* | 19.1 ± 0.93*           | 18.5 ± 1.21*        |
| Serum level of AGE (U)           | 9.5 ± 0.4    | 17.1 ± 0.5*  | 15.6 ± 0.7*            | 15.3 ± 0.8*         |
| Heart rate (ex vivo, bpm)        | 231 ± 47     | 259 ± 51     | 263 ± 48               | 237 ± 64            |
| LVDP (mmHg)                      | 73.2 ± 5.6   | 37.4 ± 4.8*  | 57.5 ± 4.3*            | 33.4 ± 4.6*         |
| +dP/dt (mmHg/sec.)               | 3453 ± 511   | 1087 ± 187*  | 2750 ± 213#            | 997 ± 102*          |
| −dP/dt dt (mmHg/sec.)            | −2304 ± 168  | −1001 ± 184* | −1939 ± 117#           | −1039 ± 103*        |

siRNA-NT: siRNA non-target control, mean ± S.E.M., n = 19 mice per group, *P < 0.05 versus control group; #P < 0.05 versus diabetic group.

shortening (TPS), time-to-90% relengthening (TR90), maximal velocity of shortening and relengthening (±dL/dt).

Measurement of mitochondrial membrane potential (MMP)

Cardiomyocytes were suspended in HEPES-saline buffer and MMP (∆Ψm) was detected as described [27]. Briefly, after pre-incubation with 5 μmol/l JC-1 for 10 min. at 37°C, cells were washed two times by sedimentation using HS buffer free of JC-1 and resuspended in MEM supplemented with MG-AGE or BSA (2.5 μmol/l) at 37°C for 4 hrs. During the incubation period, cardiomyocytes were examined periodically under confocal laser scanning microscope (Leica TCS SP2) at excitation wavelength of 490 nm and emission fluorescence was recorded at 530 nm (monomer form of JC-1, green) and at 590 nm (aggregate form of JC-1, red). Alternatively, fluorescence of each sample was read at excitation wavelength of 490 nm and emission wavelength of 530 nm and 590 nm using a spectrofluorimeter (Spectra MaxGeminiXS, Spectra Max, Atlanta, GA, USA) at an interval of 10 sec. Results in fluorescence intensity were expressed as 590-to-530-nm emission ratio. The mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μmol/l) was used as a positive control for MMP measurement.

Statistical analysis

Data were mean ± S.E.M. Differences were evaluated by analysis of variance (ANOVA) using a Tukey post hoc test. A P-value less than 0.05 was deemed statistically significant.

Results

General feature and whole heart function of control and diabetic mice with or without RAGE gene knockdown

Diabetic mice displayed significantly reduced body weight gain and heart weight, enlarged liver and kidney size (organ weight normalized to body weight) associated with hyperglycaemia compared with control mice. Diabetes did not exert any effect on liver and kidney weight or heart size. Both fasting blood glucose and serum AGE levels were markedly elevated in STZ-induced diabetic mice. The diabetes-induced changes in biometrics, levels of AGE and glucose were not affected by siRNA-RAGE or siRNA non-target control (siRNA-NT). Isolated heart function analysis revealed significantly reduced LVDP, maximal velocity of pressure development and decline (± dP/dt) in diabetic hearts, which was ablated by RAGE gene knockdown but not siRNA-NT. There was no difference in ex vivo heart rate among all groups studied (Table 1).

Levels of methylglyoxal, AGE formation and RAGE in cardiac tissues of diabetic mice

Increased methylglyoxal levels, the precursor of AGE formation, have been reported in diabetes, which may contribute to the pathogenesis
of diabetic complications [28, 29]. However, it is still not clear if elevated methylglyoxal level persists in diabetic heart and contributes to diabetic cardiomyopathy. Our HPLC data revealed that methylglyoxal level in diabetic hearts was ~57% higher than that of control hearts. RAGE expression was significantly up-regulated in diabetic hearts compared to control group, which was nullified by RAGE gene knockdown but not siRNA-NT (Fig. 1). Consistently, AGE accumulation was markedly augmented in diabetic hearts evaluated by immunofluorescent localization (Fig. 2A and B) and ELISA (control: 2.11 ± 0.17 versus diabetic: 4.08 ± 0.14 A450/mg protein, n = 10, P < 0.05 between control and diabetic groups) using an anti-AGE antibody 1H7G5 against methylglyoxal-derived imidazolone [30].

**Colocalization of MG-AGE and RAGE with immunocytochemistry in cardiomyocytes**

AGE-RAGE interaction is permissive to cumulative AGE-induced diabetic complications [31]. To correlate if MG-AGE and RAGE are simultaneously present in single cardiomyocytes, an immunocytochemistry assay was performed to localize MG-AGE and RAGE in isolated cardiomyocytes. Our result shown in Fig. 2C–F suggested simultaneous elevations in both MG-AGE formation and RAGE expression in diabetic cardiomyocytes, indicating colocalization of MG-AGE and RAGE.

**Effect of benfotiamine on cardiac methylglyoxal and MG-AGE in control and diabetic hearts**

To determine the causal link between elevated AGE levels and diabetic cardiomyocyte dysfunction, benfotiamine, an inhibitor of AGE formation, was used to interrupt AGE pathway [32, 33]. Consistent with our earlier report that benfotiamine protects against cardiomyocyte contractile dysfunction in diabetic hearts [16], results shown in Fig. 3 depicted that benfotiamine significantly attenuated or ablated diabetes-induced elevation in cardiac levels of methylglyoxal, RAGE, MG-AGE formation and collagen...
Fig. 2 Effect of diabetes on cardiac AGE formation and AGE-RAGE colocalization. (A, B) Representative immunofluorescent images depicting AGE distribution in control and diabetic ventricular tissues; (C, D) AGE-RAGE colocalization in control mouse cardiomyocytes; (E, F) AGE-RAGE colocalization in diabetic mouse cardiomyocytes. For panel C through F, myocytes were probed with an anti-AGE antibody followed by a fluorescein isothiocyanate-conjugated anti-mouse IgG for AGE detection. The cover slips were then incubated with a rabbit anti-RAGE antiserum and a rhodamine-conjugated goat anti-rabbit IgG for RAGE localization.
cross-linking (shown as increased ventricular collagen solubility). However, benfotiamine treatment did not significantly alter STZ-induced elevation in plasma levels of triglycerides and cholesterol. The cardiac levels of methylglyoxal, RAGE, MG-AGE formation and collagen cross-linking as well as plasma levels of triglycerides and cholesterol were not affected by benfotiamine treatment in non-diabetic mice. Benfotiamine did not affect diabetes-associated hyperglycaemia and reduced body weight gain (data not shown).

**Effect of RAGE gene knockdown on cardiomyocyte mechanical function**

To better understand the role of RAGE in the pathogenesis of diabetic cardiomyopathy, we examined the effect of RAGE gene knockdown using siRNA silence. Results presented in Fig. 4 indicated that STZ diabetes-induced decrease in PS and prolongation in TPS and TR90 were significantly attenuated by RAGE gene knockdown. RAGE gene silence did not have any effect in control mouse cardiomyocytes. In addition, cardiomyocyte mechanical function was not affected by siRNA-NT or sham-operation used for siRNA delivery (data not shown).

**Effect of MG-AGE on cardiomyocyte function and cardiac RAGE mRNA expression**

To better understand the role of AGE in the pathogenesis of diabetic cardiomyopathy, we examined the direct impact of AGE incubation (2 hrs) on cardiomyocyte mechanical function by constructing a concentration-dependent response of MG-AGE (0–5 μmol/l). The concentrations of MG-AGE used here were chosen based on previous reports [24, 34] and our ELISA measurement of serum AGE level (0.4–1.0 and 1.6–2.8 μmol/l in control and diabetic mice, respectively). Therefore, the levels of MG-AGE used here are considered within supra-physiological ranges. Results presented in Table 2 indicated that control myocytes treated with high levels of MG-AGE (2.5 and 5 μmol/l) displayed reduced PS and -$\Delta$L/dt as well as prolonged TPS and TR90, reminiscent of STZ-induced diabetic cardiomyocyte defects [16]. Moreover, diabetic cardiomyocytes were more susceptible to AGE-induced mechanical alteration. The -$\Delta$L/dt and TR90 from diabetic group were deteriorated at much lower concentrations of MG-AGE compared with those from control group. In addition, MG-AGE (0–5 μmol/l) significantly up-regulated cardiac RAGE mRNA expression with a more pronounced fold increase in control group (Fig. 4A). To evaluate if AGE-RAGE interaction is permissive to MG-AGE-elicited cardiomyocyte contractile dysfunction, cardiomyocytes from control mice were coincubated with a specific RAGE antibody to neutralize RAGE therefore nullifying interaction between AGE and RAGE. Data displayed in Fig. 4B–D indicated that the anti-RAGE antibody negated MG-AGE-induced decrease in PS as well as prolongation of TPS and TR90. The anti-RAGE antibody itself did not have any effect on PS, TPS and TR90 in the absence of MG-AGE treatment (data not shown). The anti-RAGE-elicited beneficial effects against MG-AGE were not reproduced by the non-immune IgG, which served as a control for the anti-RAGE antibody.

**Effect of MG-AGE and siRNA-RAGE on MMP and GSK-3β phosphorylation**

Given that mitochondrial function is essential to cardiomyocyte viability and function [35, 36], the cationic lipophilic probe JC-1 was employed to monitor MMP ($\Delta$Ψm) in response to MG-AGE or siRNA treatment. The dynamic change of $\Delta$Ψm was displayed by change in the ratio between red (aggregated JC-1) and green (monomeric form of JC-1) fluorescence (Fig. 5A and B). Quantitative analysis showed a significant reduction in the ratio between red and green fluorescence in response to the 2-hr MG-AGE (2.5 μmol/l) treatment, indicating a fall in $\Delta$Ψm. Interestingly, MG-AGE-induced fall in $\Delta$Ψm was rescued by
siRNA-RAGE. RAGE gene knockdown itself did not exert any significant effect on ΔΨm (Fig. 5E). Temporal and kinetic change of ΔΨm was verified with the positive control CCCP (Fig. 5C and E). Measurement of ΔΨm also revealed a significant drop in MMP in diabetic cardiomyocytes compared with those from control group, the effect of which was ablated by siRNA-RAGE but not siRNA-NT (Fig. 5D and F). As a key factor in the regulation of mitochondrial permeability transition, GSK-3β participates in the mitochondrial-mediated myocardial dysfunction [37, 38]. In agreement with the dynamic change of ΔΨm in response to MG-AGE treatment, MG-AGE elicited a transient rise in GSK-3β phosphorylation followed by a delayed dephosphorylation of GSK-3β. Further assessment of GSK-3β phosphorylation in hearts from control and diabetic mice also indicated a significant loss in GSK-3β phosphorylation in diabetic hearts, the effect of which was restored by siRNA-RAGE but not siRNA-NT (Fig. 6).

### Discussion

Results from our current study demonstrated that STZ-induced diabetes is associated with colocalized AGE formation and enhanced RAGE expression in cardiomyocytes. Benfotiamine, an AGF formation inhibitor, attenuated diabetes-induced elevation in AGE, RAGE and collagen cross-linking without affecting hypertriglyceridaemia and hypercholesterolaemia in diabetic mice. More importantly, RAGE gene knockdown obliterated diabetes-induced cardiac contractile dysfunction, consistent with the finding that MG-AGE directly up-regulated cardiac RAGE mRNA and deteriorated cardiomyocyte contractile function reminiscent of diabetes. The MG-AGE-induced cardiomyocyte dysfunction was nullified by an anti-RAGE antibody to neutralize AGE-RAGE interaction but not by the non-immune IgG. Our study also revealed a change in the responsiveness of diabetic cardiomyocytes to MG-AGE-elicted mechanical dysfunction compared with those from the control group. Interestingly, MG-AGE-induced cardiomyocyte dysfunction was associated with MMP depolarization and reduced GSK-3β inactivation), in a manner similar to in vivo diabetes. These effects were interrupted by RAGE gene knockdown, supporting the premise that interaction between AGE and RAGE may trigger diabetes-like cardiac contractile dysfunction.

In this study, diabetic mice developed typical signs of diabetes, such as hyperglycaemia, hyperlipidaemia and waste syndrome, similar to earlier observations in chemically induced and genetically predisposed diabetes [15, 17, 39]. Hyperglycaemia is closely associated with elevated levels of methylglyoxal, a highly reactive dicarbonyl α-ketoaldehyde derived from glycolytic intermediate

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Table 2 Effect of methylglyoxal-modified BSA (MG-AGE) on cell shortening and relengthening properties in cardiomyocytes from control and STZ-induced diabetic mice (2 hrs of treatment)

|                         | Control   | 0.1 µmol/l | 0.5 µmol/l | 2.5 µmol/l | 5.0 µmol/l |
|-------------------------|-----------|------------|------------|------------|------------|
| CL (µm)                 | 105.6 ± 3.6 | 102.6 ± 3.1 | 103.9 ± 2.7 | 101.9 ± 2.7 | 101.2 ± 2.8 |
| PS (% CL)               | 5.39 ± 0.48 | 5.15 ± 0.47 | 5.37 ± 0.34 | 4.73 ± 0.33 | 4.10 ± 0.22 |
| +dL/dt (µm/sec.)        | 134.3 ± 10.1 | 136.3 ± 10.7 | 132.3 ± 10.2 | 139.3 ± 8.5 | 116.5 ± 5.7 |
| dL/dt (µm/sec.)         | −144.7 ± 11.7 | −139.9 ± 11.4 | −140.0 ± 7.8 | −135.9 ± 7.9 | −112.2 ± 4.9 |
| TPS (ms)                | 81 ± 4     | 77 ± 3     | 82 ± 3     | 96 ± 5     | 93 ± 7     |
| TR90 (ms)               | 118 ± 6    | 124 ± 4    | 120 ± 5    | 183 ± 10   | 176 ± 12   |

CL = cell length; PS = peak shortening; +dL/dt = maximal velocity of shortening; dL/dt = maximal velocity of relengthening; TPS = time-to-PS; TR90 = time-to 90% relengthening. Mean ± S.E.M., n = 50 cells per group, *P < 0.05 versus respective control (2.5 µmol/l BSA), #P < 0.05 versus control mice.
fragmentation (triose phosphates) or acetone oxidation predominant in diabetic condition [12, 40]. Increased methylglyoxal formation has been implicated in the development of essential hypertension and type 2 diabetes [28, 30]. In line with the notion that pathogenesis of diabetes is associated with an accentuated methylglyoxal metabolism [40, 41], our current study demonstrated that diabetic cardiac tissues produced a greater amount of methylglyoxal in parallel with a marked increase in serum AGE levels independent of RAGE expression. This finding favours the predisposing role of methylglyoxal in AGE formation [12]. Data from our study revealed that benfotiamine reduced formation of cardiac methylglyoxal, MG-AGE and RAGE in diabetic mice, in agreement with our earlier report that benfotiamine alleviates cardiomyocyte contractile dysfunction in STZ-induced diabetic hearts [16]. Our data that benfotiamine negated diabetes-induced AGE accumulation are consistent with reports from non-cardiac tissues [42, 43].

Our results indicated that MG-AGE decreased PS and±dL/dt, as well as prolonged TPS and TR90. TPS was prolonged by 19% and 38% and TR90 was prolonged by 55% and 80%, respectively, in control and diabetic groups. These mechanical alterations are reminiscent of diabetic cardiomyopathy seen in whole heart [44] and cardiomyocytes [15–17]. Given that the MG-AGE and diabetes-elicited alterations were nullified by the anti-RAGE antibody and siRNA-RAGE, respectively, it is plausible to speculate that the
AGE-RAGE interaction is essential to cardiac dysfunction and the onset of diabetic cardiomyopathy. Several mechanisms have been postulated for a role of AGE-RAGE interaction in diabetic heart dysfunction. The AGE-RAGE axis has been depress sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) function and contribute to diabetic cardiomyopathy [45]. Bidasee and colleagues demonstrated that AGE leads to post-modification of SERCA protein and leads to impaired cardiac relaxation in diabetes [8]. The dampened SERCA function is consistent with the aberrant cardiomyocyte intracellular Ca\(^{2+}\) homeostasis in response to AGE-RAGE interaction [46]. In addition to diabetes-induced increase in cardiac AGE, RAGE and their colocalization in cardiomyocytes, serum AGE was also elevated in our diabetic model. Serum AGE levels are known to positively correlate with left ventricular diastolic dysfunction in patients with both type 1 and type 2 diabetes [47, 48]. The diabetes-induced change in MG-AGE-induced

**Fig. 5** Cardiomyocyte mitochondrial membrane potential (MMP) from control cardiomyocytes incubated with BSA (A, 2.5 μmol/l), MG-AGE (B, 2.5 μmol/l) or CCCP (C, 10 μmol/l) for 1 and 4 hrs as well as STZ-induced diabetic myocytes (D, E) Kinetic changes of MMP in response to MG-AGE or siRNA treatment using JC-1 fluorochrome (ratio of red to green fluorescence); (F) MMP between control and diabetic mice with siRNA-RAGE or siRNA-NT treatment. Mean ± S.E.M., n = 4, *P < 0.05 versus BSA or control group, #P < 0.05 versus diabetic or MG-AGE group.
cardiomyocyte contractile response seen in our study indicates diabetic cardiomyocytes may be prone to the MG-AGE-induced culprit effect. Diabetes is associated with reduced myocardial compliance partially due to AGE accumulation on myocardial collagen, which results in a significant decrease in myocardial collagen solubility, an index of increase formation of collagen cross-link \[9, 10\]. Our result that benfotiamine managed to negate diabetes-induced reduction in myocardial collagen solubility suggests inhibition of AGE-induced collagen cross-link may contribute, at least in part, to the beneficial effect of benfotiamine against diabetic cardiac dysfunction \[16\].

MMP has been considered an essential indicator for mitochondrial and ultimately cardiomyocyte physiological function \[36, 49\]. GSK-3β, on the other hand, plays an integral role in the

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**Fig. 6** Time-dependent phosphorylation of GSK-3β (serine-9) in response to MG-AGE and control BSA (2.5 μmol/l). (A) GSK-3β phosphorylation by BSA and MG-AGE (0–4 hrs); (B) Pooled data of GSK-3β phosphorylation in control myocytes; (C) pooled data of GSK-3β phosphorylation in control and diabetic hearts treated with siRNA-RAGE or siRNA-NT. Inset: Representative gels of GSK-3β phosphorylation using specific antibodies. Mean ± S.E.M., n = 4, *P < 0.05 versus BSA or control group, #P < 0.05 versus MG-AGE at time 0 or diabetic group.
convergence of signals to determine the mitochondrial permeability transition pore [37]. In our study, RAGE gene knockdown nullified MG-AGE-induced depolarization of cardiomyocyte MMP and diabetes associated with GSK-3β dephosphorylation, supporting a role of mitochondrial dysfunction in MG-AGE- and diabetes-induced cardiomyocyte dysfunction. Further study is warranted to elucidate the mechanism behind the AGE-RAGE interaction-triggered cardiac dysfunction.

Experimental limitations: Although our results favour an important role of methylglyoxal in AGE formation and subsequent impairment of insulin signalling [50], methylglyoxal may exert deleterious effects on cardiac mitochondrial respiration independent of AGE formation [51]. Our current study did not characterize the cardiomyocyte contractile response of methylglyoxal; therefore, a possible contribution of methylglyoxal to diabetic or MG-AGE-induced myopathic alteration cannot be ruled out at this time. In addition, non-imidazolones formed through reaction of methylglyoxal with protein lysine residues may play a role in the MG-AGE-elicited cardiac response in a RAGE-independent manner. Besides the imidazolone adducts formed through methylglyoxal reaction with arginine residues, non-imidazolones including Nε-(carboxyethyl)lysine (CEL) and the imidazolium cross-link methylglyoxal-lysine dimer (MOLD) are reported to be elevated in plasma of diabetic animals [52]. Increase of CEL and MOLD may facilitate protein cross-link such as augmented collagen cross-link and altered cardiac function. Future study is warranted to examine the effects of these non-imidazolones on cardiomyocyte mechanical function. Although our study suggests that benfotiamine alleviates diabetes-induced cardiomyocyte dysfunction [16] through inhibition of the AGE-RAGE axis, caution has to be taken for data interpretation because benfotiamine also blocks the hexosamine and diacylglycerol-protein kinase C pathways in addition to the AGE pathway [30]. Inhibition of either hexosamine or diacylglycerol-protein kinase C pathway is known to benefit diabetic heart function [12]. Last but not the least, it is worth mentioning that interrupted AGE-RAGE interaction inhibits angiogenic signal, which may exert a secondary effect on diabetic cardiac complication through altered angiogenesis [53].

In summary, our data suggest a possible link between the AGE-RAGE interaction and cardiac contractile dysfunction in diabetes. Given what we know about the cardiac contractile function in diabetes, the clinical value of employing either AGE formation inhibitors such as benfotiamine, or disturbing AGE-RAGE interaction (such as silencing RAGE) in alleviating diabetes-induced cardiac dysfunction may have a promising future.

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