Methylglyoxal increases cardiomyocyte ischemia-reperfusion injury via glycate inhibitory of thioredoxin activity

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DM is a major risk factor for ischemic heart disease development (3), directly adversely affecting ischemic cardiomyocytes, resulting in larger infarct size and more severe heart failure after ischemia-reperfusion. Although many signaling pathways relating diabetic cellular injury and cardiac dysfunction have been reported, the specific molecular basis linking DM with increased vulnerability to ischemia-reperfusion injury and resultant mortality has not been established.

Methylglyoxal (MG), a highly reactive dicarbonyl, is a natural metabolite in glucose metabolism. It is capable of inducing the nonenzymatic reaction glycation, or glycosylation, between reducing sugars and proteins and other biomolecules, yielding irreversible advanced glycation end products (AGEs) (5, 28). The concentration of MG is increased not only in diabetic animal tissues (37) but also in the plasma of diabetic patients (4, 11). Elevated MG levels are believed to contribute to complications seen in poorly controlled diabetic states. Indeed, recent investigations have demonstrated that MG induces apoptosis of rat Schwann cells (12) and human vascular endothelial cells (2), buttressing evidence to the significant role MG plays in the etiology of diabetic complications. However, the role of MG in ischemic injury endured in the diabetic cardiomyocyte and any potentially involved mechanisms (apoptotic or otherwise) remain unidentified.

Thioredoxin (Trx), a 12-kDa protein ubiquitously expressed in all living cells, fulfills a variety of biological functions related to regulation of cellular proliferation and apoptosis (41) and cytoprotection against oxidative stress (46). Clinical and experimental results have demonstrated that inhibition of Trx promotes apoptosis (24). Recent in vitro studies have demonstrated that Trx interacts directly with and inhibits the activity of apoptosis-regulating kinase-1 (ASK1), a mitogen-activated protein kinase (MAPK) that activates two proapoptotic kinases, p38 MAPK and JNK (26). These results give mechanistic insight as to how Trx may critically regulate the balance between cell proliferation and cell death.

Recent studies have demonstrated that besides upregulation or downregulation of Trx expression at the gene level, Trx activity is regulated by posttranslational modification. Five forms of posttranslational modifications of Trx have been identified previously, with each modification affecting Trx differently. These include oxidation, glutathionylation, S-nitrosylation, nitration, and glycation. We have demonstrated recently that Trx is susceptible to nonenzymatic glycation via lipopolysaccharide (LPS) exposure (47) and consequent inactivation and is furthermore unable to provide protection against LPS-induced liver toxicity. However, whether Trx activity is altered in the presence of prolonged MG exposure and any functional consequence of such alteration with respect to car-
diomyocyte protection against simulated ischemia-reperfusion has never been investigated.

Therefore, the aims of the present study were 1) to determine whether long-term treatment with MG can enhance the injury of cultured H9c2 cardiomyocytes subjected to simulated ischemia-reperfusion and, if so, 2) to investigate whether Trx activity was reduced after long-term treatment with MG, 3) to determine the signaling mechanism(s) by which reduced Trx activity leads to increased cardiomyocyte death after prolonged MG exposure, and 4) to investigate whether administration of the glycation inhibitor aminoguanidine (AG) might be a therapeutic intervention reversing the observed phenomena related to MG exposure of cardiomyocytes.

MATERIALS AND METHODS

Cell culture and experimental protocol. H9c2 cardiomyoblasts (referred to as cardiomyocytes thereafter), an embryonic rat heart-derived cell line (American Type Culture Collection, Manassas, VA), were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% calf bovine serum (CBS; MP Biomedical, Solon, OH), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were maintained at 37°C under a water-saturated atmosphere of 95% ambient air and 5% CO2 (normoxic conditions). Stock cultures were passaged at 2- to 3-day intervals. Cells were seeded at a density of 3 × 10^5 cells/35-mm well of six-well plates for 24-h culture and were made quiescent by overnight serum starvation (0% CBS). After 6 days of treatment with MG (200 μM; Sigma-Aldrich, St. Louis, MO) or MG (200 μM) plus AG hemisulfate (100 μM; Sigma-Aldrich), the cells were subjected to simulated ischemia-reperfusion, as described previously (30). Briefly, the cells were incubated in slightly hypotonic Hanks’ balanced saline solution (1.3 mm CaCl2, 5 mm KCl, 0.3 mm KH2PO4, 0.5 mm MgCl2, 0.4 mm MgSO4, 69 mm NaCl, 4 mm NaHCO3, and 0.3 mm NaH2PO4) without glucose or serum and transferred in an airtight incubator from the controls received water only. The reaction was terminated by adding 125 μl of stopping solution (0.2 M Tris-Cl, 10 M guanidine-HCl, and 1.7 mM 3-carboxy-4-nitrophenyl disulfide). Absorption measurement occurred at 412 nm.

Immunocytochemical detection of AGEs. Cardiomyocytes, seeded on glass coverslips in six-well plates, were treated as described above. Briefly, cells were fixed with polyformaldehyde (4% in PBS) for 1 h, washed with PBS, blocked with 10% normal goat serum, and incubated with rabbit anti-AGE polyclonal antibody (Abcam, Cambridge, MA). Immunostaining was performed utilizing a Vectastain ABC kit (Vector Laboratories, Burlingame, CA), and examined under light microscopy.

Detection of Trx-ASK1 interaction. Cells were homogenized with lysis buffer. Immunoprecipitation and immunoblotting were performed using a procedure described by Vandenhout et al. (43). In brief, endogenous Trx was immunoprecipitated with a monoclonal anti-murine Trx antibody (Redox Bioscience). After sample separation, the Trx-ASK1 interaction was determined by Western blot analysis using a monoclonal antibody against ASK1 (Upstate Biotechnology, Lake Placid, NY) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA).

p38 MAPK activity assay. The p38 MAPK activity assay was performed utilizing a p38 MAPK assay kit (Cell Signaling Technology) with substrate activating transcription factor-2 (ATF-2) per the manufacturer’s instructions (14). In brief, cells were homogenized in ice-cold lysis buffer. Lysates were sonicated on ice and centrifuged at 12,000 g for 10 min at 4°C. Immunoprecipitation was performed by adding 20 μl of resuspended immobilized monoclonal antibody against phospho-p38 MAPK (Thr180/Tyr182) to 100 μl of cell lysate containing 150 μg of protein. The mixture was incubated with gentle rocking overnight at 4°C. After a 10,000-g centrifugation at 4°C for 2 min, the pellets were washed twice with lysis buffer and twice with kinase buffer. The kinase reactions occurred in the presence of 200 μM ATP and 2 μg ATF-2 fusion protein at 30°C for 30 min. After incubation the samples were separated by SDS-PAGE, and ATF-2 phosphorylation was measured by Western immunoblotting using a monoclonal antibody against phosphorylated ATF-2, followed by enhanced chemiluminescent detection.

In vitro incubation of recombinant Trx with MG or MG ± AG. To investigate the effects of MG on Trx directly, 1 μg of recombinant human Trx (hTrx; Sigma-Aldrich) was incubated with 500 μM MG at 37°C for 8 days. Activity of treated Trx (0.3 μg) was then determined as described above. Control Trx was incubated with deionized water.
under the same conditions. For the antiglycation treatment, AG (300 
\mu\text{M}) was added at the initiation of incubation.

Western blot analysis. Cultured cardiomyocyte cells were collected in lysis buffer after treatment. Aliquots containing 30–60 \mu g of protein were separated by electrophoresis through 8–12% SDS-polyacrylamide gel and transferred to positively charged nylon membranes. The membranes were blocked with 5% dry fat-free milk in Tris-buffered saline containing 0.1% Tween-20 and then incubated with primary antibodies against Trx (Redox Biosciences), p38, phospho-p38, GAPDH (Cell Signaling Technology), and N\(^{-}\)-carboxymethyl lysine (CML; Abcam). Positively charged nylon membranes were then incubated with HRP-conjugated anti-rabbit immunoglobulin G antibody (Cell Signaling) for 1 h. The blot was developed with a Supersignal Chemiluminescence Detection Kit (Pierce, Rockford, IL). Bands were visualized with a Kodak Images Station 400 (Rochester, NY), and the band densities were analyzed with Kodak 1-Dimensional software (version 3.6).

Statistical analysis. All values in the text, table, and figures are presented as means ± SE of \(n\) independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc \(t\)-test. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn’s posttest. Probabilities of 0.05 or less were considered to be statistically significant.

RESULTS

Preculturing cardiomyocytes with high MG increased their susceptibility to SI-R injury. Cardiomyocytes subjected to SI-R injury manifested significant cellular injury including necrotic and apoptotic cell death, as evidenced by increased LDH release, TUNEL staining, and caspase-3 activity (Fig. 1). Under basal conditions, cardiomyocytes treated with MG developed normally without apparent injury (MG + Sham SI-R groups in Fig. 1). However, when subjected to SI-R, the cells treated with MG for 6 days (200 \mu M) exhibited significantly greater cellular injury compared with control in the parameters listed above.

![Diagram](https://via.placeholder.com/150)

**Fig. 1.** Simulated ischemia-reperfusion (SI-R) injury is increased significantly in methylglyoxal (MG)-precultured cardiomyocytes, as measured by lactate dehydrogenase (LDH) release (A), apoptotic index by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive staining cells (B), and caspase-3 activity (C). D: MG preculture results in cardiomyocyte glycation prior to being subjected to SI-R, as measured by total advanced glycation end product (AGE) content; \(n = 6\) independent experiments. **\(P < 0.01\) vs. Sham SI-R; ##\(P < 0.01\) vs. vehicle + SI-R. AFC, 7-amino-4-trifluoromethyl-coumarin.
candidates, Trx was selected because 1) Trx is a critical antiapoptotic and cell survival molecule, and its inactivation has been causatively related to cardiovascular injury, and 2) we recently demonstrated that the Trx is susceptible to glycative modification by LPS and consequent activity inhibition. To directly test a novel hypothesis that MG may cause Trx-glycative inactivation, rendering cardiomyocytes more susceptible to SI-R injury, we first determined whether Trx can be glycatively modified by MG with subsequent activity inhibition. Recombinant hTrx (human thioredoxin-1) was incubated with MG in a cell-free system, and Trx glycation and activity were determined. As summarized in Fig. 4A, in vitro incubation of hTrx caused Trx glycation, as evidenced by abundant production of CML, a biomarker of AGE formation. MG incubation markedly inhibited Trx activity (60.4% reduction, \( P < 0.01 \) vs. vehicle-incubated Trx; Fig. 4B). More importantly, addition of AG in the system completely abolished MG-induced Trx glycation (Fig. 4A) and significantly attenuated MG-induced Trx inactivation (Fig. 4B).

Trx is glycatively inhibited in MG-pretreated cardiomyocytes prior to SI-R injury. Having demonstrated that MG is capable of causing Trx-glycative inhibition in a cell-free system, we further determined whether cellular Trx activity/expression might be reduced after MG exposure, leaving the cells more vulnerable to reperfusion injury. Compared with control, Trx activity was significantly decreased in the MG-treated cells prior to SI-R (Fig. 5A). However, Trx expression was slightly increased in MG-treated cells (Fig. 5B), indicating that the observed reduction in Trx activity in the MG-treated cells is not from reduced expression of the protein but rather its posttranslational modification. More importantly, cotreatment with AG significantly attenuated MG inhibition of Trx (Fig. 5A).

Preculturing cardiomyocytes with MG promoted SI-R-induced Trx-ASK1 dissociation and subsequent p38 MAPK activation, which were attenuated by cotreatment with AG. Recent in vitro studies have demonstrated that the binding and resultant inhibition of ASK1 is the primary mechanism by which Trx exerts its antiapoptotic effect (38). Moreover, the increased ratio of ASK1/Trx-ASK1 correlates with the increased basal activity of the p38 MAPK pathway (18). To determine whether MG inhibition of Trx may alter the Trx-ASK1 interaction and consequently activate downstream proapoptotic kinases, two additional experiments were performed. Via anti-Trx-1 immunoprecipitation and anti-ASK1 immunoblotting, Fig. 5A illustrates that Trx is physically associated with ASK1 in normal cultured cardiomyocytes, and this protein-protein interaction was significantly decreased after SI-R. Consequently, the activity of p38 MAPK, a proapoptotic downstream molecule for ASK1, was significantly enhanced in the MG-treated cardiomyocyte compared with control (Fig. 5B). More importantly, this SI-R-induced disassociation of Trx-ASK1 was further significantly enhanced when cells were precultured with MG, and p38 MAPK activity was further significantly increased (Fig. 5B, far right bar). Treatment with
aminoguanidine restored Trx-ASK1 interaction (Fig. 5C) and reduced p38 MAPK phosphorylation (Fig. 5D) in MG-treated cardiomyocytes.

**DISCUSSION**

Ischemic heart disease continues to gain prevalence as a cause of disability and death in the United States and is costly in terms of patient morbidity and mortality as well as financial resources utilized in acute and chronic treatment. The specific molecular mechanisms underlying why diabetes mellitus directly increases ischemic heart disease risk remain elusive. Accumulating evidence has indicated that MG, a reactive dicarbonyl compound produced mainly from cellular glycolytic intermediates, is often found at high circulating blood levels in diabetic patients (4, 11, 39). Evidence suggests elevated MG levels may play a role in the development of a number of diabetic complications (32). Elucidation of the effects of MG and other AGE precursors upon the preischemic...
heart, and the involved underlying mechanisms, could yield improved preventative and therapeutic treatment of the diabetic heart at risk for and undergoing ischemic injury, respectively. Our current study provided evidence that protein glycation is a new mechanism through which MG aggravates SI-R injury. This notion is supported by our observations that 1) preincubating cardiomyocytes with MG for 6 days caused a greater than 30-fold increase in AGE production, which was dramatically reduced by cotreatment with AG, a strong AGE formation inhibitor, and 2) preincubating cardiomyocytes with MG for 6 days made cardiomyocytes more susceptible to SI-R, as evidenced by increased LDH release, more cardiac caspase-3 activation, and greater percentage of TUNEL-positive staining, all of which were also markedly inhibited by AG cotreatment. Two experimental limitations should be discussed. First, the MG concentration present in the culture medium is much higher than that found in diabetic patient plasma (31, 35). However, it must be indicated that clinical situations are much more complicated, and actual MG concentrations to which in vivo cells are exposed remain uncertain. The intracellular MG level is likely much higher than the plasma MG level in the diabetic condition because diabetic tissues are chronically (months to years) exposed to high MG levels, which can cause dramatic intracellular MG accumulation ($\leq$300 $\mu$M) (7). In contrast, cultured cells were only transiently (days) exposed to high concentrations of MG, which may limit intracellular MG accumulation (8). Additionally, actual diabetic tissues are concomitantly exposed to high levels of plasma glucose, whereas cultured cells in this study were exposed to normal glucose concentration. Furthermore, because MG is formed during glycosylation, clinical diabetes, often causing tissue hypoperfusion and hypoxia, may stimulate intracellular MG production. In contrast, normal oxygen was present during the 6-day preincubating period of our study, and cellular glycosylation was minimal. For these reasons, high concentrations of MG ranging from 200 $\mu$M to 1.5 mM were typically used in previously published studies by many investigators (10, 19, 23, 25, 45). Second, besides its strong antiglycation property, AG is also a potent inducible nitric oxide synthase (iNOS) inhibitor. Therefore, the protective effect of AG against MG-enhanced SI-R injury could be attributed to its anti-iNOS effect. Although theoretically possible, our experimental results do not support this possibility because 1) we have demonstrated previously that significant iNOS upregulation begins 2 h after reperfusion, but AG was washed out from the culturing system before the cells were subjected to SI-R; and 2) MG preincubating did not cause any significant cell injury (sham SI-R group; Fig. 1) unless the cells were subjected to SI-R (SI-R group; Fig. 1), and pretreatment with AG alone during the preincubating period (washed out before SI-R) had no effect on cellular injury before or after SI-R (data not shown).

Discovered 40 years ago in bacteria, Trx’s influence in human cells has only recently begun to be appreciated as the diverse gamut of processes (including cellular redox balance, cell growth promotion, apoptosis inhibition, and inflammation modulation) regulated by Trx continue to be discovered (34). Therefore, it is not surprising to behold the role Trx plays in a wide range of human diseases and conditions, including cancer, viral pathology, and ischemia-reperfusion injury (9). Emerging evidence suggests that Trx plays critical roles in promoting cell proliferation/survival and reducing cell death. Trx and its reductase protein (TrxR) are upregulated in cancer tissues; molecules inhibiting Trx or TrxR promote apoptosis and reduce cancer development (36). In contrast, Trx activity is reduced in diseased tissues where pathological apoptosis is increased (27). Recent studies have demonstrated that besides upregulation or downregulation of Trx expression at the gene level, Trx activity is differentially regulated by posttranslational modifications. Oxidation of the thiols groups of Cys32 and -35 forms a disulfide bond and reversibly inhibits Trx’s antioxidative activity. Glutathionylation, occurring at Cys73, significantly inhibits Trx’s antioxidative activity (6). S-nitrosylation occurs at Cys69 or Cys73 and has been shown to markedly enhance Trx’s antioxidative, antiapoptotic, and organ-protective activity (15, 16, 41). Nitration, occurring at Tyr49, causes significant irreversible inhibition of Trx’s antioxidative and cellular protection.

Protein glycation, also known as nonenzymatic glycosylation, is a protein modification reaction between proteins and reducing sugars (42). Glycation occurs in several steps. In an initial step that is completed in a short period of time (minutes to hours), the reducing sugar reacts with the protein chain and produces Schiff reaction primary-glycated products (e.g., fructosamine). After several days or weeks, amadorial rearrangement commences, and AGEs are formed (22). In recent years, the pathogenic roles of AGEs have been investigated extensively. Increased AGE accumulation and subsequent tissue injury have been found in many human diseases, such as type 2 diabetes, and during the aging process (1, 13, 44). However, whether the early modification of protein by sugar prior to AGE formation may alter protein function remains largely unknown. A study by McCarthy et al. (29) reported that incubation of alkaline phosphatase with reducing sugars reduced enzyme activity associated with an increase in fructosamine levels, indicating that early glycation may alter protein function. In two more recent studies, it was reported that human Cu-Zn-superoxide dismutase (20) and esterase (40) can be glycated by MG, and their activities are subsequently inhibited. MG reacts with the free amino groups of lysine and arginine and with cysteine thiol groups to form AGEs. In a recent study, we have demonstrated that Trx is susceptible to nonenzymatic glycation via LPS exposure (47) and consequent inactivation, and furthermore, it is unable to provide protection against LPS-induced liver toxicity.

Our current study demonstrated for the first time that preincubating cardiomyocytes significantly inhibited cellular Trx activity before the cells were subjected to SI-R and caused greater dissociation of Trx-ASK1 and p38 MAPK activation after SI-R. The MG inhibition of Trx is likely attributable to Trx-glycative modification and partially responsible for MG enhancement of SI-R injury. This novel hypothesis is supported by the following observations. First, MG preculture significantly increased Trx expression, indicating that posttranslational modification, rather than Trx gene expression, is responsible for reduced Trx activity in MG-preincubated cardiomyocytes (Fig. 5). Second, MG caused significant recombinant hTrx-1 glycation and inactivation in a cell-free incubation system, and cotreatment with AG blocked Trx glycation and preserved Trx activity (Fig. 4). Third, addition of AG significantly attenuated the inhibitory effect of MG on cellular Trx activity (Fig. 5). Finally, treatment with AG only during the preincubating period significantly improved Trx-ASK1 associa-
tion and inhibited proapoptotic p38 MAPK activation after SI-R (Fig. 7).

It should be indicated that Trx is also susceptible to nitrative inhibition. AG, as an iNOS inhibitor, may preserve Trx activity in MG-treated cells by blocking iNOS expression. However, our current study supports that Trx-glycative modification is a more likely mechanism responsible for MG inactivation of Trx because 1) preculturing cells with MG significantly reduced Trx activity even before cells were subjected to SI-R, whereas significant iNOS expression was not observed until 2 h after reperfusion; 2) treatment with AG only during the preculturing period where no significant iNOS is present significantly attenuated MG inactivation of Trx; and 3) in a cell-free system where no iNOS is present, AG blocked MG glycation and preserved Trx activity after MG incubation.

Finally, some limitations should be addressed. First, the specific amino acid residues of Trx-1 responsible for glycative modification remain unknown and are currently under investigation. However, our preliminary data indicated that cysteine residues are not involved in glycative modification, because mutations of any or all of the five Trx cysteine residues failed to block Trx-1 glycation. Second, we were unable to directly measure cardiomyocyte Trx glycation after MG incubation, because a method sensitive enough to detect early protein glycation in cells is currently unavailable. Nonetheless, our cell-free experimental results demonstrating that Trx function is glycatively inhibited, together with our cellular experimental results showing that cardiomyocyte Trx-1 activity is reduced in MG-treated cells and preserved by AG, summarily suggest that glycative Trx inactivation may contribute to MG enhancement of cardiomyocyte SI-R injury. Third, H9c2 cells are neonatal myoblasts and may have some differences from the adult cardiomyocyte SI-R injury. Third, H9c2 cells are neonatal myoblasts and may have some differences from the adult cardiomyocyte SI-R injury. However, this cell line has been used extensively as an experimental cardiomyocyte model, especially in those experiments where cells need to be cultured for a long period of time.

In conclusion, our results demonstrated that Trx activity was decreased due to posttranslational glycative modification in the cardiomyocytes treated with MG. Blocking AGE production inhibited Trx inactivation and significantly protected the cardiomyocytes from SI-R injury. These results suggest that clinical therapeutic interventions preserving Trx activity or scavenging MG in the diabetic setting may be novel modalities for attenuating injury endured in myocardial ischemia-reperfusion processes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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