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Xiaoliang Wang
Thomas Jefferson University

Wayne B. Lau
Thomas Jefferson University

Yue-Xing Yuan
Thomas Jefferson University

Ya-Jing Wang
Thomas Jefferson University

Wei Yi
Thomas Jefferson University

See next page for additional authors

Authors

Xiaoliang Wang, Wayne B. Lau, Yue-Xing Yuan, Ya-Jing Wang, Wei Yi, Theodore A. Christopher, Bernard L. Lopez, Hui-Rong Liu, and Xin-Liang Ma

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Methylglyoxal Increases Cardiomyocyte Ischemia/Reperfusion Injury via Glycative Inhibition of Thioredoxin Activity

Xiao-Liang Wang, MD, PhD, Wayne B. Lau, MD, Yue-Xing Yuan, PhD,
Ya-Jing Wang, MD, PhD, Wei Yi, MD, Theodore A. Christopher, MD,
Bernard L. Lopez, MD, Hui-Rong Liu, MD, PhD[#], Xin-Liang Ma, MD, PhD*

Department of Emergency Medicine
Thomas Jefferson University
1025 Walnut Street
Philadelphia, PA 19107

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Address proofs to:
*Xin L Ma, M.D., Ph.D.
Department of Emergency Medicine
1020 Sansom Street
Thompson Building, Room 239
Philadelphia, PA 19107

Tel: (215)955-4994
Fax: (215)923-6225
E-mail: Xin.Ma@Jefferson.edu

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[#]Department of Pathophysiology, Capital Medical University, Beijing, China.

Abstract

Diabetes (DM) is closely related to cardiovascular morbidity and mortality, but the specific molecular basis linking DM with increased vulnerability to cardiovascular injury remains incompletely understood. Methylglyoxal (MG), a precursor to advanced glycation end-products (AGEs), is increased in diabetic patient plasma but its role in diabetic cardiovascular complications is unclear. Thioredoxin (Trx), a cytoprotective molecule with anti-apoptotic function, has been demonstrated to be vulnerable to glycative inhibition, but whether Trx is glycatively inhibited by MG thus contributing to increased cardiac injury has never been investigated. Cultured H9c2 cardiomyocytes were treated with MG (200μM) for 6 days. The following were determined pre- and post-simulated ischemia/reperfusion (SI/R) (8 hours hypoxia followed by 3 hours of reoxygenation): cardiomyocyte death/apoptosis, Trx expression and activity, AGEs formation, Trx-apoptosis-regulating kinase-1 (Trx-ASK1) complex formation, and p38 mitogen-activated protein kinase (MAPK) phosphorylation and activity. Compared to vehicle, MG significantly increased SI/R-induced cardiomyocyte LDH release and apoptosis ($P<0.01$). Prior to SI/R, Trx activity was reduced in MG-treated cells, but Trx expression was moderately increased. Moreover, Trx-ASK1 complex formation was reduced, and both p38 MAPK activity and phosphorylation were increased. To investigate the effects of MG on Trx directly, recombinant human Trx (hTrx) was incubated with MG in vitro. Compared to vehicle, MG incubation markedly increased CML formation (a glycation footprint), and inhibited Trx activity. Finally, glycation inhibitor aminoguanidine (AG) administration during MG-treatment of cultured cells reduced AGEs formation, increased Trx activity, restored Trx-ASK1 interaction, and reduced p38 MAPK phosphorylation and activity, caspase-3 activation, and LDH release ($p<0.01$). We demonstrated for the first time that methylglyoxal sensitized cultured cardiomyocytes to SI/R injury by post-translational modification of Trx via glycation. Therapeutic interventions scavenging AGEs precursors may attenuate ischemic/reperfusion injury in hyperglycemic state diseases such as diabetes**.**

Keywords: Methylglyoxal; thioredoxin; cardiomyocyte; hypoxia and reoxygenation; apoptosis

Diabetes mellitus (DM) is a leading metabolic disorder in developed society, and causes devastating systemic consequences if poorly managed in the clinical setting. Considerable experimental and clinical data has demonstrated the close association between diabetes and significant cardiovascular morbidity and mortality. Recent studies have demonstrated that 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 non-enzymatic reaction glycation, or glycosylation, between reducing sugars and proteins and other biomolecules, yielding irreversible advanced glycation end-products (AGEs)(5; 28). The concentration of methylglyoxal 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 methylglyoxal 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

demonstrate 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 c-Jun N-terminal kinase (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 post-translational modification. Five forms of post-translational modifications of Trx have been previously identified, each modification affecting Trx differently. These include oxidation, glutathionylation, S-nitrosylation, nitration, and glycation. We have recently demonstrated that Trx is susceptible to non-enzymatic glycation via lipopolysaccharide (LPS) exposure (Free Radical Biology and Medicine, in revision), 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 methylglyoxal exposure, and any functional consequence of such alteration with respect to cardiomyocyte 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 (SI/R), (2) if so, 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 glycation inhibitor aminoguanidine (AG) might be a therapeutic intervention reversing the observed phenomena related to methylglyoxal exposure of cardiomyocytes.

Material and methods

Cell culture and experimental protocol: H9c2 cardiomyoblast cells (referred to as cardiomyocytes thereafter), an embryonic rat heart-derived cell line (the American Type Culture Collection, Manassas, VA), was 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 per 35-mm well of 6-well plates for 24-hour culture and were made quiescent by overnight serum-starvation (0% CBS). After 6 days treatment with MG (200µM; Sigma-Aldrich, St. Louis, MO) or MG (200µM) + aminoguanidine hemisulfate (AG, 100µM; Sigma-Aldrich, St. Louis, MO), the cells were subject to simulated ischemia/reperfusion (SI/R) as described previously(30). Briefly, the cells were incubated in slightly hypotonic Hanks' balanced saline solution (1.3 mmCaCl2, 5 mm KCl, 0.3 mmKH2PO4, 0.5 mm MgCl2, 0.4 mm MgSO4, 69 mm NaCl, 4 mm NaHCO3, and 0.3 mmNa2HPO4) without glucose or serum, and transferred in an airtight incubator from which oxygen was removed and replaced by nitrogen for 8 hours at 37°C. The incubator oxygen concentration (1%) was adjusted to simulate hypoxic conditions. Following hypoxia, the cells were re-oxygenated for 3 hours in DMEM with 1% serum at 37°C. Sham-cells incubated in DMEM with 1% serum were not subjected to hypoxia. MG and AG were dissolved in double distilled H2O (ddH2O). Cells received ddH2O as vehicle in control experiments. MG or AG was only present during the 6-day pre-incubation period, but not during SI/R period. Concentrations of MG and incubation time was established based on dose and time-dependent pilot experiments.

Lactate dehydrogenase (LDH) activity assay: Post-treatment completion, all conditioned medium was collected, and the cells were lysed(21). In short, a 100 µl medium was added to

100 µl of a solution containing 100 mM Tris buffer, pH 8.2, 1.35 mM tetrazolium salt, and 0.58 mM phenazine methosulphate, and 2.7 mM NADH. The OD values were read at 0 and 30 minutes using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at 490 nm. The percentage of LDH release was calculated as follows: (A-B)/(C-B) × 100, where A = LDH activity in conditioned media; B = LDH activity in culture media (without cells); C = LDH activity in cell lysates.

Assessment of cardiomyocyte apoptosis: Cardiomyocyte apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and caspase-3 activity, as we reported previously(41). TUNEL assay was performed utilizing the In Situ Cell Death Detection Kit (Roche, Palo Alto, CA). Briefly, cells were fixed with 10% paraformaldehyde and incubated with the TUNEL reaction mixture containing TdT-mediated dUTP. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Samples were visualized on an Olympus BX51 Fluorescence Microscope, and digital images were acquired with IP Lab Imagine Analysis Software (version 3.5, Scanalytics, Fairfax, VA). Apoptotic index (number of TUNEL positively stained nuclei/total number of nuclei × 100) was automatically calculated and exported for further analysis. Assays were performed in a blinded manner. The caspase-3 activity assay utilized the fluorogenic substrate DEVD-7-amino-4-trifluoromethyl-coumarin. Briefly, cells were lysed using caspase-3 lysis buffer (50mM HEPES PH 7.4, 0.1% Chaps, 5mM DTT, 0.1 mM EDTA, 0.1% Triton-X100), and total protein concentration was determined by the Bradford method. To each well of a 96-well plate, supernatant containing 50 µg of protein was loaded and incubated with 3.645 µg Ac-DEVD-AFC at 37°C for 1.5 hours. AFC was cleaved from DEVD by activated caspase-3, and the free AFC was quantified with a Biotek FL600 microplate fluorescence reader (excitation wavelength, 400 nm; emission wavelength, 508 nm). Caspase-3 activity was expressed as nanomoles of AFC formation per hour per milligram of protein.

Trx activity assay: Trx activity was determined via the insulin disulfide reduction assay(17). In brief, 40 μg of cellular protein extracts were pre-incubated at 37°C for 15 minutes with 2 μl activation buffer (100 mM Hepes, 2 mMEDTA, 1 mg/ml BSA, and 2 mM dithiothreitol) to reduce thioredoxin. After addition of 20 μl reaction buffer (100 mM Hepes, 2.0 mM EDTA, 0.2 mM NADPH, and 140 μM insulin), the reaction was initiated by addition of mammalian Trx reductase (1 μl, 15 mU, Sigma), and samples were incubated for 30 minutes at 37°C (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 1.7mM 3-carboxy-4-nitrophenyl disulfide). Absorption measurement occurred at 412 nm.

Immunocytochemical detection of advanced glycated end products: Cardiomyocytes, seeded on glass coverslips in 6 well plates, were treated as described above. Cells were fixed with polyformaldehyde (4% in PBS) for 1 hour, washed with PBS, blocked with 10% normal goat serum, and incubated with rabbit anti-AGE polyclonal antibody (Abcam Inc., Cambridge, MA). Immunostaining was developed with 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 Ischiropoulos and colleagues(43). In brief, endogenous Trx was immunoprecipitated with a monoclonal anti-murine Trx antibody (Redox Bioscience, Japan). After sample separation, the Trx–ASK-1 interaction was determined by Western blot analysis using a monoclonal antibody against ASK-1 (Upstate Biotechnology, Lake Placid, NY) and HRP-conjugated anti-mouse IgG antibody (Cell Signaling, Danvers, MA).

p38 MAPK activity assay: The p38 MAPK activity assay was performed utilizing a p38 MAPK assay kit (Cell Signaling Technology Inc., Danvers, MA) with substrate ATF-2, per manufacturer's instructions(14). In brief, cells were homogenized in ice-cold lysis buffer.

Lysates were sonicated on ice, and centrifuged at 12,000g for 10 minutes at 4°C. Immunoprecipitation was performed by adding 20 μl of resuspended immobilized monoclonal antibody against phospho-p38 MAPK (Thr180/Tyr182) to 100 μl cell lysate containing 150 μg of protein. The mixture was incubated with gentle rocking overnight at 4°C. After 10,000g centrifugation at 4°C for 2 minutes, 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 minutes. 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, St. Louis, MO) 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 same conditions. For the anti-glycation treatment, AG (300 μM) was added at the initiation of incubation.

Western blot analysis: Cultured Cardiomyocytes cells were collected in lysis buffer after treatment. Aliquots containing 30–60 μg 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 Bioscience, Japan), p38, phospho-p38, GAPDH (Cell Signaling Technology Inc., Danvers, MA), and CML(Abcam Inc., Cambridge, MA), respectively. Positively charged nylon membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G antibody (Cell Signaling, Danvers, MA) for 1 hour. The blot was developed with a Supersignal

229 Chemiluminescence Detection Kit (Pierce, Rockford, IL). Bands were visualized with a Kodak
230 Images Station 400 (Rochester, NY), and the band densities were analyzed with Kodak
231 1-Dimensional software (version 3.6).
232 *Statistical analysis*: All values in the text, table, and figures are presented as means+SEM of n
233 independent experiments. All data (except western blot density) were subjected to ANOVA
234 followed by Bonferroni correction for post hoc t test. Western blot densities were analyzed
235 with the Kruskal–Wallis test followed by Dunn's post test. Probabilities of 0.05 or less were
236 considered to be statistically significant.

Results

Pre-culturing cardiomyocytes with high MG increased their susceptibility to simulated ischemia/reperfusion 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 (Figure 1). Under basal conditions, cardiomyocytes treated with MG developed normally without apparent injury (MG+Sham SI/R groups in Figure 1). However, when subjected to SI/R, the cells treated with 6 days of MG (200 μM) exhibited significantly greater cellular injury compared to control in the parameters listed above.

Pre-culturing cardiomyocytes with high MG caused significant protein glycation. As a highly reactive α-oxoaldehyde, MG may modify proteins and other substrates via glycation. To determine whether pre-culturing cardiomyocytes with MG may cause cardiomyocyte protein glycation, AGE content in vehicle or MG-cultured cardiomyocytes was determined by immunohistological staining. As illustrated in Figure 2A and summarized in Figure 2B, exposure of cardiomyocytes to MG caused a concentration-dependent increase in AGE content with a 30.4-fold increase in AGEs formation observed at 200 μM MG. Moreover, co-treatment of cardiomyocytes with aminoguanidine, a potent protein glycation inhibitor, markedly reduced high concentration MG-induced protein glycation (Figure 2, last bar).

Treatment with aminoguanidine protected MG-treated cardiomyocytes from simulated ischemia/reperfusion injury. Having demonstrated that pre-culturing cardiomyocytes with high concentration of MG significantly increased AGE formation and enhanced SI/R injury, we further determined whether increased protein glycation may play a causative role in enhanced cardiomyocyte SI/R injury. As summarized in Figure 3, co-treatment of cardiomyocytes with aminoguanidine, an advanced glycation end product formation inhibitor(33), significantly reduced LDH release (A), TUNEL staining (B), and caspase-3 activation (C) in high MG (200

μM) pre-treated cells subjected to SI/R. These results demonstrated that blockade of MG-induced protein glycation significantly protected cardiomyocytes from SI/R injury.

MG incubation induced recombinant hTrx glycation and significantly decreased its activity. Our experimental results presented above demonstrated that MG is capable of initiating protein glycation reactions and that blockade of protein glycation reduced SI/R injury in MG pretreated cardiomyocytes. However, the specific proteins that are glycated by MG potentially contributive to increased SI/R injury remain unknown. After a thorough literature review identifying feasible protein candidates, Trx was selected because 1) Trx is a critical anti-apoptotic and cell survival molecule, and its inactivation has been causatively related to cardiovascular injury; and 2) we recently demonstrated the Trx is susceptible to glycative modification by lipopolysaccharide 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 Trx activity were determined. As summarized in Figure 4A, in vitro incubation of hTrx caused Trx glycation as evidenced by abundant production of N^{ε}-carboxymethyl lysine (CML), a biomarker of AGEs formation. MG incubation markedly inhibited Trx activity (60.4% reduction, $P<0.01$ vs. vehicle-incubated Trx)(Figure 4B). More importantly, addition of AG in the system completely abolished MG-induced Trx glycation (Figure 4A) and significantly attenuated MG-induced Trx inactivation (Figure 4B).

Trx is glycatively inhibited in MG pre-treated 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 to control, Trx

activity was significantly decreased in the MG-treated cells prior to SI/R (Figure 5A). However, Trx expression was slightly increased in MG-treated cells (Figure 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, co-treatment with AG significantly attenuated MG inhibition of Trx (Figure 5A).

Pre-culturing cardiomyocytes with MG promoted SI/R-induced Trx-ASK1 dissociation and subsequent p38 MAPK activation, which were attenuated by co-treatment 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 anti-apoptotic 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 pro-apoptotic kinases, two additional experiments were performed. Via anti-Trx-1 immunoprecipitation and anti-ASK1 immunoblotting, Figure 6A illustrates 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 pro-apoptotic downstream molecule for ASK1, was significantly enhanced in the MG-treated cardiomyocyte compared to control (Figure 6B). More importantly, this SI/R-induced disassociation of Trx-ASK1 was significantly further enhanced when cells were pre-cultured with MG, and p38 MAPK activity was significantly further increased (Figure 6). Treatment with aminoguanidine restored Trx-ASK1 interaction (Figure 7A), reduced p38 MAPK phosphorylation (Figure 7B), and inhibited p38 MAPK activity (Figure 7C) in MG-treated cardiomyocytes.

Discussion

Ischemic heart disease (IHD) 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 IHD 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 AGEs precursors upon the pre-ischemic heart, and the involved underlying mechanisms, could yield improved preventative and therapeutic treatment of the diabetic heart respectively at risk for and undergoing ischemic injury.

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) pre-culturing cardiomyocytes with MG for 6 days caused a greater than 30-fold increase in AGE production, which was dramatically reduced by co-treatment with AG, a strong AGE formation inhibitor; and 2) pre-culturing 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 co-treatment. 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 (up to 300 μM)(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, as MG is formed during glycolysis, clinical diabetes, often causing tissue hypoperfusion and hypoxia, may stimulate intracellular MG production. In contrast, normal oxygen was present during the 6-day pre-culturing period of our study, and cellular glycolysis was minimal. For these reasons, high concentrations of MG ranging from 200 μM to 1.5 mM were typically used in previously published studies by many investigators(10; 19; 23; 25; 45). Second, besides its strong anti-glycation property, AG is also a potent 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 previously demonstrated that significant iNOS upregulation begins 2 hours after reperfusion, but AG was washed out from the culturing system before the cells were subjected to SI/R; and 2) MG pre-culturing did not cause any significant cell injury (Figure 1, sham SI/R group) unless the cells were subjected to SI/R (Figure 1, SI/R group), and pre-treatment with AG alone during the pre-culturing 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, thioredoxin'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 thioredoxin continue to be discovered(34). It is not surprising, therefore, 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 pathologic 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 post-translational modifications. Oxidation of the thiol groups of Cys-32 and -35 forms a disulfide bond, and reversibly inhibits Trx's anti-oxidative activity. Glutathionylation, occurring at Cys-73, significantly inhibits Trx's antioxidant activity(6). S-nitrosylation occurs at Cys-69 or Cys-73, and has been shown to markedly enhance Trx's anti-oxidant, anti-apoptotic and organ protective activity(15; 16; 41). Nitration, occurring at Ty-49, causes significant irreversible inhibition of Trx's anti-oxidative and cellular protection.

Protein glycation, also know as non-enzymatic glycosylation, is a protein modification reaction between proteins and reducing sugars(42). Glycation occurs in several steps. In an initial step that completes 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 advanced glycated end-products (AGEs) are formed(22). In recent years, the pathogenic roles of AGEs have been extensively investigated. Increased AGEs accumulation and subsequent tissue injury have been found in many human diseases, such as type 2 diabetes and the aging process(1; 13; 44). However, whether the early modification of protein by sugar prior to AGEs formation may alter protein function remains largely unknown. A study by McCarthy et al(29) reported that incubation of alkaline phosphatase (ALP) 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 methylglyoxal, and their

activities are subsequently inhibited. Methylglyoxal 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 non-enzymatic glycation via lipopolysaccharide (LPS) exposure (Free Radical Biology and Medicine, in revision), consequent inactivation, and is furthermore unable to provide protection against LPS-induced liver toxicity.

Our current study demonstrated for the first time that pre-culturing cardiomyocytes significantly inhibited cellular thioredoxin 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. Firstly, MG pre-culture slightly increased Trx expression, indicating that posttranslational modification, rather than Trx gene expression is responsible for reduced Trx activity in MG pre-cultured cardiomyocytes (Figure 5). Secondly, MG caused significant recombinant hTrx-1 glycation and inactivation in a cell-free incubation system, and co-treatment with AG blocked Trx glycation and preserved Trx activity (Figure 4). Thirdly, addition of AG significantly attenuated the inhibitory effect of MG on cellular Trx activity (Figure 5). Finally, treatment with AG only during the pre-culturing period significantly improved Trx-ASK1 association and inhibited pro-apoptotic p38 MAPK activation after SI/R (Figure 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) pre-culturing cells with MG significantly reduced Trx activity even before cells were subjected to SI/R, whereas significant iNOS expression was not observed until 2 hours after reperfusion; 2) treatment with AG only

during the pre-culturing 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 Trx glycation and preserved Trx activity after MG incubation.

Finally, some limitations should be addressed. Firstly, 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 5 Trx cysteine residues failed to block Trx-1 glycation. Secondly, 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. Thirdly, H9c2 cells are neonatal myoblasts and may have some differences from the adult cardiomyocytes. However, this cell line has been extensively used as an experimental cardiomyocyte model, specially in those experiments that cells needs to be cultured for a long period of time.

In conclusion, our results demonstrated that thioredoxin activity was decreased due to posttranslational glycative modification in the cardiomyocytes treated with methylglyoxal. Blocking AGEs 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 methylglyoxal in the diabetic setting may be novel modalities for attenuating injury endured in myocardial ischemia/reperfusion processes.

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Disclosures None

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574 **Figure Legends**

575 Figure 1. SI/R injury is significantly increased in MG pre-cultured cardiomyocytes as
576 measured by LDH release (A), apoptotic index by TUNEL positive staining cells (B), and
577 caspase-3 activity (C). MG pre-culture results in cardiomyocyte glycation prior to being
578 subject to SI/R as measured by total AGEs content (D). n=6 independent experiments.
579 **P<0.01 vs. Sham SI/R, $^{\#\#}$P<0.01 vs. vehicle + SI/R.

580

581 Figure 2. Effect of AG treatment on MG enhanced LDH release (A) and caspase-3 activity (B)
582 after SI/R. n=5–6 independent experiments.

583

584 Figure 3. Effect of AG treatment on MG enhanced apoptotic cell death determined by TUNEL
585 positive staining after SI/R. n=5–6 independent experiments.

586

587 Figure 4. Effects of MG on CML formation (A) and Trx activity of recombinant hTrx (B) in the
588 absence and presence of AG, an AGE formation inhibitor. n=6 independent experiments. Effect
589 of AG treatment on MG-exposed Trx inactivation (C) and Trx expression (D) in
590 cardiomyocytes prior to being subject to SI/R. Insets: representative Western blots; bar graphs:
591 density analysis (n=5–8 independent experiments).

592

593 Figure 5. MG pre-culture decreases Trx-ASK1 binding (A), and increases p38 MAPK
594 activation (B). Insets: representative Western blots; bar graphs: density analysis (n=5–7
595 independent experiments). Effect of AG treatment on MG enhanced Trx-ASK1 dissociation (C)
596 and p38 MAPK phosphorylation (D) in cardiomyocytes subjected to SI/R. Insets:
597 representative Western blots; bar graphs: density analysis (n=5–6 independent experiments).

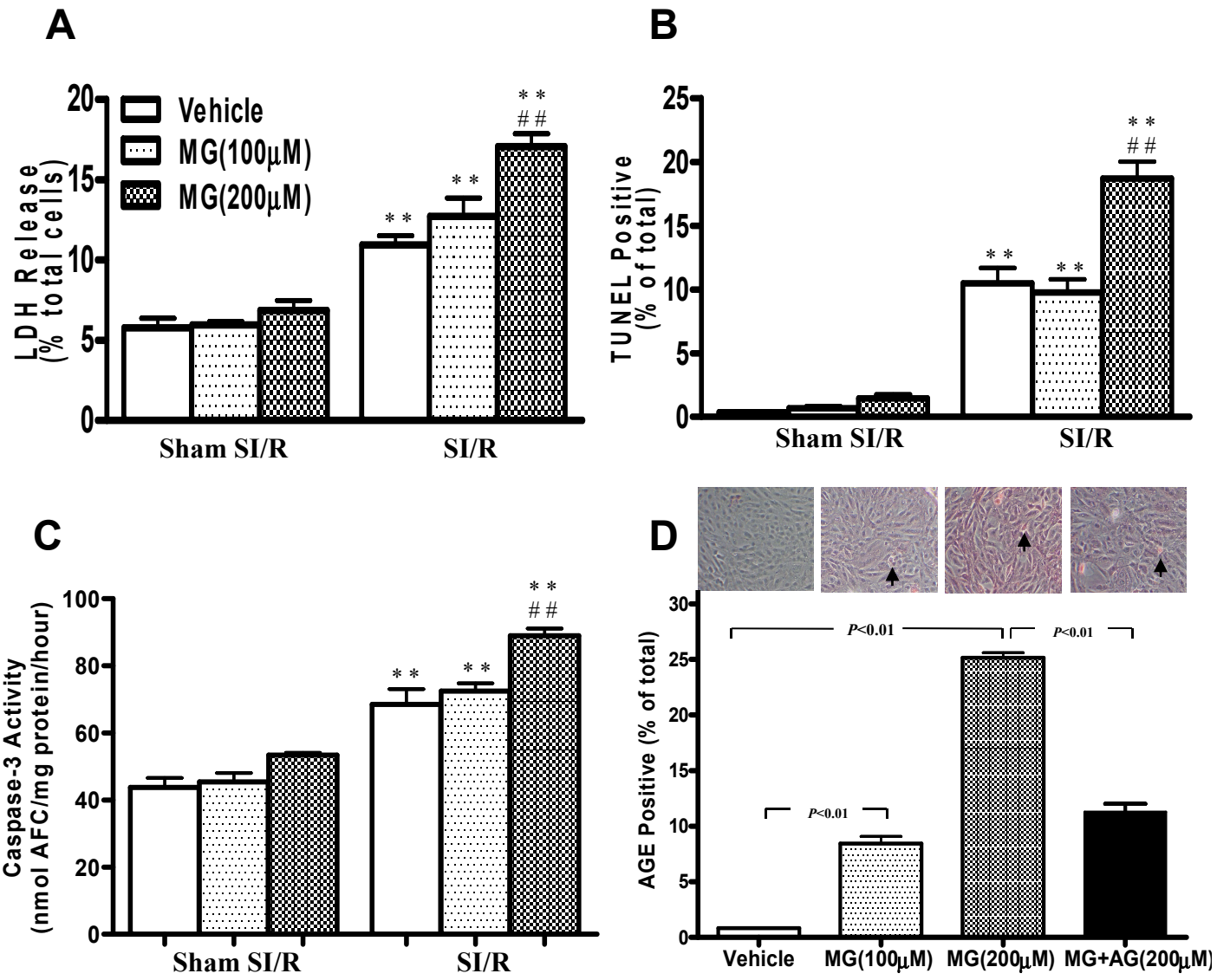
A
Vehicle
MG(100μM)
MG(200μM)
LDH Release (% total cells)
Sham SI/R
SI/R
B
TUNEL Positive (% of total)
Sham SI/R
SI/R
C
Caspase-3 Activity (nmol AFC/mg protein/hour)
Sham SI/R
SI/R
D
AGE Positive (% of total)
P<0.01
P<0.01
P<0.01
Vehicle
MG(100μM)
MG(200μM)
MG+AG(200μM)

Figure 1

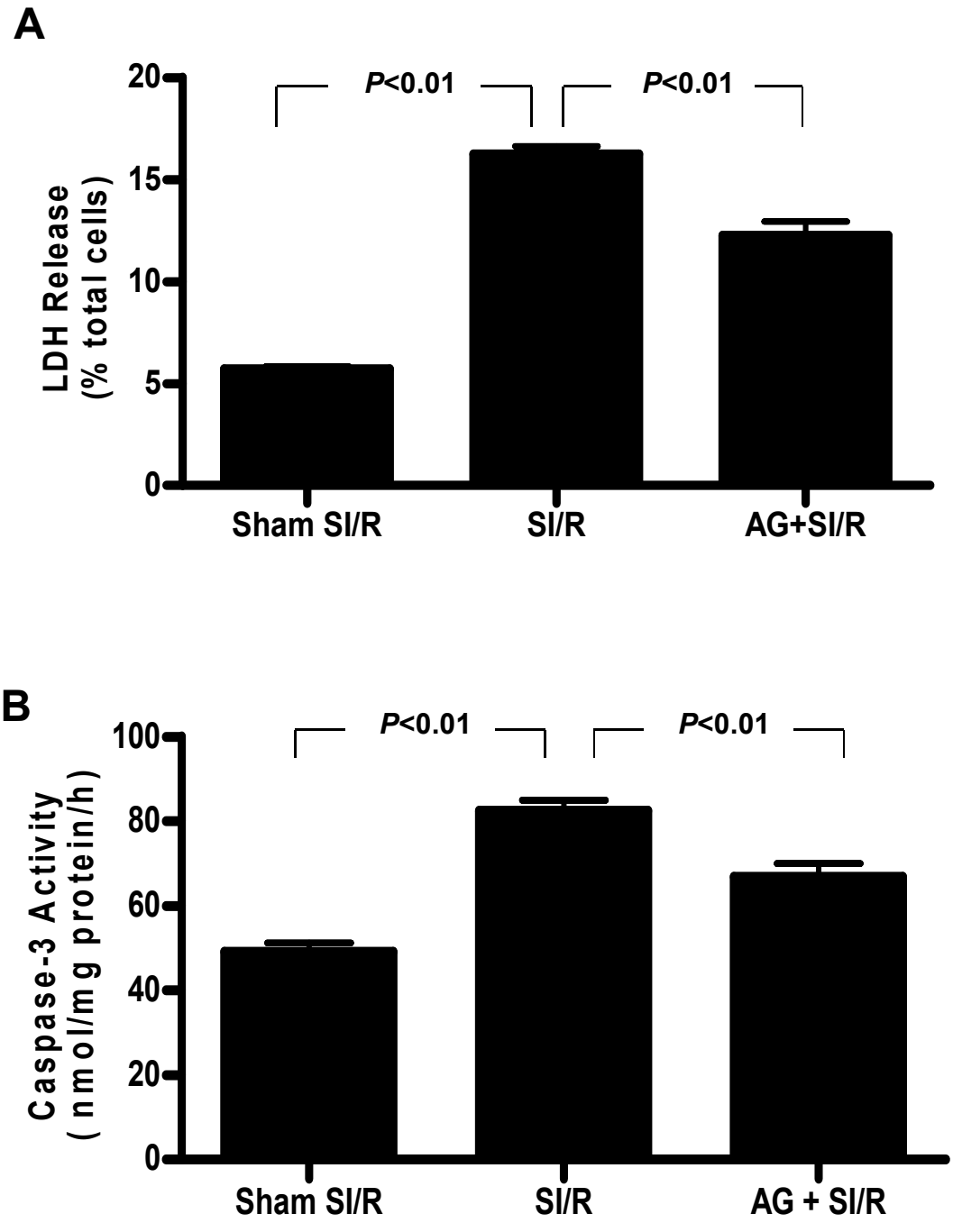
A
P<0.01
P<0.01
LDH Release
(% total cells)
20
15
10
5
0
Sham SI/R
SI/R
AG+SI/R
B
P<0.01
P<0.01
Caspase-3 Activity
(nmol/mg protein/h)
100
80
60
40
20
0
Sham SI/R
SI/R
AG + SI/R

Figure 2

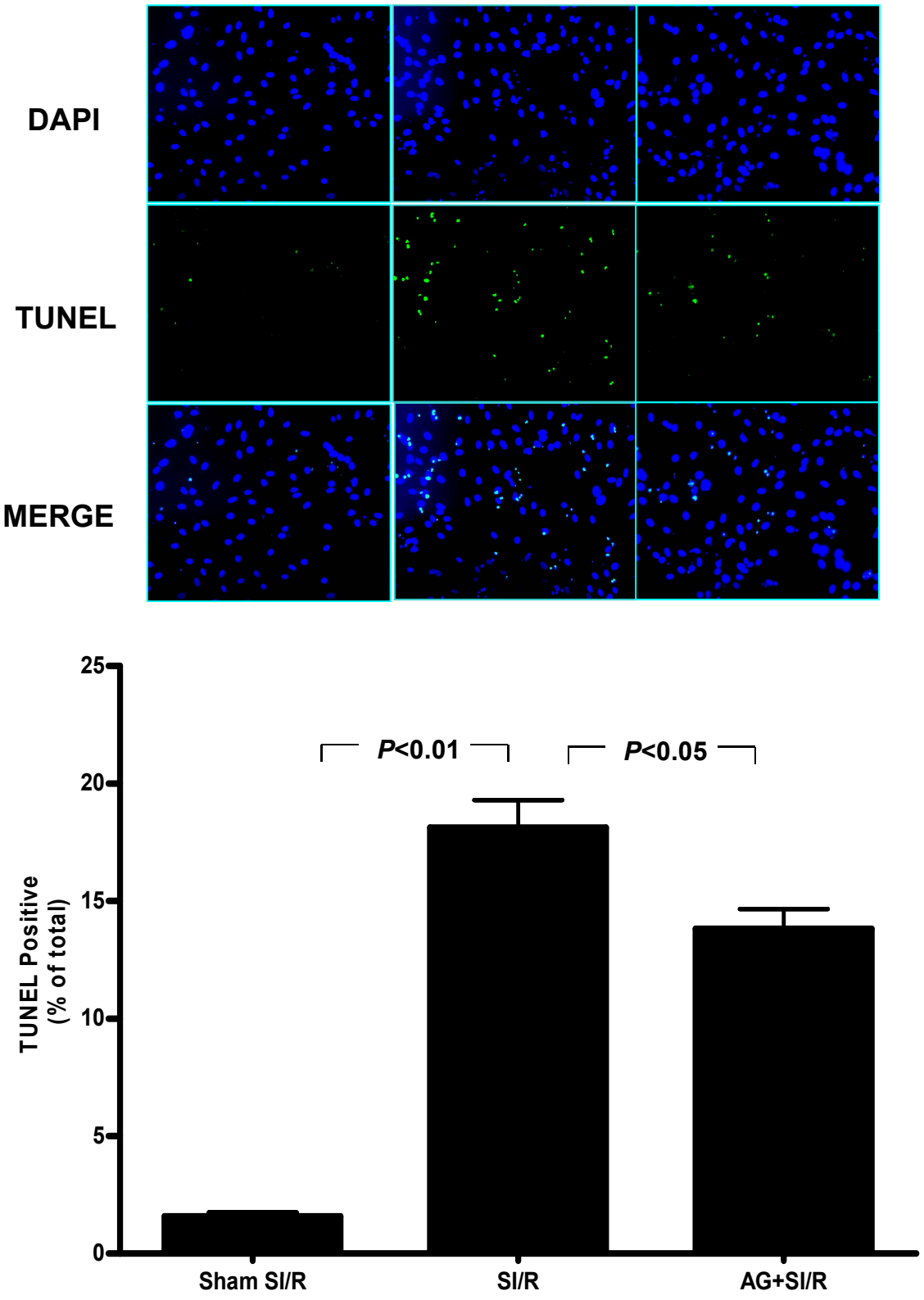
DAPI
TUNEL
MERGE
25
20
15
10
5
0
TUNEL Positive
(% of total)
P<0.01
P<0.05
Sham SI/R
SI/R
AG+SI/R

Figure 3

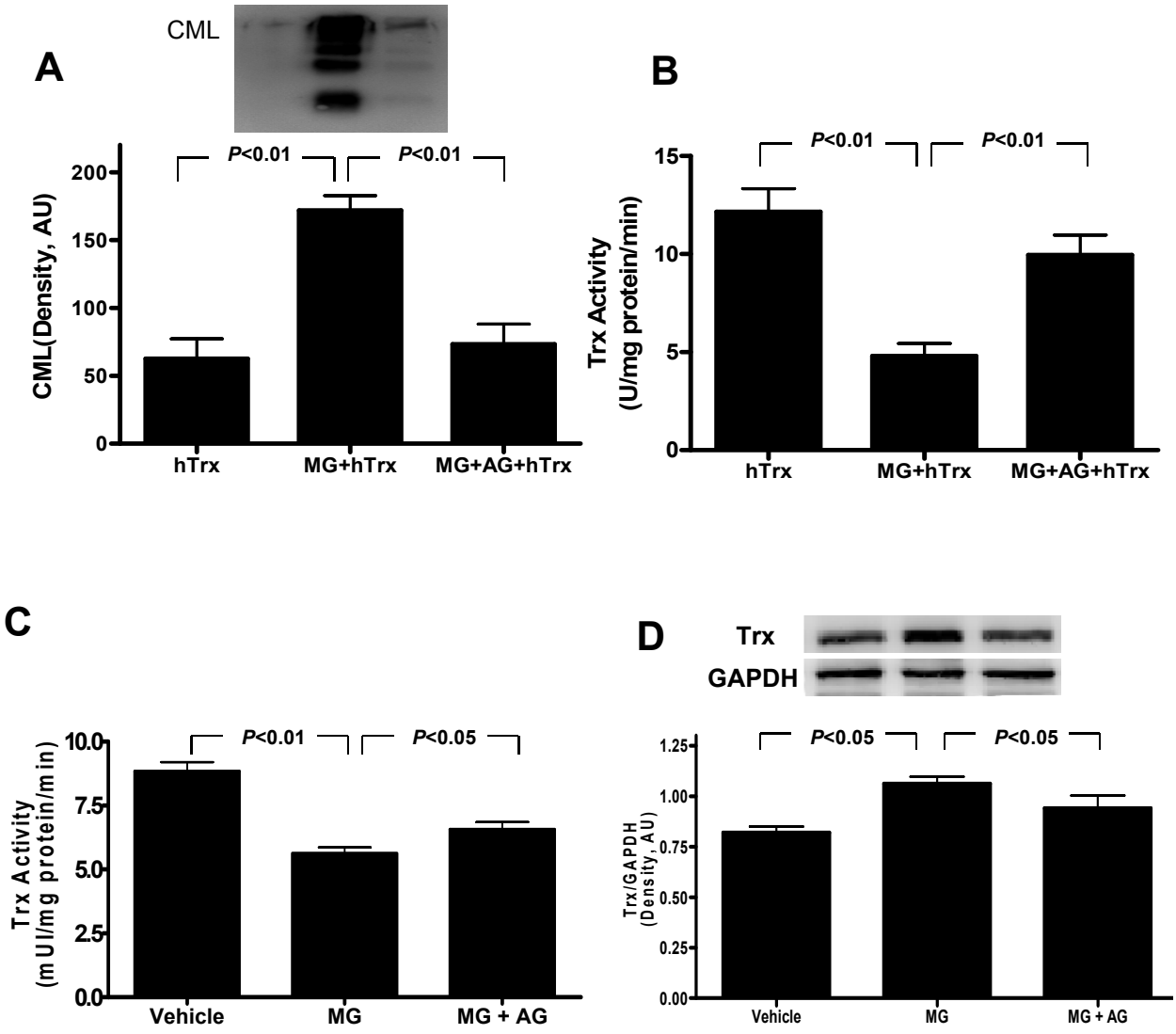
A
CML
CML(Density, AU)
P<0.01
P<0.01
200
150
100
50
0
hTrx
MG+hTrx
MG+AG+hTrx
B
Trx Activity
(U/mg protein/min)
P<0.01
P<0.01
15
10
5
0
hTrx
MG+hTrx
MG+AG+hTrx
C
Trx Activity
(mUl/mg protein/min)
P<0.01
P<0.05
10.0
7.5
5.0
2.5
0.0
Vehicle
MG
MG + AG
D
Trx
GAPDH
Trx/GAPDH
(Density, AU)
P<0.05
P<0.05
1.25
1.00
0.75
0.50
0.25
0.00
Vehicle
MG
MG + AG

Figure 4

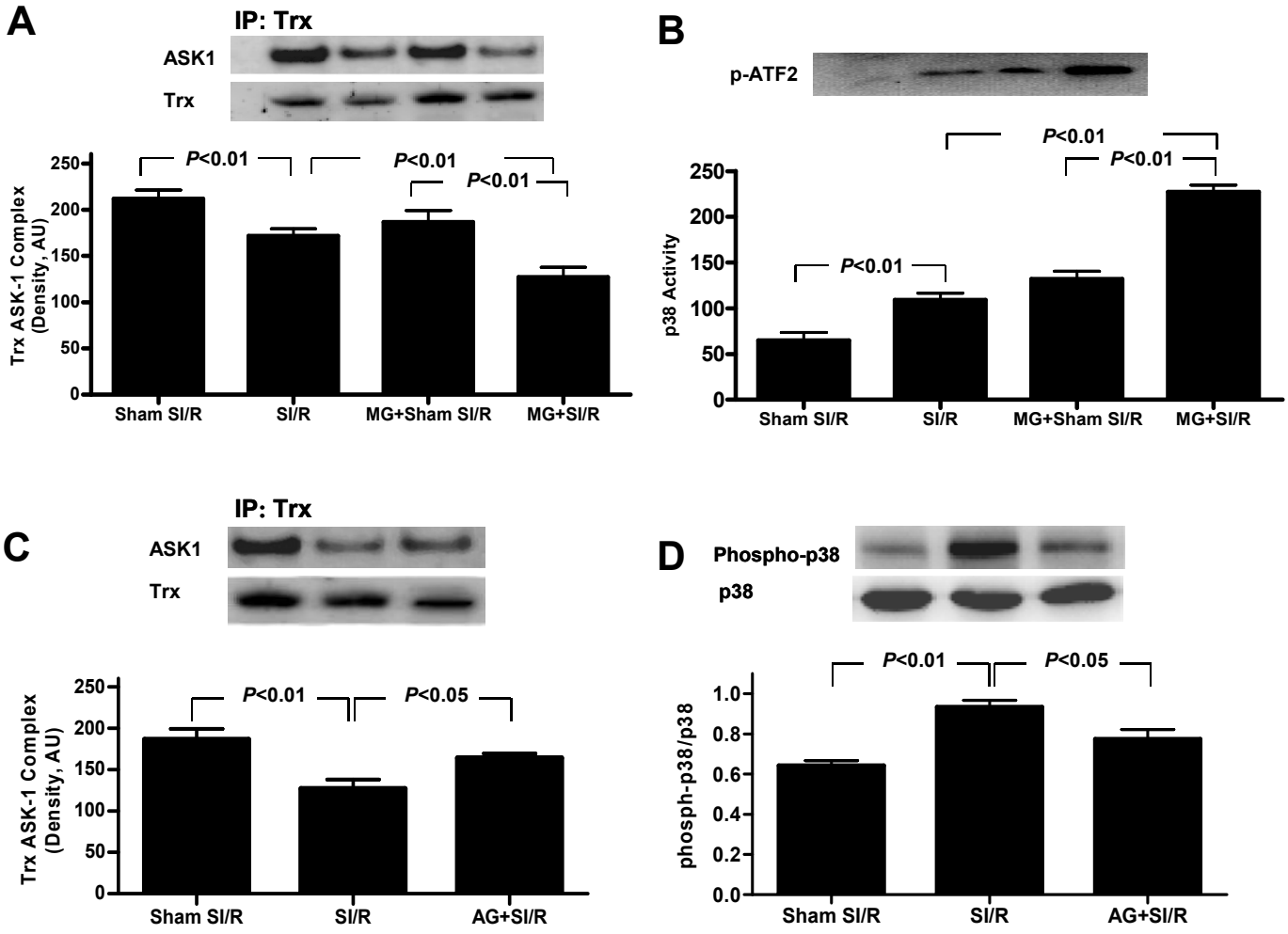
A
IP: Trx
ASK1
Trx
Trx ASK-1 Complex (Density, AU)
P<0.01
P<0.01
P<0.01
Sham SI/R
SI/R
MG+Sham SI/R
MG+SI/R
B
p-ATF2
p38 Activity
P<0.01
P<0.01
P<0.01
Sham SI/R
SI/R
MG+Sham SI/R
MG+SI/R
C
IP: Trx
ASK1
Trx
Trx ASK-1 Complex (Density, AU)
P<0.01
P<0.05
Sham SI/R
SI/R
AG+SI/R
D
Phospho-p38
p38
phosph-p38/p38
P<0.01
P<0.05
Sham SI/R
SI/R
AG+SI/R

Figure 5