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## Methylglyoxal increases cardiomyocyte ischemia-reperfusion injury via glycative inhibition of thioredoxin activity.

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

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## Abstract

49

50

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

76

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

78

79 Diabetes mellitus (DM) is a leading metabolic disorder in developed society, and causes  
80 devastating systemic consequences if poorly managed in the clinical setting. Considerable  
81 experimental and clinical data has demonstrated the close association between diabetes and  
82 significant cardiovascular morbidity and mortality. Recent studies have demonstrated that DM  
83 is a major risk factor for ischemic heart disease development (3), directly adversely affecting  
84 ischemic cardiomyocytes, resulting in larger infarct size and more severe heart failure after  
85 ischemia/reperfusion. Although many signaling pathways relating diabetic cellular injury and  
86 cardiac dysfunction have been reported, the specific molecular basis linking DM with  
87 increased vulnerability to ischemia/reperfusion injury and resultant mortality has not been  
88 established.

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

100 Thioredoxin (Trx), a 12-kDa protein ubiquitously expressed in all living cells, fulfills a  
101 variety of biological functions related to regulation of cellular proliferation and apoptosis(41),  
102 and cytoprotection against oxidative stress(46). Clinical and experimental results have  
103 demonstrated that inhibition of Trx promotes apoptosis(24). Recent in vitro studies

104 demonstrate that Trx interacts directly with and inhibits the activity of apoptosis-regulating  
105 kinase-1 (ASK1), a mitogen activated protein kinase (MAPK) that activates two proapoptotic  
106 kinases, p38 MAPK and c-Jun N-terminal kinase (JNK)(26). These results give mechanistic  
107 insight as to how Trx may critically regulate the balance between cell proliferation and cell  
108 death.

109         Recent studies have demonstrated that besides upregulation or downregulation of Trx  
110 expression at the gene level, Trx activity is regulated by post-translational modification. Five  
111 forms of post-translational modifications of Trx have been previously identified, each  
112 modification affecting Trx differently. These include oxidation, glutathionylation,  
113 S-nitrosylation, nitration, and glycation. We have recently demonstrated that Trx is susceptible  
114 to non-enzymatic glycation via lipopolysaccharide (LPS) exposure (Free Radical Biology and  
115 Medicine, in revision), consequent inactivation, and is furthermore unable to provide  
116 protection against LPS-induced liver toxicity. However, whether Trx activity is altered in the  
117 presence of prolonged methylglyoxal exposure, and any functional consequence of such  
118 alteration with respect to cardiomyocyte protection against simulated ischemia/reperfusion has  
119 never been investigated.

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

128

129 **Material and methods**

130 Cell culture and experimental protocol: H9c2 cardiomyoblast cells (referred to as  
131 cardiomyocytes thereafter), an embryonic rat heart-derived cell line (the American Type  
132 Culture Collection, Manassas, VA), was cultured in Dulbecco's modified Eagle's medium  
133 (DMEM; Invitrogen, Carlsbad, CA) containing 10% calf bovine serum (CBS; MP Biomedical,  
134 Solon, OH), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were maintained at  
135 37°C under a water-saturated atmosphere of 95% ambient air and 5% CO<sub>2</sub> (normoxic  
136 conditions). Stock cultures were passaged at 2- to 3-day intervals. Cells were seeded at a  
137 density of  $3 \times 10^5$  cells per 35-mm well of 6-well plates for 24-hour culture and were made  
138 quiescent by overnight serum-starvation (0% CBS). After 6 days treatment with MG (200µM;  
139 Sigma-Aldrich, St. Louis, MO) or MG (200µM) + aminoguanidine hemisulfate (AG, 100µM;  
140 Sigma-Aldrich, St. Louis, MO), the cells were subject to simulated ischemia/reperfusion (SI/R)  
141 as described previously(30). Briefly, the cells were incubated in slightly hypotonic Hanks'  
142 balanced saline solution (1.3 mmCaCl<sub>2</sub>, 5 mm KCl, 0.3 mmKH<sub>2</sub>PO<sub>4</sub>, 0.5 mm MgCl<sub>2</sub>, 0.4 mm  
143 MgSO<sub>4</sub>, 69 mm NaCl, 4 mm NaHCO<sub>3</sub>, and 0.3 mmNa<sub>2</sub>HPO<sub>4</sub>) without glucose or serum, and  
144 transferred in an airtight incubator from which oxygen was removed and replaced by nitrogen  
145 for 8 hours at 37°C. The incubator oxygen concentration (1%) was adjusted to simulate  
146 hypoxic conditions. Following hypoxia, the cells were re-oxygenated for 3 hours in DMEM  
147 with 1% serum at 37°C. Sham-cells incubated in DMEM with 1% serum were not subjected to  
148 hypoxia. MG and AG were dissolved in double distilled H<sub>2</sub>O (ddH<sub>2</sub>O). Cells received ddH<sub>2</sub>O  
149 as vehicle in control experiments. MG or AG was only present during the 6-day pre-incubation  
150 period, but not during SI/R period. Concentrations of MG and incubation time was established  
151 based on dose and time-dependent pilot experiments.

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

154 100 µl of a solution containing 100 mM Tris buffer, pH 8.2, 1.35 mM tetrazolium salt, and 0.58  
155 mM phenazine methosulphate, and 2.7 mM NADH. The OD values were read at 0 and 30  
156 minutes using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices,  
157 Sunnyvale, CA) at 490 nm. The percentage of LDH release was calculated as follows:  
158  $(A-B)/(C-B) \times 100$ , where A = LDH activity in conditioned media; B = LDH activity in culture  
159 media (without cells); C = LDH activity in cell lysates.

160 Assessment of cardiomyocyte apoptosis: Cardiomyocyte apoptosis was determined by terminal  
161 deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and  
162 caspase-3 activity, as we reported previously(41). TUNEL assay was performed utilizing the In  
163 Situ Cell Death Detection Kit (Roche, Palo Alto, CA). Briefly, cells were fixed with 10%  
164 paraformaldehyde and incubated with the TUNEL reaction mixture containing TdT-mediated  
165 dUTP. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Samples were  
166 visualized on an Olympus BX51 Fluorescence Microscope, and digital images were acquired  
167 with IP Lab Imagine Analysis Software (version 3.5, Scanalytics, Fairfax, VA). Apoptotic  
168 index (number of TUNEL positively stained nuclei/total number of nuclei  $\times$  100) was  
169 automatically calculated and exported for further analysis. Assays were performed in a blinded  
170 manner. The caspase-3 activity assay utilized the fluorogenic substrate  
171 DEVD-7-amino-4-trifluoromethyl-coumarin. Briefly, cells were lysed using caspase-3 lysis  
172 buffer (50mM HEPES PH 7.4, 0.1% Chaps, 5mM DTT, 0.1 mM EDTA, 0.1% Triton-X100),  
173 and total protein concentration was determined by the Bradford method. To each well of a  
174 96-well plate, supernatant containing 50 µg of protein was loaded and incubated with 3.645 µg  
175 Ac-DEVD-AFC at 37°C for 1.5 hours. AFC was cleaved from DEVD by activated caspase-3,  
176 and the free AFC was quantified with a Biotek FL600 microplate fluorescence reader  
177 (excitation wavelength, 400 nm; emission wavelength, 508 nm). Caspase-3 activity was  
178 expressed as nanomoles of AFC formation per hour per milligram of protein.



179 Trx activity assay: Trx activity was determined via the insulin disulfide reduction assay(17). In  
180 brief, 40 µg of cellular protein extracts were pre-incubated at 37°C for 15 minutes with 2 µl  
181 activation buffer (100 mM Hepes, 2 mM EDTA, 1 mg/ml BSA, and 2 mM dithiothreitol) to  
182 reduce thioredoxin. After addition of 20 µl reaction buffer (100 mM Hepes, 2.0 mM EDTA, 0.2  
183 mM NADPH, and 140 µM insulin), the reaction was initiated by addition of mammalian Trx  
184 reductase (1 µl, 15 mU, Sigma), and samples were incubated for 30 minutes at 37°C (the  
185 controls received water only). The reaction was terminated by adding 125 µl of stopping  
186 solution (0.2 M Tris-CL, 10 M guanidine-HCl, and 1.7mM 3-carboxy-4-nitrophenyl disulfide).  
187 Absorption measurement occurred at 412 nm.

188 Immunocytochemical detection of advanced glycated end products: Cardiomyocytes, seeded on  
189 glass coverslips in 6 well plates, were treated as described above. Cells were fixed with  
190 polyformaldehyde (4% in PBS) for 1 hour, washed with PBS, blocked with 10% normal goat  
191 serum, and incubated with rabbit anti-AGE polyclonal antibody (Abcam Inc., Cambridge, MA).  
192 Immunostaining was developed with a Vectastain ABC kit (Vector Laboratories, Burlingame,  
193 CA), and examined under light microscopy.

194 Detection of Trx-ASK1 interaction: Cells were homogenized with lysis buffer.  
195 Immunoprecipitation and immunoblotting were performed using a procedure described by  
196 Ischiropoulos and colleagues(43). In brief, endogenous Trx was immunoprecipitated with a  
197 monoclonal anti-murine Trx antibody (Redox Bioscience, Japan). After sample separation, the  
198 Trx-ASK-1 interaction was determined by Western blot analysis using a monoclonal antibody  
199 against ASK-1 (Upstate Biotechnology, Lake Placid, NY) and HRP-conjugated anti-mouse IgG  
200 antibody (Cell Signaling, Danvers, MA).

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

204 Lysates were sonicated on ice, and centrifuged at 12,000g for 10 minutes at 4°C.  
205 Immunoprecipitation was performed by adding 20 µl of resuspended immobilized monoclonal  
206 antibody against phospho-p38 MAPK (Thr180/Tyr182) to 100 µl cell lysate containing 150 µg  
207 of protein. The mixture was incubated with gentle rocking overnight at 4°C. After 10,000g  
208 centrifugation at 4°C for 2 minutes, the pellets were washed twice with lysis buffer, and twice  
209 with kinase buffer. The kinase reactions occurred in the presence of 200 µM ATP and 2 µg  
210 ATF-2 fusion protein at 30°C for 30 minutes. After incubation, the samples were separated by  
211 SDS-PAGE, and ATF-2 phosphorylation was measured by Western immunoblotting using a  
212 monoclonal antibody against phosphorylated ATF-2, followed by enhanced chemiluminescent  
213 detection.

214 *In vitro incubation of recombinant Trx with MG or MG+AG:* To investigate the effects of MG  
215 on Trx directly, 1 µg of recombinant human Trx (hTrx, Sigma-Aldrich, St. Louis, MO) was  
216 incubated with 500 µM MG at 37°C for 8 days. Activity of treated Trx (0.3 µg) was then  
217 determined as described above. Control Trx was incubated with deionized water under same  
218 conditions. For the anti-glycation treatment, AG (300 µM) was added at the initiation of  
219 incubation.

220 *Western blot analysis:* Cultured Cardiomyocytes cells were collected in lysis buffer after  
221 treatment. Aliquots containing 30–60 µg of protein were separated by electrophoresis through  
222 8–12% SDS-polyacrylamide gel, and transferred to positively charged nylon membranes. The  
223 membranes were blocked with 5% dry fat-free milk in Tris-buffered saline containing 0.1%  
224 Tween 20 and then incubated with primary antibodies against Trx (Redox Bioscience, Japan),  
225 p38, phospho-p38, GAPDH (Cell Signaling Technology Inc., Danvers, MA), and CML(Abcam  
226 Inc., Cambridge, MA), respectively. Positively charged nylon membranes were then incubated  
227 with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G antibody (Cell  
228 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.

237 **Results**

238 Pre-culturing cardiomyocytes with high MG increased their susceptibility to simulated  
239 ischemia/reperfusion injury. Cardiomyocytes subjected to SI/R injury manifested significant  
240 cellular injury including necrotic and apoptotic cell death, as evidenced by increased LDH  
241 release, TUNEL staining, and caspase-3 activity (Figure 1). Under basal conditions,  
242 cardiomyocytes treated with MG developed normally without apparent injury (MG+Sham SI/R  
243 groups in Figure 1). However, when subjected to SI/R, the cells treated with 6 days of MG  
244 (200  $\mu$ M) exhibited significantly greater cellular injury compared to control in the parameters  
245 listed above.

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

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

262  $\mu$ M) pre-treated cells subjected to SI/R. These results demonstrated that blockade of  
263 MG-induced protein glycation significantly protected cardiomyocytes from SI/R injury.

264 *MG incubation induced recombinant hTrx glycation and significantly decreased its activity.*

265 Our experimental results presented above demonstrated that MG is capable of initiating protein  
266 glycation reactions and that blockade of protein glycation reduced SI/R injury in MG  
267 pretreated cardiomyocytes. However, the specific proteins that are glycated by MG potentially  
268 contributive to increased SI/R injury remain unknown. After a thorough literature review  
269 identifying feasible protein candidates, Trx was selected because 1) Trx is a critical  
270 anti-apoptotic and cell survival molecule, and its inactivation has been causatively related to  
271 cardiovascular injury; and 2) we recently demonstrated the Trx is susceptible to glycative  
272 modification by lipopolysaccharide and consequent activity inhibition. To directly test a novel  
273 hypothesis that MG may cause Trx glycative inactivation, rendering cardiomyocytes more  
274 susceptible to SI/R injury, we first determined whether Trx can be glycatively modified by MG  
275 with subsequent activity inhibition. Recombinant hTrx (human thioredoxin-1) was incubated  
276 with MG in a cell-free system, and Trx glycation and Trx activity were determined. As  
277 summarized in Figure 4A, in vitro incubation of hTrx caused Trx glycation as evidenced by  
278 abundant production of N<sup>ε</sup>-carboxymethyl lysine (CML), a biomarker of AGEs formation. MG  
279 incubation markedly inhibited Trx activity (60.4% reduction, P<0.01 vs. vehicle-incubated  
280 Trx)(Figure 4B). More importantly, addition of AG in the system completely abolished  
281 MG-induced Trx glycation (Figure 4A) and significantly attenuated MG-induced Trx  
282 inactivation (Figure 4B).

283 *Trx is glycatively inhibited in MG pre-treated cardiomyocytes prior to SI/R injury.* Having  
284 demonstrated that MG is capable of causing Trx glycative inhibition in a cell-free system, we  
285 further determined whether cellular Trx activity/expression might be reduced after MG  
286 exposure, leaving the cells more vulnerable to reperfusion injury. Compared to control, Trx

287 activity was significantly decreased in the MG-treated cells prior to SI/R (Figure 5A). However,  
288 Trx expression was slightly increased in MG-treated cells (Figure 5B), indicating that the  
289 observed reduction in Trx activity in the MG-treated cells is not from reduced expression of the  
290 protein, but rather its posttranslational modification. More importantly, co-treatment with AG  
291 significantly attenuated MG inhibition of Trx (Figure 5A).

292 Pre-culturing cardiomyocytes with MG promoted SI/R-induced Trx-ASK1 dissociation and  
293 subsequent p38 MAPK activation, which were attenuated by co-treatment with AG. Recent in  
294 vitro studies have demonstrated that the binding and resultant inhibition of ASK1 is the  
295 primary mechanism by which Trx exerts its anti-apoptotic effect(38). Moreover, the increased  
296 ratio of ASK1/Trx-ASK1 correlates with the increased basal activity of the p38 MAPK  
297 pathway(18). To determine whether MG inhibition of Trx may alter the Trx-ASK1 interaction,  
298 and consequently activate downstream pro-apoptotic kinases, two additional experiments were  
299 performed. Via anti-Trx-1 immunoprecipitation and anti-ASK1 immunoblotting, Figure 6A  
300 illustrates Trx is physically associated with ASK1 in normal cultured cardiomyocytes, and this  
301 protein-protein interaction was significantly decreased after SI/R. Consequently, the activity of  
302 p38 MAPK, a pro-apoptotic downstream molecule for ASK1, was significantly enhanced in the  
303 MG-treated cardiomyocyte compared to control (Figure 6B). More importantly, this  
304 SI/R-induced disassociation of Trx-ASK1 was significantly further enhanced when cells were  
305 pre-cultured with MG, and p38 MAPK activity was significantly further increased (Figure 6).  
306 Treatment with aminoguanidine restored Trx-ASK1 interaction (Figure 7A), reduced p38  
307 MAPK phosphorylation (Figure 7B), and inhibited p38 MAPK activity (Figure 7C) in  
308 MG-treated cardiomyocytes.

309

310 **Discussion**

311 Ischemic heart disease (IHD) continues to gain prevalence as a cause of disability and  
312 death in the United States, and is costly in terms of patient morbidity and mortality, as well as  
313 financial resources utilized in acute and chronic treatment. The specific molecular mechanisms  
314 underlying why diabetes mellitus directly increases IHD risk remain elusive. Accumulating  
315 evidence has indicated that MG, a reactive dicarbonyl compound produced mainly from  
316 cellular glycolytic intermediates, is often found at high circulating blood levels in diabetic  
317 patients(4; 11; 39). Evidence suggests elevated MG levels may play a role in the development  
318 of a number of diabetic complications(32). Elucidation of the effects of MG and other AGEs  
319 precursors upon the pre-ischemic heart, and the involved underlying mechanisms, could yield  
320 improved preventative and therapeutic treatment of the diabetic heart respectively at risk for  
321 and undergoing ischemic injury.

322 Our current study provided evidence that protein glycation is a new mechanism through  
323 which MG aggravates SI/R injury. This notion is supported by our observations that 1)  
324 pre-culturing cardiomyocytes with MG for 6 days caused a greater than 30-fold increase in  
325 AGE production, which was dramatically reduced by co-treatment with AG, a strong AGE  
326 formation inhibitor; and 2) pre-culturing cardiomyocytes with MG for 6 days made  
327 cardiomyocytes more susceptible to SI/R as evidenced by increased LDH release, more cardiac  
328 caspase-3 activation, and greater percentage of TUNEL positive staining, all of which were  
329 also markedly inhibited by AG co-treatment. Two experimental limitations should be discussed.  
330 First, the MG concentration present in the culture medium is much higher than that found in  
331 diabetic patient plasma(31; 35). However, it must be indicated that clinical situations are much  
332 more complicated, and actual MG concentrations to which in vivo cells are exposed remain  
333 uncertain. The intracellular MG level is likely much higher than the plasma MG level in the  
334 diabetic condition because diabetic tissues are chronically (months to years) exposed to high

335 MG levels, which can cause dramatic intracellular MG accumulation (up to 300  $\mu$ M)(7). In  
336 contrast, cultured cells were only transiently (days) exposed to high concentrations of MG,  
337 which may limit intracellular MG accumulation(8). Additionally, actual diabetic tissues are  
338 concomitantly exposed to high levels of plasma glucose, whereas cultured cells in this study  
339 were exposed to normal glucose concentration. Furthermore, as MG is formed during  
340 glycolysis, clinical diabetes, often causing tissue hypoperfusion and hypoxia, may stimulate  
341 intracellular MG production. In contrast, normal oxygen was present during the 6-day  
342 pre-culturing period of our study, and cellular glycolysis was minimal. For these reasons, high  
343 concentrations of MG ranging from 200  $\mu$ M to 1.5 mM were typically used in previously  
344 published studies by many investigators(10; 19; 23; 25; 45). Second, besides its strong  
345 anti-glycation property, AG is also a potent iNOS inhibitor. Therefore, the protective effect of  
346 AG against MG-enhanced SI/R injury could be attributed to its anti-iNOS effect. Although  
347 theoretically possible, our experimental results do not support this possibility because 1) we  
348 have previously demonstrated that significant iNOS upregulation begins 2 hours after  
349 reperfusion, but AG was washed out from the culturing system before the cells were subjected  
350 to SI/R; and 2) MG pre-culturing did not cause any significant cell injury (Figure 1, sham SI/R  
351 group) unless the cells were subjected to SI/R (Figure 1, SI/R group), and pre-treatment with  
352 AG alone during the pre-culturing period (washed out before SI/R) had no effect on cellular  
353 injury, before or after SI/R (data not shown).

354 Discovered 40 years ago in bacteria, thioredoxin's influence in human cells has only  
355 recently begun to be appreciated, as the diverse gamut of processes (including cellular redox  
356 balance, cell growth promotion, apoptosis inhibition, and inflammation modulation) regulated  
357 by thioredoxin continue to be discovered(34). It is not surprising, therefore, to behold the role  
358 Trx plays in a wide range of human diseases and conditions, including cancer, viral pathology,  
359 and ischemia/reperfusion injury(9). Emerging evidence suggests that Trx plays critical roles in



360 promoting cell proliferation/survival and reducing cell death. Trx and its reductase protein  
361 (TrxR) are upregulated in cancer tissues; molecules inhibiting Trx or TrxR promote apoptosis,  
362 and reduce cancer development(36). In contrast, Trx activity is reduced in diseased tissues  
363 where pathologic apoptosis is increased(27). Recent studies have demonstrated that besides  
364 upregulation or downregulation of Trx expression at the gene level, Trx activity is differentially  
365 regulated by post-translational modifications. Oxidation of the thiol groups of Cys-32 and -35  
366 forms a disulfide bond, and reversibly inhibits Trx's anti-oxidative activity. Glutathionylation,  
367 occurring at Cys-73, significantly inhibits Trx's antioxidant activity(6). S-nitrosylation occurs  
368 at Cys-69 or Cys-73, and has been shown to markedly enhance Trx's anti-oxidant,  
369 anti-apoptotic and organ protective activity(15; 16; 41). Nitration, occurring at Ty-49, causes  
370 significant irreversible inhibition of Trx's anti-oxidative and cellular protection.

371 Protein glycation, also know as non-enzymatic glycosylation, is a protein modification  
372 reaction between proteins and reducing sugars(42). Glycation occurs in several steps. In an  
373 initial step that completes in a short period of time (minutes to hours), the reducing sugar reacts  
374 with the protein chain and produces Schiff-reaction primary-glycated products (e.g.,  
375 fructosamine). After several days or weeks, Amadorial rearrangement commences, and  
376 advanced glycated end-products (AGEs) are formed(22). In recent years, the pathogenic roles  
377 of AGEs have been extensively investigated. Increased AGEs accumulation and subsequent  
378 tissue injury have been found in many human diseases, such as type 2 diabetes and the aging  
379 process(1; 13; 44). However, whether the early modification of protein by sugar prior to AGEs  
380 formation may alter protein function remains largely unknown. A study by McCarthy et al(29)  
381 reported that incubation of alkaline phosphatase (ALP) with reducing sugars reduced enzyme  
382 activity associated with an increase in fructosamine levels, indicating that early glycation may  
383 alter protein function. In two more recent studies, it was reported that human  
384 Cu-Zn-superoxide dismutase(20) and esterase(40) can be glycated by methylglyoxal, and their

385 activities are subsequently inhibited. Methylglyoxal reacts with the free amino groups of lysine  
386 and arginine, and with cysteine thiol groups to form AGEs. In a recent study, we have  
387 demonstrated that Trx is susceptible to non-enzymatic glycation via lipopolysaccharide (LPS)  
388 exposure (Free Radical Biology and Medicine, in revision), consequent inactivation, and is  
389 furthermore unable to provide protection against LPS-induced liver toxicity.

390 Our current study demonstrated for the first time that pre-culturing cardiomyocytes  
391 significantly inhibited cellular thioredoxin activity before the cells were subjected to SI/R, and  
392 caused greater dissociation of Trx-ASK1 and p38 MAPK activation after SI/R. The MG  
393 inhibition of Trx is likely attributable to Trx glycative modification and partially responsible  
394 for MG enhancement of SI/R injury. This novel hypothesis is supported by the following  
395 observations. Firstly, MG pre-culture slightly increased Trx expression, indicating that  
396 posttranslational modification, rather than Trx gene expression is responsible for reduced Trx  
397 activity in MG pre-cultured cardiomyocytes (Figure 5). Secondly, MG caused significant  
398 recombinant hTrx-1 glycation and inactivation in a cell-free incubation system, and  
399 co-treatment with AG blocked Trx glycation and preserved Trx activity (Figure 4). Thirdly,  
400 addition of AG significantly attenuated the inhibitory effect of MG on cellular Trx activity  
401 (Figure 5). Finally, treatment with AG only during the pre-culturing period significantly  
402 improved Trx-ASK1 association and inhibited pro-apoptotic p38 MAPK activation after SI/R  
403 (Figure 7).

404 It should be indicated that Trx is also susceptible to nitrate inhibition. AG, as an iNOS  
405 inhibitor, may preserve Trx activity in MG-treated cells by blocking iNOS expression.  
406 However, our current study supports that Trx glycative modification is a more likely  
407 mechanism responsible for MG inactivation of Trx because 1) pre-culturing cells with MG  
408 significantly reduced Trx activity even before cells were subjected to SI/R, whereas significant  
409 iNOS expression was not observed until 2 hours after reperfusion; 2) treatment with AG only

410 during the pre-culturing period where no significant iNOS is present significantly attenuated  
411 MG inactivation of Trx, and 3) in a cell-free system where no iNOS is present, AG blocked Trx  
412 glycation and preserved Trx activity after MG incubation.

413 Finally, some limitations should be addressed. Firstly, the specific amino acid residues  
414 of Trx-1 responsible for glycative modification remain unknown, and are currently under  
415 investigation. However, our preliminary data indicated that cysteine residues are not involved  
416 in glycative modification, because mutations of any or all of the 5 Trx cysteine residues failed  
417 to block Trx-1 glycation. Secondly, we were unable to directly measure cardiomyocyte Trx  
418 glycation after MG incubation, because a method sensitive enough to detect early protein  
419 glycation in cells is currently unavailable. Nonetheless, our cell-free experimental results  
420 demonstrating that Trx function is glycatively inhibited, together with our cellular experimental  
421 results showing that cardiomyocyte Trx-1 activity is reduced in MG-treated cells and preserved  
422 by AG, summarily suggest that glycative Trx inactivation may contribute to MG enhancement  
423 of cardiomyocyte SI/R injury. Thirdly, H9c2 cells are neonatal myoblasts and may have some  
424 differences from the adult cardiomyocytes. However, this cell line has been extensively used as  
425 an experimental cardiomyocyte model, specially in those experiments that cells needs to be  
426 cultured for a long period of time.

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

433

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438

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440

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573

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).

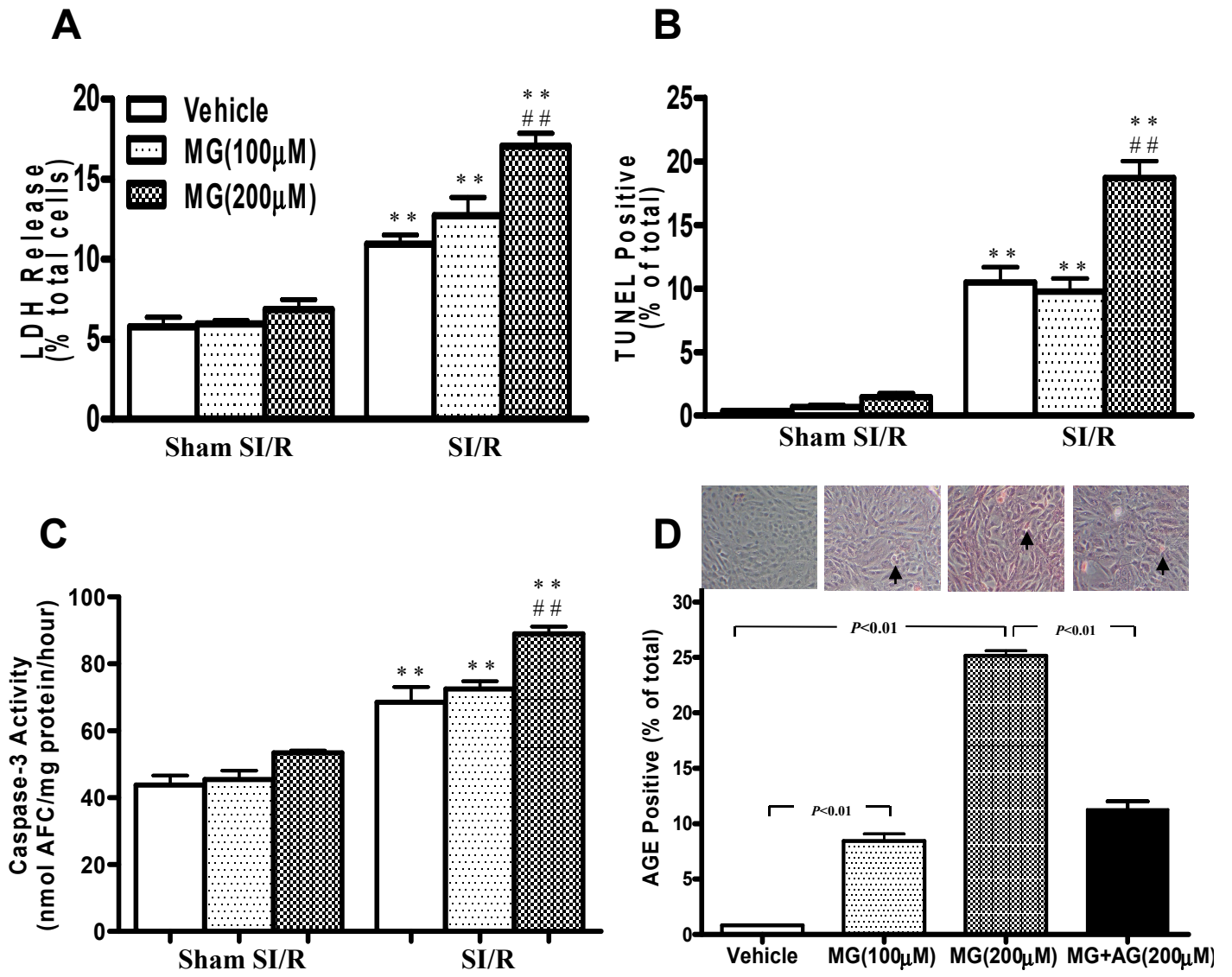


Figure 1

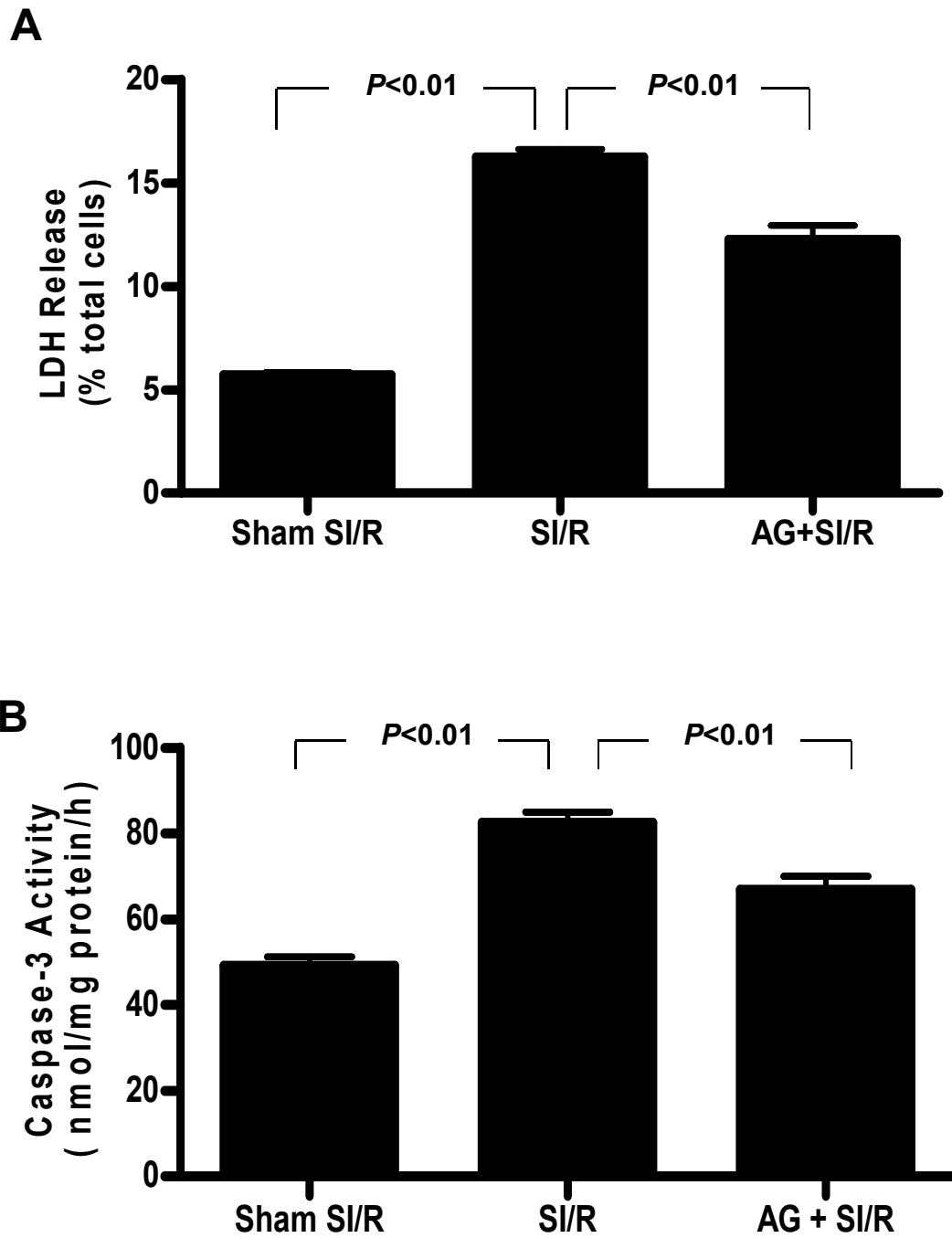


Figure 2

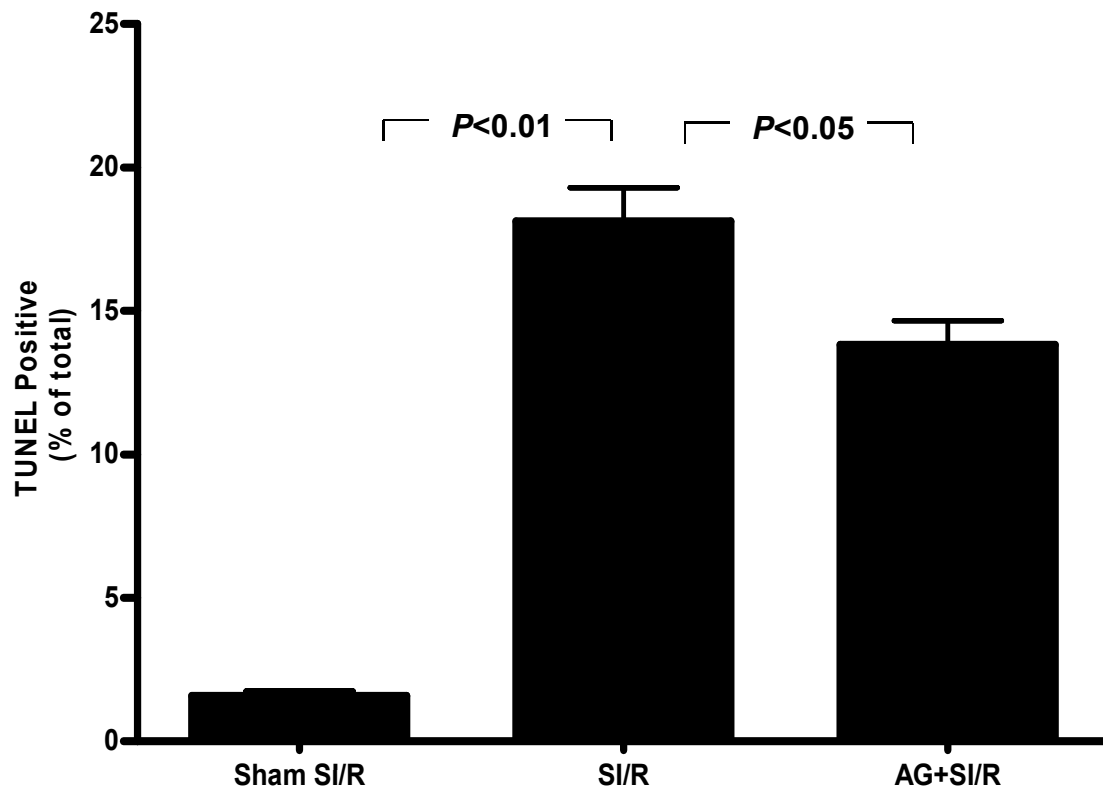
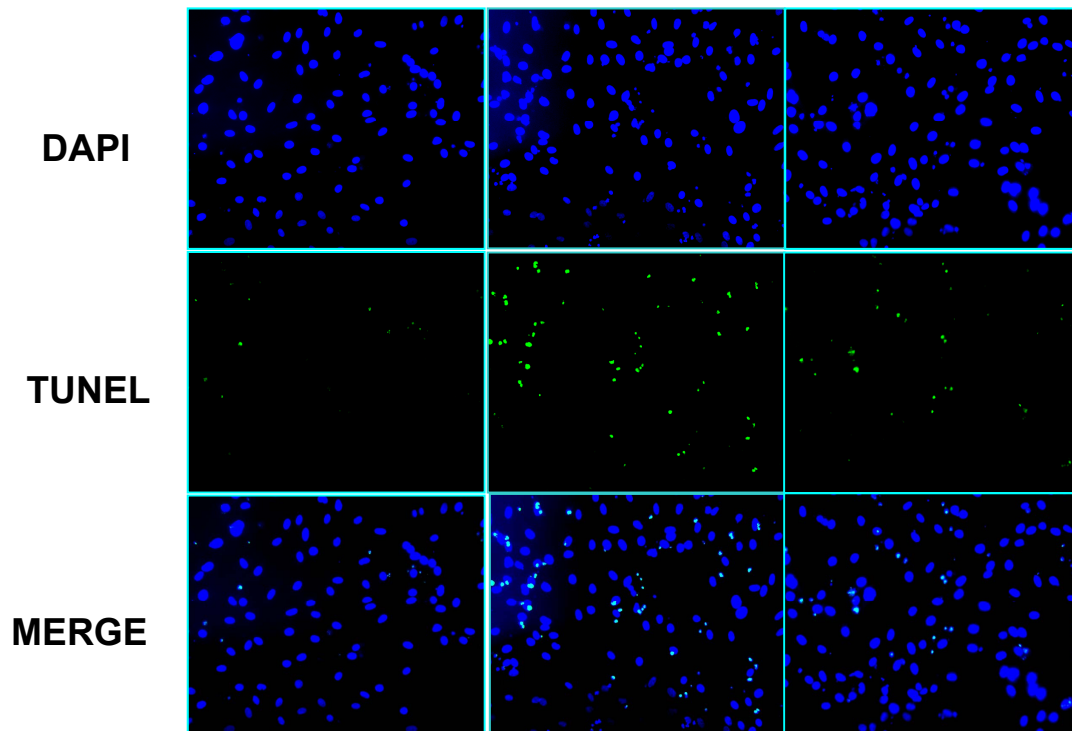


Figure 3

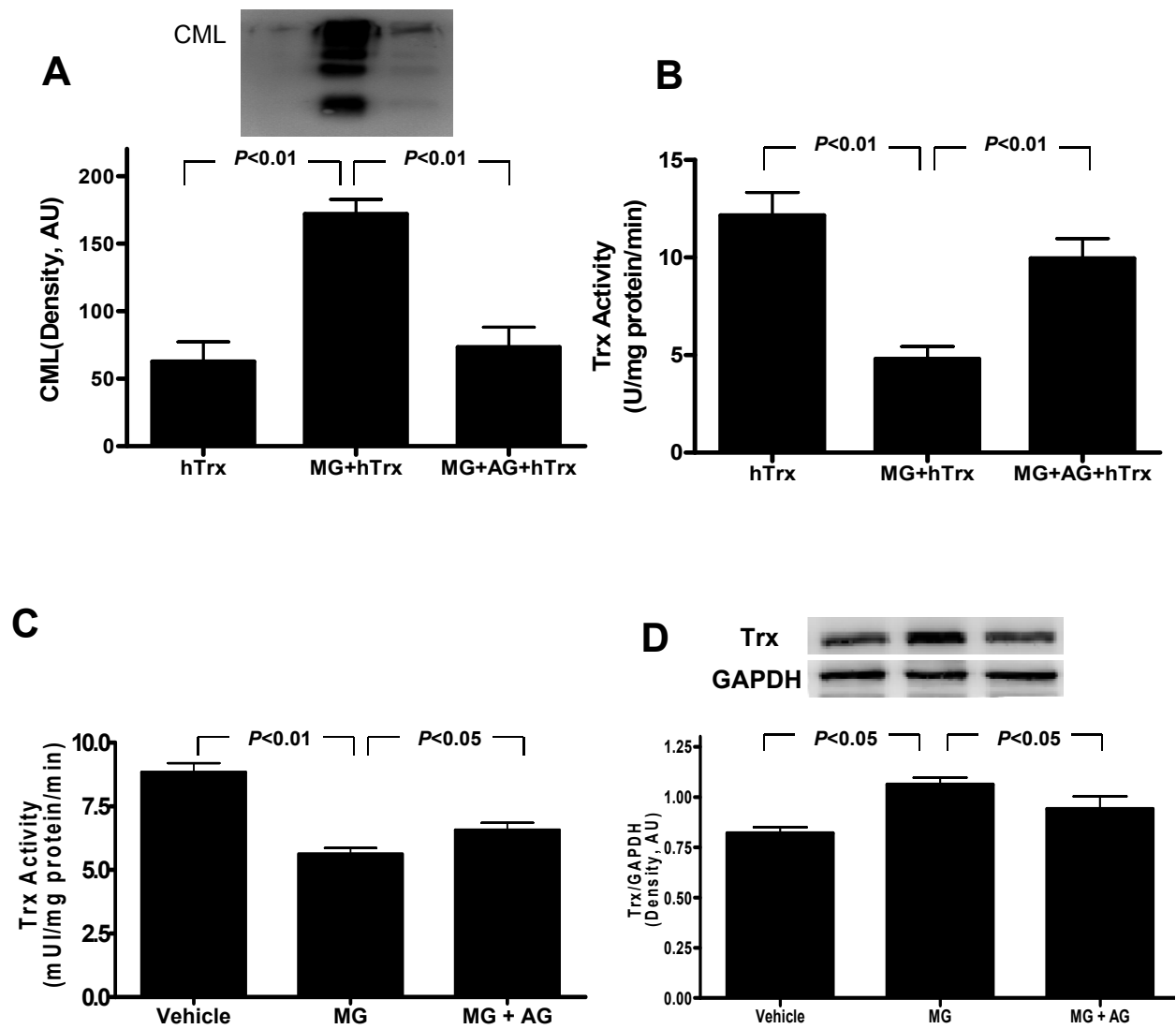


Figure 4

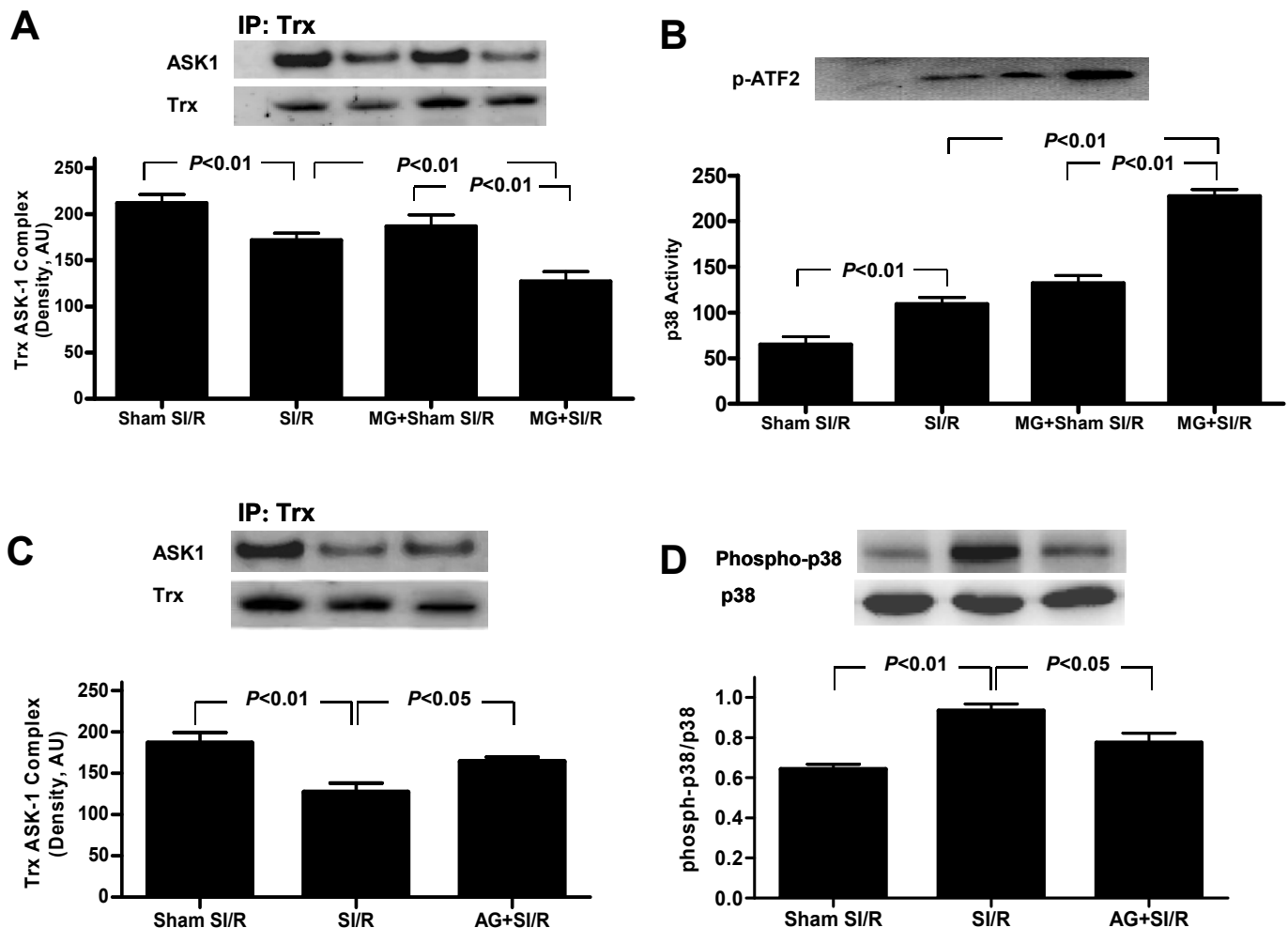


Figure 5