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**The Alternative Crosstalk between RAGE and Nitrative Thioredoxin
Inactivation during Diabetic Myocardial Ischemia/Reperfusion injury**

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Running Title: RAGE and nitrative Trx inactivation in diabetes

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43 **Abstract**

44 The receptor for advanced glycation end products (RAGE) and thioredoxin (Trx)
45 play opposing roles in diabetic myocardial ischemia/reperfusion (MI/R) injury. We
46 recently demonstrated nitrative modification of Trx leads to its inactivation and loss
47 of cardioprotection. The present study is to determine the relationship between
48 augmented RAGE expression and diminished Trx activity pertaining to exacerbated
49 MI/R injury in the diabetic heart. The diabetic state was induced in mice by multiple
50 intraperitoneal low-dose streptozotocin (STZ) injections. RAGE small-interfering
51 RNA or soluble RAGE (sRAGE, a RAGE decoy) was via intramyocardial and
52 intraperitoneal injection before MI/R, respectively. Mice were subjected to 30 minutes
53 of MI followed by 3 hours or 24 hours of reperfusion. At 10 min before reperfusion,
54 diabetic mice were randomized to receive EUK134 (peroxynitrite scavenger),
55 recombinant human Trx-1 (rhTrx-1), nitrated Trx-1 (N-Trx-1), apocynin (a NADPH
56 oxidase inhibitor) or 1400W (an iNOS inhibitor) administration. The diabetic heart
57 manifested increased RAGE expression and N ϵ -(carboxymethyl)lysine(CML, major
58 AGE subtype) content, reduced Trx-1 activity, increased Trx nitration after MI/R.
59 RAGE siRNA or administration of sRAGE in diabetic mice decreased MI/R-induced
60 iNOS and gp91phox expression, reduced Trx nitration, preserved Trx activity, and
61 decreased infarct size. Apocynin or 1400W significantly decreased the nitrotyrosine
62 production and restored the thioredoxin activity. Conversely, administration of either
63 EUK134 or reduced hTrx, but not nitrated hTrx, attenuated MI/R-induced superoxide
64 production, RAGE expression and CML content, and decreased cardiomyocytes
65 apoptosis in diabetic mice. Collectively, we demonstrate that RAGE modulates the
66 MI/R injury in a Trx nitrative inactivation fashion. Conversely, nitrative modification
67 of Trx blocked its inhibitory effect upon RAGE expression in the diabetic heart. This
68 is the first direct evidence demonstrating the alternative crosstalk between RAGE
69 overexpression and nitrative Trx inactivation, suggesting that interventions interfering
70 with their interaction may be novel means of mitigating diabetic MI/R injury.

71 **Keywords**

72 Diabetes; Ischemia/reperfusion; thioredoxin; protein nitration; RAGE

73 **Introduction**

74 Cardiovascular disease is the most significant cause of morbidity and mortality
75 among diabetic patients (11). Diabetics suffer augmented incidence and severity of
76 myocardial infarction (MI), with increased susceptibility to heart failure post MI
77 compared to non-diabetics. However, despite enormous efforts in recent years, the
78 defined molecular basis linking diabetes with exacerbated susceptibility to ischemic
79 injury, and resultant higher mortality, remains elusive.

80 The receptor for advanced glycation end products (RAGE) is a multi-ligand
81 receptor of the immunoglobulin superfamily with diverse functions. In addition to
82 AGEs, RAGE also binds distinct families of ligands, such as S100/calgranulins high
83 mobility group box 1(18). RAGE therefore plays a comprehensive role in signal
84 transduction activation and gene expression modulation. Initially, RAGE was
85 demonstrated to be involved in diabetic vascular injury(26). Subsequent studies have
86 demonstrated that RAGE also modulates acute nerve injury and cellular death in
87 advanced keratectomy (9, 29). Recently, knockdown or blockade of RAGE by soluble
88 RAGE (sRAGE, the extracellular binding ligand RAGE decoy) has been
89 demonstrated to attenuate myocardial ischemic injury through modulation of
90 comprehensive signaling pathways including TGF- β , NF- κ B, JNK, STAT and
91 GSK3 β pathway(1, 5, 23, 36).

92 We recently demonstrate that the protein nitrative modification play a crucial role
93 in I/R- induced cardiomyocyte injury (34). Our and others studies also demonstrate
94 that RAGE elicits nitrotyrosine production (the footprint of protein nitration) (6, 22),
95 which indicates that RAGE acts as a modulator of protein nitration; however the
96 mechanism by which RAGE cause the protein nitration is completely unknown. More
97 importantly, whether RAGE-induced protein modification has any influence upon
98 MI/R injury has not been investigated. Thioredoxin(Trx), a small 12 kD protein
99 expressed in nearly all living cells with various biological functions, is protective
100 against oxidative stress and is anti-apoptotic(19). Susceptible to up- or
101 down-regulation at the gene level, Trx activity is also regulated by posttranslational
102 modification (17, 33). We have previously demonstrated nitrative modification of

103 Trx-1 results in its irreversible inactivation during MI/R (33, 39). However, whether
104 RAGE might posttranslationally affect Trx with consequences upon the latter's
105 protective effects against MI/R injury remains completely unknown.

106 Upregulation of RAGE augments reactive oxidative species (ROS) production.
107 ROS furthermore contributes to AGE generation and enhanced RAGE expression,
108 ultimately perpetuating a vicious cycle potentially resulting in cellular and tissue
109 injury (15). Trx functions as key antioxidant in the living body. We recently
110 demonstrate that nitrative inactivation of Trx-1 is exacerbated in diabetic mice,
111 enhancing cardiac vulnerability to I/R injury (39). In the diabetic state, both RAGE
112 expression and oxidative stress are increased (4). Whether Trx might directly or
113 indirectly affect ROS generation or RAGE expression remains unknown. More
114 importantly, the relationship between augmented RAGE expression and diminished
115 Trx activity pertaining to exacerbated MI/R injury in the diabetic heart has not been
116 investigated.

117 Therefore, the aims of the present study were (1) to determine the effects of
118 myocardial ischemic injury upon AGE/RAGE levels and nitrative Trx inactivation in
119 the diabetic condition; 2) to determine the specific mechanism by which AGEs and
120 RAGE exacerbate myocardial ischemia injury; and 3) investigate potential therapeutic
121 modalities capable of attenuating exacerbated MI/R injury in the diabetic state
122 utilizing mechanistic information gleaned from Aims 1 and 2.

123

124 **Materials and Methods**

125 **Experimental Protocols**

126 All experiments were performed in adherence with the National Institutes of Health
127 Guidelines on the Use of Laboratory Animals, and were approved by the Fourth
128 Military Medical University Committee on Animal Care. C57BL/6 mice (aged
129 8–10 weeks) were used for the present study. The diabetic state was induced by
130 intraperitoneal injection of 40 mg/kg STZ (Sigma, St. Louise, MO) diluted in citrate
131 buffer (pH 4.5) for 5 consecutive days. Age-matched control mice were injected with
132 an equal volume of citrate buffer. Diabetes onset was confirmed by hyperglycemia
133 exceeding 300 mg/dl 10 days after initial STZ administration. Mice were sacrificed
134 after 3 weeks of established diabetes.

135 Two methods were utilized for blocking RAGE signaling in diabetic mice: Firstly,
136 small-interfering RNA (siRNA) gene silencing technique was used to knockdown
137 RAGE expression. Predesigned RAGE-specific siRNA (catalog# sc-36375, Santa
138 Cruz) or control scrambled siRNA (catalog# sc-37007, Santa Cruz) were diluted in
139 5% glucose and mixed with in vivo jet PEI (polyethyleneimine; Genesee Scientific,
140 San Diego, CA). Diabetic mice were anesthetized with 2% isoflurane, and the heart
141 was exposed via left fifth intercostal space thoracotomy. RAGE siRNA or scrambled
142 siRNA was delivered via three separate intramyocardial injections, temporarily
143 blanching the left ventricular free wall. Hearts were subjected myocardial
144 ischemia/reperfusion (MI/R) 48 hours after siRNA injection (24). Secondly, murine
145 soluble RAGE (sRAGE, a RAGE decoy; 500 µg/day, Adipobioscience) was
146 administered via intraperitoneal (IP) injection for 3 days duration prior to MI/R.

147 In vivo MI/R procedure was performed as previously described(14). Briefly, male
148 mice were anesthetized with 2% isoflurane, and MI was induced by temporarily
149 exteriorizing the heart via a left thoracic incision, and placement of a 6-0 silk suture
150 slipknot around the left anterior descending coronary artery. After 30 minutes of MI,
151 the slipknot was released, and the myocardium was reperfused for 3 hours (for
152 apoptosis determination) or 24 hours reperfusion (for infarct size and cardiac function
153 assays). Additionally, 10 min before reperfusion, the non-RAGE siRNA and

154 non-sRAGE treated diabetic mice were randomized to receive vehicle (PBS, pH 7.5)
155 or reduced human Trx (rhTrx-1, 2 mg/kg; Sigma), EUK-134(a peroxynitrite
156 decomposition catalyst, 5 mg/kg; Cayman Chemical), nitratively modified hTrx
157 (N-hTrx), 1400W(a selective iNOS inhibitor, 2mg/kg), apocynin (a selective
158 NADPH oxidase inhibitor, 5 mg/kg) via IP injection. Sham-operated control mice
159 (sham MI/R) underwent identical surgical procedures, except the suture placed under
160 the left coronary artery was not tied. At the conclusion of the reperfusion period, the
161 ligature around the coronary artery was retied, and 2% Evans Blue dye was injected
162 into the left ventricular cavity. Cardioectomy was swiftly performed, and the
163 ischemic/reperfused cardiac tissue was isolated and processed per below protocols.

164 **In vitro nitration of Trx-1**

165 Human Trx-1 (Sigma, St. Louise, MO) was subjected to in vitro nitration with a
166 modified procedure recently described previously (16). In brief, Purified human Trx-1
167 (dissolved in 0.1 μ M phosphate buffer, pH 7.4, final concentration 50 μ M) was
168 incubated with SIN-1 (final concentration of 100 μ M; Cayman Chemical, Ann Arbor,
169 Michigan) at 37 ° 30 minutes. Unreacted SIN-1 was removed by ultrafiltration over
170 membranes with a 5-kDa cutoff.

171 **Determination of cardiac function and myocardial infarct size**

172 Twenty-four hours after reperfusion, mice were anesthetized, and cardiac function
173 was determined by echocardiography (VisualSonics VeVo 2100 imaging system).
174 After assessment, cardiectomies were performed. Myocardial infarct size was
175 determined by using Evans blue/2, 3, 5-triphenyltetrazolium chloride (TTC) staining
176 as previously described (8).

177 **Determination of myocardial apoptosis**

178 Myocardial apoptosis was determined via terminal
179 deoxynucleotidyltransferase-mediated dUTP nick end labeling staining (TUNEL,
180 Roche Ltd. Switzerland) and caspase-3 activity assay(Beyotime Company,
181 Shanghai, China), inclusive of the entire ischemic/reperfused region termed
182 "area-at-risk" as previously described (33).

183 **Quantification of superoxide production, nitrotyrosine content, CML content**

184 Superoxide production, an index of oxidative stress, was measured by
185 lucigenin-enhanced chemiluminescence as previously described (17), and expressed
186 as relative light units (RLU) per second per milligram protein (RLU/s/mg protein). In
187 situ superoxide detection was performed with dihydroethidium staining (DHE,
188 Beyotime Company, Shanghai, China) as described previously(35). Nitrotyrosine
189 content, an established index of protein nitration and nitrative stress, was determined
190 by Millipore nitrotyrosine assay per commercial manufacturer protocol kit (Millipore).
191 N^ε-(carboxymethyl)lysine (CML) content, the predominant AGE subtype, was
192 measured per commercial manufacturer protocol kit(gersion Bio-Technology, Beijing,
193 China).

194 **Trx activity assay**

195 Trx activity was determined via insulin disulfide reduction assay(19). In brief,
196 40μg of cellular protein extracts were incubated at 37°C for 15 minutes with 2 mL
197 activation buffer (100 mM HEPES, 2 mM EDTA, 1 mg/mL BSA, and 2 mM DTT) to
198 reduce thioredoxin. After addition of 20 uL reaction buffer (100 mM HEPES, 2.0 mM
199 EDTA, 0.2 mM NADPH, and 140 mM insulin), the reaction was initiated by addition
200 of mammalian Trx reductase (1 mL, 15 mU, Sigma, St. Louis, USA) or distilled water
201 to controls. After incubation for 30 minutes at 37°C, the reaction was terminated by
202 125uL stopping solution (0.2m Tris-CL, 10M guanidine-HCl, and 1.7mM
203 3-carboxy-4-nitrophenyl disulfide, DTNB), followed by absorption measurement (412
204 nm). Trx-1 activity was expressed as oxidized NADPH micromol per minute per
205 milligram (μmol/min/mg) of protein.

206 **Immunoblotting**

207 Cardiac tissue homogenate proteins were separated on SDS-PAGE gels, transferred
208 to nitrocellulose membranes, and Western blotted with monoclonal antibody against
209 RAGE (Abcam, Cambridge, MA), gp91phox(Santa Cruz, Delaware, USA)),
210 iNOS(cell signaling, Boston, USA). Nitrocellulose membranes were then incubated
211 with HRP-conjugated immunoglobulin G antibody (Santa Cruz Biotechnology, Inc)
212 for 1 hour. The blot was developed with an ECL-Plus chemiluminescence reagent kit
213 and visualized via UVP Bio-Imaging Systems. Blot densities were analyzed with

214 Vision Works LS Acquisition and Analysis Software.

215 **Detection of Trx-1 nitration**

216 The cardiac tissue or cardiomyocytes were homogenized with lysis buffer.
217 Endogenous Trx-1 was immunoprecipitated with a monoclonal anti-murine Trx-1
218 antibody (Redox Bioscience, Kyoto, Japan). After sample separation, Trx-1 nitration
219 was detected with a monoclonal antibody (Upstate, Charlottesville, VA, USA) against
220 nitrotyrosine. The blot was developed with an ECL-Plus chemiluminescence reagent
221 kit (Amersham, Corston, UK) and visualized via UVP Bio-Imaging Systems. Blot
222 densities were analyzed with Vision Works LS Acquisition and Analysis Software.

223 **Statistical analysis**

224 All values in the text and figures are presented as mean $\pm$ SEM of n independent
225 experiments. All data (except Western blot density) were subjected to ANOVA
226 followed by Bonferroni correction for post hoc t test. Western blot densities were
227 analyzed with the Kruskal-Wallis test followed by Dunn post hoc test. P values less
228 than 0.05 were considered statistically significant.

229

230 **Results**

231 **MI/R induced elevated AGE/RAGE expression and increased nitrative** 232 **thioredoxin inactivation to greater extent in diabetic mice versus control**

233 RAGE expression is upregulated in diabetic tissues subjected to MI/R injury (4).
234 Our recent study demonstrated nitrative inactivation of Trx-1 is significantly
235 increased in diabetic hearts (39). However, the relationship between increased RAGE
236 expression and reduced Trx activity in the diabetic heart pertaining to enhanced I/R
237 injury in diabetic hearts has not been investigated. To approach this question, we
238 evaluated the pre- and post-MI/R injury levels of both AGE/RAGE expression and
239 nitrative thioredoxin inactivation (evidenced by Trx activity and nitrated Trx content).
240 Compared to control, diabetic mice harbored significantly elevated
241 N^ε-carboxymethyl-lysine (CML, the prevailing AGE subtype, 266.02±28.74 pg/mg
242 protein VS 139.57±18.45 pg/mg protein, $P < 0.05$, Figure 1A) and RAGE (0.37±0.048
243 VS 0.16±0.03, $P < 0.05$, Figure 1B) in sham group. After MI/R, both CML content
244 (473.9±51.3 pg/mg protein VS 289.04± 39.93 pg/mg protein, $P < 0.01$, Figure 2A) and
245 RAGE expression (0.76±0.087 VS 0.49± 0.07, $P < 0.01$, Figure 1B) were amplified in
246 diabetic mice compared to control in MI/R group. The diabetic condition increased
247 nitrotyrosine content (the well accepted footprint of protein nitration, compared to
248 control, sham conditions, $P < 0.05$, Figure 1C), Trx nitration (compared to control,
249 sham conditions, $P < 0.01$, Figure 1E), and attenuated Trx activity (compared to
250 control, sham conditions, $P < 0.05$, Figure 1D). Compared to control, MI/R further
251 increased nitrotyrosine content ($P < 0.001$, Figure 1C), Trx nitration levels ($P < 0.001$,
252 Figure 1E), decreased Trx activity ($P < 0.001$, Figure 1D) to greater extent in diabetic
253 mice. However, Compared to control, MI/R has no effect on the Trx expression
254 (Figure 6) in diabetic mice.

255 **RAGE siRNA attenuated MI/R-induced oxidative/nitrative stress and nitrative** 256 **Trx inactivation**

257 To further evaluate the downstream mechanisms by which RAGE enhances MI/R
258 injury, siRNA gene silencing techniques were utilized to knock down RAGE. Our
259 method of intramyocardial siRNA delivery was highly successful, markedly inhibiting

260 basal RAGE expression (versus vehicle, $P<0.01$, Figure 2A). RAGE siRNA
261 significantly decreased oxidative/nitrative stress, evidenced by decreased superoxide
262 production (versus vehicle, $P<0.01$, Figure 2B), decreased nitrotyrosine content
263 (versus vehicle, $P<0.05$, Figure 2C). Most importantly, we demonstrated for the first
264 time that RAGE siRNA decreased the MI/R-induced Trx nitration (versus vehicle,
265 $P<0.01$, Figure 2E), restored I/R-induced diminished Trx activity (versus vehicle,
266 $P<0.05$, Figure 2D), but had no effect on the Trx expression (Figure 6). This data also
267 importantly demonstrate for the first time that MI/R injury mediated by RAGE is
268 closely involved with nitrative thioredoxin inactivation, supporting RAGE as a
269 modulator of nitrative thioredoxin inactivation.

270

271 **MI/R-induced nitrative thioredoxin inactivation was attenuated by iNOS or**
272 **NADPH oxidase inhibitor in diabetic mice**

273 It is well known that NO reacts with superoxide ($O_2^{\cdot-}$), resulting in enhanced toxic
274 peroxynitrite ($ONOO^{\cdot-}$) formation which is the critical contributor of protein nitrative
275 modification (34), and the NADPH oxidase is the most important cytosolic source for
276 superoxide production(12). To further determine the molecular mechanism by which
277 RAGE causes thioredoxin nitration, two additional experiments were performed.
278 Firstly, a selective NADPH oxidase inhibitor (apocynin) or a selective iNOS inhibitor
279 (1400W) was administered 10 minutes before reperfusion. Apocynin or a 1400W
280 significantly decreased MI/R nitrotyrosine production (versus vehicle, all $P<0.05$,
281 Figure 3A), attenuated the thioredoxin nitration (versus vehicle, $P<0.01$, $P<0.05$,
282 respectively, Figure 3C), restored the thioredoxin activity (versus vehicle, all $P<0.05$,
283 Figure 3B), but had no effect on the Trx expression (Figure 6). Secondly, the effect of
284 RAGE siRNA and sRAGE on the iNOS and gp91phox (a major component of
285 NADPH oxidase) were determined, respectively. We demonstrated that the
286 MI/R-induced iNOS (versus vehicle, $P<0.05$, Figure 3E) and gp91phox (a major
287 component of NADPH oxidase) expression (versus vehicle, $P<0.05$, Figure 3D) were
288 significantly attenuated by RAGE knockdown or sRAGE. These results demonstrate
289 for the first time that NADPH oxidase and iNOS are important mediator by which

290 RAGE result in the nitrative thioredoxin inactivation.

291

292 **Administration of ONOO⁻ decomposition catalyst or reduced hTrx, but not**
293 **nitrated hTrx, attenuated MI/R-induced myocardial apoptosis in diabetic mice**

294 To further investigate a potential causative relationship between nitrative
295 thioredoxin inactivation and exacerbated MI/R injury in the diabetic heart, three
296 different compounds (the peroxynitrite decomposition catalyst EUK134, reduced
297 hTrx, and nitrated hTrx) were administered via IP injection 10 minutes before
298 reperfusion. Reduced hTrx and EUK134 both attenuated I/R-induced myocardial
299 apoptosis (versus vehicle, $P<0.05$, Figure 4A, 4B) and caspase-3 activity (Figure 4C).
300 Administration of nitratively modified hTrx had no effect upon myocardial apoptosis
301 (versus vehicle, $P>0.05$, Figure 4A, 4B, 4C). Consistent with our previous study (39),
302 nitrative Trx inactivation plays a causative role in exacerbated MI/R injury in the
303 diabetic state.

304

305 **Administration of ONOO⁻ decomposition catalyst or reduced hTrx, but not**
306 **nitrated hTrx, attenuated MI/R-induced superoxide production and**
307 **AGEs/RAGE expression in diabetic mice**

308 Evidence exists that ROS could increase the RAGE expression(38), and
309 thioredoxin act as an antioxidant in the living body. Therefore we postulate that
310 thioredoxin may have effect on the expression of the AGEs/RAGE expression.
311 Administration of reduced hTrx and EUK134 dramatically attenuated MI/R-induced
312 superoxide production (versus vehicle, $P<0.01$, respectively, Figure 5C). More
313 importantly, reduced hTrx and EUK134 significantly decreased MI/R-induced CML
314 production (versus vehicle, all $P<0.05$, Figure 5A) and RAGE expression (versus
315 vehicle, all $P<0.05$, Figure 5B). However, supplementation of nitrated hTrx had no
316 effect upon I/R-induced superoxide production, CML production and RAGE
317 expression in the diabetic heart (Figure5C, 5A and 5B). As far as we know, this is the
318 first study to demonstrate that reduced hTrx can inhibit AGEs/RAGE expression, and
319 nitrative modification of Trx reduces its inhibitory effects upon AGEs/RAGE

320 expression in the diabetic state.

321

322

323

Discussion

Several novel observations were made in the present study. Firstly, we demonstrate for the first time that RAGE exacerbates myocardial ischemic injury via nitrative Trx inactivation. Secondly, we provide the first evidence that nitrative modification of Trx abrogates its inhibition of AGEs/RAGE expression in the diabetic heart. Together, this is the first direct evidence of alternative crosstalk between RAGE overexpression and nitrative Trx inactivation in the diabetic heart, providing a novel mechanism by which diabetes sensitizes the heart to ischemic injury, with potential therapeutic modality applications.

Cardiovascular disease is the cause of much morbidity and mortality among diabetic patients. It is well known that diabetes predisposes the patient to more severe cardiac ischemic injury sequelae via unknown underlying mechanisms. Advanced glycation end products (AGEs), a heterogeneous class of compounds, are non-enzymatically modified proteins or lipids that become glycated and oxidized after contact with sugars (32). In vivo AGEs form under hyperglycemic environments, contributing to vascular pathophysiology in the diabetic state, with the receptor for AGEs (RAGE) playing a central role in signal-transduction mechanisms(3). The RAGE is a multi-ligand cell surface molecule belonging to the immunoglobulin superfamily (7). RAGE can also interact, in addition to AGEs, with distinct ligand families, such as S100/calgranulins high mobility group box 1(18). RAGE therefore plays a comprehensive role in signal transduction activation and gene expression modulation. Whereas initially, RAGE was demonstrated to be involved in diabetic vascular injury(3), recent studies have demonstrated that RAGE also modulates myocardial ischemia/reperfusion injury(1, 6). Presently, we demonstrated that MI/R induced infarct size is exacerbated in diabetic mice (Figure 7, Table 1) and MI/R-induced RAGE expression and N^ε-carboxymethyl-lysine (CML, the most predominant in vivo AGE) were significantly increased in the diabetic heart. Administration of sRAGE (a decoy of RAGE) in diabetic mice significantly decreased infarct size and preserved cardiac function post MI/R (Figure 8, Table 2). Consistent with previous studies (4, 6), our results demonstrate that RAGE

354 upregulation is a factor responsible for increased susceptibility to ischemia injury in
355 the diabetic heart.

356 Previous studies demonstrated that RAGE modulates MI/R injury in part via JNK
357 and STAT, JNK and GSK-3 β , and NF- κ B pathways (1, 2, 31). Recent evidence
358 demonstrates that nitric oxide reactive nitrogen species (RNS) such as peroxynitrite
359 (ONOO⁻), a critical contributor of protein nitrative modification and cell injury, play a
360 crucial role in I/R induced cardiomyocyte injury(34). Presently, we demonstrated that
361 nitrotyrosine (the well accepted footprint of protein nitration) levels were increased
362 after MI/R, and were significantly mitigated after RAGE knockdown, which indicates
363 that RAGE may modulate MI/R injury by posttranslational modification of some key
364 protein involved in the cardioprotection. Thioredoxin (Trx), a 12 kD protein nearly
365 ubiquitously expressed in living cells, fulfills many biological functions, with
366 anti-apoptotic and anti-oxidative properties(20). Subject to regulation at the gene
367 expression level, Trx activity is also regulated by posttranslational modification (17,
368 33). We have previously demonstrated that nitrative modification of Trx-1 results in its
369 irreversible abrogation of protective activity against MI/R injury (34). Furthermore, in
370 a very recent study, we demonstrated that nitrative inactivation of Trx-1 increases
371 vulnerability of diabetic hearts to I/R injury (39). However, the specific modulator
372 causative of nitrative Trx inactivation was unidentified. Presently, we demonstrated
373 that administration of reduced hTrx or peroxynitrite decomposition catalyst EUK134
374 significantly reduced I/R-injury in the diabetic heart, but supplementation of nitrated
375 hTrx failed to produce such protective effects. Additionally, we provided evidence
376 RAGE knockdown significantly decreased MI/R-induced Trx nitration, and preserved
377 Trx activity in the diabetic heart. However RAGE knockdown had no effect on the
378 Trx expression in the diabetic heart (Figure 6). To our knowledge, this is the first
379 direct evidence of RAGE modulation of MI/R injury via nitrative Trx inactivation,
380 and that RAGE itself is a key modulator of Trx nitrative inactivation.

381 In the present study, we demonstrated that RAGE increased ONOO⁻ production,
382 which caused the nitrative modification of Trx. However the molecular sources of
383 ONOO⁻ induced by RAGE still remains to be elucidated. It is well known that NO

384 reacts with superoxide, resulting in ONOO^- , a toxic molecule that modifies proteins
385 and induces cellular injury (25, 28, 37). In chronic disease states, such as diabetes and
386 neurodegenerative disorders, upregulation of NADPH oxidase is linked to reactive
387 oxidative species (ROS) production, such as superoxide anion (O_2^-)(10, 12, 21).
388 Evidence exists that iNOS-null diabetic mice manifest attenuated I/R injury compared
389 to control, underlining the crucial role iNOS plays in diabetic I/R pathophysiology(13,
390 30). In the present study, we demonstrated that the MI/R-induced iNOS and gp91phox
391 (a major component of NADPH oxidase) expression were significantly attenuated by
392 RAGE knockdown, which indicates that the RAGE-induced nitrotyrosine production
393 possible results from the upregulation of iNOS and NADPH oxidase. To further
394 determine the causative link between of superoxide and NO overproduction caused by
395 RAGE, the NADPH oxidase and iNOS inhibitor was given 10 min before reperfusion.
396 Presently, we demonstrated that the NADPH oxidase or iNOS inhibitor were
397 significantly mitigated MI/R-induced nitrotyrosine levels. Furthermore, the NADPH
398 oxidase or iNOS inhibitor significantly restored MI/R-attenuated thioredoxin activity.
399 From this data, we concluded that RAGE-induced protein nitrative modification is in
400 a NADPH oxidase/iNOS dependent fashion.

401 It has been demonstrated that ROS contributes to AGEs generation and
402 subsequent additional RAGE expression (27, 38). It is well known that Trx functions
403 as a crucial antioxidant in vivo(20). Whether Trx might directly affect RAGE
404 expression remains unknown. We demonstrate in the current study that RAGE is
405 causative of Trx inactivation. Supplementation of either reduced hTrx or EUK134
406 attenuates I/R-induced superoxide production. Furthermore, hTrx or EUK134 RAGE
407 significantly decreased the RAGE expression in the diabetic heart, but nitrated Trx
408 has no such effect. This is the first direct evidence that Trx affects the RAGE
409 expression possibly via its antioxidant action.

410 In summary, our study presents several novel findings. Firstly, RAGE modulates
411 MI/R injury via nitrative Trx inactivation. Secondly, reduced, but not nitrated, hTrx
412 supplementation directly inhibits MI/R-induced RAGE expression. Taken together,
413 we offer the first direct evidence of existent alternative crosstalk between RAGE

414 overexpression and nitrative Trx inactivation. Interventions capable of blocking this
415 crosstalk may mitigate diabetic MI/R injury with broad clinical application potential.

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597 **Figure legends**

598 **Figure 1. MI/R-induced elevated AGE/RAGE expression and increased nitrative**
599 **thioredoxin inactivation to greater extent in diabetic mice.** A:
600 N^ε-(carboxymethyl)lysine(CML) by ELISA assay. B: RAGE expression by Western
601 blot. C: nitrotyrosine content by ELISA assay. D: Trx-1 activity by insulin disulfide
602 reduction assay. E: Trx-1 nitration by immunoprecipitation (IP). Representative
603 immunoblot (IB) graphs are shown. Nty, nitrotyrosine; DM, Diabetic Mice; MI/R,
604 Myocardial Ischemia/ Reperfusion; **P*<0.05, ***P*<0.01 vs. Control+Sham; ^{##}*P*<0.01,
605 ^{###}*P*<0.001 vs. DM+Sham; n=5 to 10 hearts/group.

606 **Figure 2. RAGE siRNA attenuated MI/R-induced oxidative/nitrative stress and**
607 **nitrative Trx inactivation.** A: RAGE expression by Western blot. B: superoxide
608 production in situ superoxide detection by dihydroethidium staining (top) and
609 lucigenin-enhanced luminescence (bottom). C: nitrotyrosine content by ELISA assay.
610 D: Trx-1 activity by insulin disulfide reduction assay. E: Trx-1 nitration by
611 immunoprecipitation (IP). Representative immunoblot (IB) graphs are shown. Nty,
612 nitrotyrosine; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; ^{##}*P*<0.01,
613 vs. DM+Vehicle; **P*<0.05, ***P*<0.01 vs. DM+MI/R+Vehicle; n=5 to 10 hearts/group.

614 **Figure3. MI/R-induced nitrative thioredoxin inactivation was attenuated by**
615 **iNOS or NADPH oxidase inhibitor in diabetic mice.** A: nitrotyrosine content by
616 ELISA assay. B: Trx-1 activity by insulin disulfide reduction assay. C: Trx-1 nitration
617 by immunoprecipitation (IP). Representative immunoblot (IB) graphs are shown. D:

gp91phox expression by Western blot. E: iNOS expression by Western blot. Nty, nitrotyrosine; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. DM+MI/R+Vehicle; n=5 to 10 hearts/group.

Figure 4. Administration of EUK134 (an ONOO⁻ decomposition catalyst) or reduced hTrx, but not nitrated hTrx, attenuated MI/R-induced myocardial apoptosis in diabetic mice. A: Myocardial apoptosis by TUNEL staining. Representative photographs of heart sections are shown. TUNEL staining (green) indicates apoptotic nuclei; DAPI counterstaining (blue) indicates total nuclei. B: Quantification of apoptotic nuclei. TUNEL-positive nuclei are expressed as a percentage of the total number of nuclei, automatically counted and calculated by Image-Pro Plus software. C: Myocardial apoptosis by caspase-3 activity by ELISA assay; N-Trx, nitrated hTrx; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; * $P<0.05$, ** $P<0.01$ vs. DM+MI/R+Vehicle; n=8 to 10 hearts/group.

Figure 5. Administration of EUK134 (an ONOO⁻ decomposition catalyst) or reduced hTrx, but not nitrated hTrx, attenuated MI/R-induced superoxide production and AGEs/RAGE expression in diabetic mice. A: N^ε-(carboxymethyl)lysine(CML) by ELISA assay. B: RAGE expression by Western blot. C: superoxide production in situ superoxide detection by dihydroethidium staining (top) and lucigenin-enhanced luminescence (bottom). N-Trx, nitrated hTrx; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; * $P<0.05$, ** $P<0.01$, vs. DM+MI/R+Vehicle; n=5 to 10 hearts/group.

Figure 6 . Trx expression in the whole groups. RAGE expression by Western blot.

1, Control+Sham; 2, Control+MI/R; 3, DM+ Sham; 4, DM+MI/R; 5, DM+MI/R+Vehicle; 6, DM+MI/R+Scrambed siRNA; 7, DM+MI/R+RAGE siRNA; 8, DM+MI/R+Apocynin; 9, DM+MI/R+1400W; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; * $P<0.05$ vs. Control+Sham; n=4 hearts/group.

Figure 7. MI/R induced infarct size is exacerbated in diabetic mice. A: Myocardial infarct size assessed by Evans blue/TTC double staining. Representative photographs of heart sections are shown (top). Blue-stained areas indicate non-ischemic/normal regions; red-stained areas indicate ischemic/reperfused, but viable regions; negatively stained areas indicate ischemic/reperfused and infarcted regions. Quantification of infarct size was expressed as the ratio of infarct area (Inf) to total ischemic/reperfused area (area-at-risk, AAR) (bottom). B: Cardiac function assessed by echocardiography; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; LVEF, Left Ventricular Ejection Fraction. * $P<0.05$ vs. Control+Sham; $^{##}P<0.05$ vs. DM+Sham; n=10 hearts/group.

Figure 8. MI/R-induced myocardial injury was attenuated by sRAGE in diabetic mice. B, C: Myocardial infarct size assessed by Evans blue/TTC double staining. A, D: Cardiac function assessed by echocardiography; DM, Diabetic Mice; MI/R, Myocardial Ischemia/ Reperfusion; LVEF, Left Ventricular Ejection Fraction. * $P<0.05$, ** $P<0.01$ vs. DM+MI/R+Vehicle; n=10 hearts/group.

Table 1. Echocardiography of control and diabetic mice after MI/R. Values are means $\pm$ SE. LVEDD, Left Ventricular End Diastolic Diameter; LVESD, Left

661 Ventricular End Systolic Diameter; FS, Fractional Shortening. $*P<0.05$ vs.
662 Control+MI/R; n=10 hearts/group.

663 **Table 2. Echocardiography of diabetic mice treated with sRAGE.** Values are
664 means $\pm$ SE. LVEDD, Left Ventricular End Diastolic Diameter; LVESD, Left
665 Ventricular End Systolic Diameter; FS, Fractional Shortening. $*P<0.05$ vs.
666 Control+MI/R; n=10 hearts/group.

667

Table 1

	Sham		MI/R	
	Control	DM	Control	DM
FS(%)	51.30±1.74	46.61±1.851	33.37±3.65	20.72±2.99 [*]
LVEDD(mm)	3.19±0.14	3.54±0.08	3.72±0.06	3.85±0.11
LVESD(mm)	1.73±0.20	1.88±0.10	2.47±0.13	3.06±0.16 [*]
Heart rate	352±11	358±13	364±15	343±14

Table 2

	DM+Sham	DM+MI/R+Vehicle	DM+MI/R+sRAGE
FS(%)	46.61±1.85	20.72±3.00	28.41±2.77
LVEDD(mm)	3.54±0.08	3.86±0.11	3.62±0.09
LVESD(mm)	1.88±0.09	3.06±0.16	2.58±0.10 [*]
Heart rate	352±11	340±9	361±15

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