The alternative crosstalk between RAGE and nitrative thioredoxin inactivation during diabetic myocardial ischemia-reperfusion injury.

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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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Abstract

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

Keywords
Diabetes; Ischemia/reperfusion; thioredoxin; protein nitration; RAGE
Introduction

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

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor of the immunoglobulin superfamily with diverse functions. In addition to AGEs, RAGE also binds distinct families of ligands, such as S100/calgranulins high mobility group box 1 (18). RAGE therefore plays a comprehensive role in signal transduction activation and gene expression modulation. Initially, RAGE was demonstrated to be involved in diabetic vascular injury (26). Subsequent studies have demonstrated that RAGE also modulates acute nerve injury and cellular death in advanced keratectomy (9, 29). Recently, knockdown or blockade of RAGE by soluble RAGE (sRAGE, the extracellular binding ligand RAGE decoy) has been demonstrated to attenuate myocardial ischemic injury through modulation of comprehensive signaling pathways including TGF-β, NF-κB, JNK, STAT and GSK3β pathway (1, 5, 23, 36).

We recently demonstrate that the protein nitrative modification play a crucial role in I/R-induced cardiomyocyte injury (34). Our and others studies also demonstrate that RAGE elicits nitrotyrosine production (the footprint of protein nitration) (6, 22), which indicates that RAGE acts as a modulator of protein nitration; however the mechanism by which RAGE cause the protein nitration is completely unknown. More importantly, whether RAGE-induced protein modification has any influence upon MI/R injury has not been investigated. Thioredoxin (Trx), a small 12 kD protein expressed in nearly all living cells with various biological functions, is protective against oxidative stress and is anti-apoptotic (19). Susceptible to up- or down-regulation at the gene level, Trx activity is also regulated by posttranslational modification (17, 33). We have previously demonstrated nitrative modification of
Trx-1 results in its irreversible inactivation during MI/R (33, 39). However, whether RAGE might posttranslationally affect Trx with consequences upon the latter’s protective effects against MI/R injury remains completely unknown.

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

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

Experimental Protocols

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

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

In vivo MI/R procedure was performed as previously described(14). Briefly, male mice were anesthetized with 2% isoflurane, and MI was induced by temporarily exteriorizing the heart via a left thoracic incision, and placement of a 6-0 silk suture slipknot around the left anterior descending coronary artery. After 30 minutes of MI, the slipknot was released, and the myocardium was reperfused for 3 hours (for apoptosis determination) or 24 hours reperfusion (for infarct size and cardiac function assays). Additionally, 10 min before reperfusion, the non-RAGE siRNA and
non-sRAGE treated diabetic mice were randomized to receive vehicle (PBS, pH 7.5) or reduced human Trx (rhTrx-1, 2 mg/kg; Sigma), EUK-134 (a peroxynitrite decomposition catalyst, 5 mg/kg; Cayman Chemical), nitratively modified hTrx (N-hTrx), 1400W (a selective iNOS inhibitor, 2 mg/kg), apocynin (a selective NADPH oxidase inhibitor, 5 mg/kg) via IP injection. Sham-operated control mice (sham MI/R) underwent identical surgical procedures, except the suture placed under the left coronary artery was not tied. At the conclusion of the reperfusion period, the ligature around the coronary artery was retied, and 2% Evans Blue dye was injected into the left ventricular cavity. Cardioectomy was swiftly performed, and the ischemic/reperfused cardiac tissue was isolated and processed per below protocols.

In vitro nitration of Trx-1

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

Determination of cardiac function and myocardial infarct size

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

Determination of myocardial apoptosis

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

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

**Trx activity assay**

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

**Immunoblotting**

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

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

Statistical analysis

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

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

RAGE expression is upregulated in diabetic tissues subjected to MI/R injury (4). Our recent study demonstrated nitrative inactivation of Trx-1 is significantly increased in diabetic hearts (39). However, the relationship between increased RAGE expression and reduced Trx activity in the diabetic heart pertaining to enhanced I/R injury in diabetic hearts has not been investigated. To approach this question, we evaluated the pre- and post-MI/R injury levels of both AGE/RAGE expression and nitrative thioredoxin inactivation (evidenced by Trx activity and nitrated Trx content).

Compared to control, diabetic mice harbored significantly elevated Nε-carboxymethyl-lysine (CML, the prevailing AGE subtype, 266.02±28.74 pg/mg protein VS 139.57±18.45 pg/mg protein, P<0.05, Figure 1A) and RAGE (0.37±0.048 VS 0.16±0.03, P<0.05, Figure 1B) in sham group. After MI/R, both CML content (473.9±51.3 pg/mg protein VS 289.04±39.93 pg/mg protein, P<0.01, Figure 2A) and RAGE expression (0.76±0.087 VS 0.49±0.07, P<0.01, Figure 1B) were amplified in diabetic mice compared to control in MI/R group. The diabetic condition increased nitrotyrosine content (the well accepted footprint of protein nitration, compared to control, sham conditions, P<0.05, Figure 1C), Trx nitration (compared to control, sham conditions, P<0.01, Figure 1E), and attenuated Trx activity (compared to control, sham conditions, P<0.05, Figure 1D). Compared to control, MI/R further increased nitrotyrosine content (P<0.001, Figure 1C), Trx nitration levels (P<0.001, Figure 1E), decreased Trx activity (P<0.001, Figure 1D) to greater extent in diabetic mice. However, Compared to control, MI/R has no effect on the Trx expression (Figure 6) in diabetic mice.

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

To further evaluate the downstream mechanisms by which RAGE enhances MI/R injury, siRNA gene silencing techniques were utilized to knock down RAGE. Our method of intramyocardial siRNA delivery was highly successful, markedly inhibiting
basal RAGE expression (versus vehicle, \(P<0.01\), Figure 2A). RAGE siRNA significantly decreased oxidative/nitrative stress, evidenced by decreased superoxide production (versus vehicle, \(P<0.01\), Figure 2B), decreased nitrotyrosine content (versus vehicle, \(P<0.05\), Figure 2C). Most importantly, we demonstrated for the first time that RAGE siRNA decreased the MI/R-induced Trx nitration (versus vehicle, \(P<0.01\), Figure 2E), restored I/R-induced diminished Trx activity (versus vehicle, \(P<0.05\), Figure 2D), but had no effect on the Trx expression (Figure 6). This data also importantly demonstrate for the first time that MI/R injury mediated by RAGE is closely involved with nitrative thioredoxin inactivation, supporting RAGE as a modulator of nitrative thioredoxin inactivation.

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

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

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

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

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

Evidence exists that ROS could increase the RAGE expression(38), and thioredoxin act as an antioxidant in the living body. Therefore we postulate that thioredoxin may have effect on the expression of the AGEs/RAGE expression. Administration of reduced hTrx and EUK134 dramatically attenuated MI/R-induced superoxide production (versus vehicle, \( P<0.01 \), respectively, Figure 5C). More importantly, reduced hTrx and EUK134 significantly decreased MI/R-induced CML production (versus vehicle, all \( P<0.05 \), Figure 5A) and RAGE expression (versus vehicle, all \( P<0.05 \), Figure 5B). However, supplementation of nitrated hTrx had no effect upon I/R-induced superoxide production, CML production and RAGE expression in the diabetic heart (Figure5C, 5A and 5B). As far as we know, this is the first study to demonstrate that reduced hTrx can inhibit AGEs/RAGE expression, and nitrative modification of Trx reduces its inhibitory effects upon AGEs/RAGE.
expression in the diabetic state.
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 Nε-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
upregulation is a factor responsible for increased susceptibility to ischemia injury in the diabetic heart.

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

In the present study, we demonstrated that RAGE increased ONOO− production, which caused the nitrative modification of Trx. However the molecular sources of ONOO− induced by RAGE still remains to be elucidated. It is well known that NO
reacts with superoxide, resulting in ONOO−, a toxic molecule that modifies proteins
and induces cellular injury (25, 28, 37). In chronic disease states, such as diabetes and
neurodegenerative disorders, upregulation of NADPH oxidase is linked to reactive
oxidative species (ROS) production, such as superoxide anion (O2•−)(10, 12, 21).
Evidence exists that iNOS-null diabetic mice manifest attenuated I/R injury compared
to control, underlining the crucial role iNOS plays in diabetic I/R pathophysiology(13,
30). In the present study, we demonstrated that the MI/R-induced iNOS and gp91phox
(a major component of NADPH oxidase) expression were significantly attenuated by
RAGE knockdown, which indicates that the RAGE-induced nitrotyrosine production
possible results from the upregulation of iNOS and NADPH oxidase. To further
determine the causative link between of superoxide and NO overproduction caused by
RAGE, the NADPH oxidase and iNOS inhibitor was given 10 min before reperfusion.
Presently, we demonstrated that the NADPH oxidase or iNOS inhibitor were
significantly mitigated MI/R-induced nitrotyrosine levels. Furthermore, the NADPH
oxidase or iNOS inhibitor significantly restored MI/R-attenuated thioredoxin activity.
From this data, we concluded that RAGE-induced protein nitrative modification is in
a NADPH oxidase/iNOS dependent fashion.

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

In summary, our study presents several novel findings. Firstly, RAGE modulates
MI/R injury via nitrative Trx inactivation. Secondly, reduced, but not nitrated, hTrx
supplementation directly inhibits MI/R-induced RAGE expression. Taken together,
we offer the first direct evidence of existent alternative crosstalk between RAGE
overexpression and nitritative Trx inactivation. Interventions capable of blocking this crosstalk may mitigate diabetic MI/R injury with broad clinical application potential.
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Figure legends

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

Figure 2. RAGE siRNA attenuated MI/R-induced oxidative/nitrative stress and nitrative Trx inactivation. A: RAGE expression by Western blot. B: superoxide production in situ superoxide detection by dihydroethidium staining (top) and lucigenin-enhanced luminescence (bottom). C: nitrotyrosine content by ELISA assay. D: Trx-1 activity by insulin disulfide reduction assay. E: Trx-1 nitration by immunoprecipitation (IP). Representative immunoblot (IB) graphs are shown. Nty, nitrotyrosine; DM, Diabetic Mice; MI/R, Myocardial Ischemia/Reperfusion; \(##P<0.01, ###P<0.001\) vs. DM+Vehicle; \(*P<0.05, **P<0.01\) vs. DM+MI/R+Vehicle; \(n=5\) to 10 hearts/group.

Figure 3. MI/R-induced nitrative thioredoxin inactivation was attenuated by iNOS or NADPH oxidase inhibitor in diabetic mice. A: nitrotyrosine content by ELISA assay. B: Trx-1 activity by insulin disulfide reduction assay. C: Trx-1 nitration 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\(^{\epsilon}\)-(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± SE. LVEDD, Left Ventricular End Diastolic Diameter; LVESD, Left
Table 2. Echocardiography of diabetic mice treated with sRAGE. Values are means± SE. LVEDD, Left Ventricular End Diastolic Diameter; LVESD, Left Ventricular End Systolic Diameter; FS, Fractional Shortening. *$P<0.05$ vs. Control+MI/R; n=10 hearts/group.

Ventricular End Systolic Diameter; FS, Fractional Shortening. *$P<0.05$ vs. Control+MI/R; n=10 hearts/group.
### Table 1

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<td><strong>FS(%)</strong></td>
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<td><strong>LVEDD(mm)</strong></td>
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<td><strong>LVESD(mm)</strong></td>
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<td><strong>Heart rate</strong></td>
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### Table 2

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<tr>
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<th>DM+MI/R+sRAGE</th>
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<td><strong>FS(%)</strong></td>
<td>46.61±1.85</td>
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<td><strong>LVEDD(mm)</strong></td>
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<td><strong>LVESD(mm)</strong></td>
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<td><strong>Heart rate</strong></td>
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<td>340±9</td>
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