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

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Liu Y, Qu Y, Wang R, Ma Y, Xia C, Gao C, Liu J, Lian K, Xu A, Lu X, Sun L, Yang L, Lau WB, Gao E, Koch W, Wang H, Tao L. The alternative crosstalk between RAGE and nitrative thioredoxin inactivation during diabetic myocardial ischemia-reperfusion injury. Am J Physiol Endocrinol Metab 303: E841–E852, 2012. First published July 24, 2012; doi:10.1152/ajpendo.00075.2012.—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 nitrative 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 injections. RAGE small-interfering RNA (siRNA) or soluble RAGE (sRAGE, a RAGE decoy) was via intramyocardial and intraperitoneal injection before MI/R, respectively. Mice were subjected to 30 min of myocardial infarction followed by 3 or 24 h of reperfusion. At 10 min before reperfusion, diabetic mice were randomized to receive EUK134 (peroxynitrite scavenger), recombinant hTrx-1, nitrated Trx-1, apocynin (a NADPH oxidase inhibitor), or 1400W [an inducible nitric oxide synthase (iNOS) inhibitor] administration. The diabetic heart manifested increased RAGE expression and Nε-carboxymethyl)lysine (CML, major advanced glycation end product subtype) content, reduced Trx-1 activity, and increased Trx nitration after MI/R. 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 cardiomyocyte apoptosis in diabetic mice. Collectively, we demonstrate that RAGE modulates the Mi/R injury in a Trx nitrative inactivation fashion. Conversely, nitrative modification of Trx blocked its inhibitory effect upon RAGE expression in the diabetic heart. This is the first direct evidence demonstrating the alternative cross talk between RAGE overexpression and nitrative Trx inactivation, suggesting that interventions interfering with their interaction may be novel means of mitigating diabetic MI/R injury.

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 with nondiabetics. 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 multiligand receptor of the immunoglobulin superfamily with diverse functions. In addition to advanced glycation end products (AGEs), RAGE also binds distinct families of ligands, such as S100/calgranulins and 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, knock down 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 tumor necrosis factor-β, nuclear factor (NF)-κB, c-Jun NH2-terminal kinase (JNK), signal transducer and activator of transcription (STAT), and the glycogen synthase kinase (GSK) 3β pathway (1, 5, 23, 36).

We have recently demonstrated that the protein nitrative modification play a crucial role in ischemia-reperfusion (I/R)-induced cardiomyocyte injury (34). Our studies and others also demonstrate that RAGE elicits nitrotyrosine production (the footprint of protein nitrination) (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 myocardial ischemia-reperfusion (MI/R) injury has not been investigated. Thioredoxin (Trx), a small 12-kDa 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 downregulation 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 con-
sequences 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 a 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 wk) were used for the present study. The diabetic state was induced by intraperitoneal injection of 40 mg/kg streptozotocin (STZ; Sigma, St. Louis, MO) diluted in citrate buffer (pH 4.5) for five 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 killed after 3 wk of established diabetes.

Two methods were used for blocking RAGE signaling in diabetic mice. First, small-interfering RNA (siRNA) gene silencing technique was used to knock down RAGE expression. Preadesign RAGE-specific siRNA (catalog no. sc-36375; Santa Cruz) or control scrambled siRNA (catalog no. sc-37007; Santa Cruz) were diluted in 5% glucose and mixed with in vivo jet 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 to MI/R 48 h after siRNA injection (24). Second, murine sRAGE (a RAGE decoy; 500 μg/day; Adipobioscience) was administered via intraperitoneal injection for 3 days duration before 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 min of MI, the slipknot was released, and the myocardium was reperfused for 3 h (for apoptosis determination) or 24 h (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 (hTrx, 2 mg/kg; Sigma), EUK-134 [a peroxynitrite (ONOO−) decomposition catalyst, 5 mg/kg; Cayman Chemical], nitratively modified hTrx, 1400W [a selective inducible nitric oxide synthase (iNOS) inhibitor, 2 mg/kg], and apocynin (a selective NADPH oxidase inhibitor, 5 mg/kg) via intraperitoneal 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 retired, and 2% Evans Blue dye was injected in the left ventricular cavity. Cardiectomy was performed swiftly, and the ischemic-reperfused cardiac tissue was isolated and processed per the protocols described below.

In vitro nitration of Trx-1. HTrx-1 (Sigma) was subjected to in vitro nitration with a modified procedure recently described previously (16). In brief, purified hTrx-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, MI) at 37°C for 30 min. 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, cardiomeotries were performed. Myocardial infarct size was determined by using Evans blue (2,3,5-triphenyltetrazolium chloride staining as previously described (8).

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

Quantification of superoxide production, nitrotyrosine content, and N′-(carboxymethyl)lysine content. Superoxide (O2−) production, an index of oxidative stress, was measured by lucigenin-enhanced chemiluminescence as previously described (17) and expressed as relative light units per second per milligram protein. In situ O2− detection was performed with dihydroethidium staining (Beyotime) as described previously (35). Nitrotyrosine content, an established index of protein nitration and nitrative stress, was determined by Millipore nitrotyrosine assay per a commercial manufacturer protocol kit (Millipore). N′-(carboxymethyl)lysine (CML) content, the predominant AGE subtype, was measured per a commercial manufacturer protocol kit (Genesee Bio-Technology, Beijing, China).

Trx activity assay. Trx activity was determined via insulin disulfide reduction assay (19). In brief, 40 μg of cardiac protein extracts were incubated at 37°C for 15 min with 2 ml activation buffer (100 mM HEPES, 2 mM EDTA, 1 mg/ml BSA, and 2 mM dithiothreitol) to reduce Trx. 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 μM; Sigma) or distilled water to controls. After incubation for 30 min at 37°C, the reaction was terminated by 125 μl stopping solution (0.2 m Tris·Cl, 10 mM guanidine hydrochloride, and 1.7 mM 3-carboxy-4-nitrophenyl disulfide), followed by absorption measurement (412 nm). Trx-1 activity was expressed as oxidized NADPH micromol per minute per milligram 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), and iNOS (Cell Signaling, Boston, MA). Nitrocellulose membranes were then incubated with horseradish peroxidase-conjugated immunoglobulin G antibody (Santa Cruz Biotechnology) for 1 h. The blot was developed with an ECL-Plus chemiluminescence reagent kit and visualized via UVP Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS Acquisition and Analysis Software.

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) 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 Figs. 1–8 are presented as means ± SE of n independent experiments. All data (except Western blot density) were subjected to ANOVA followed by Bonferroni correction for post hoc t-test. Western blot densities were analyzed with the Kruskal-Wallis test followed by the Dunn

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**Fig. 1.** Myocardial ischemia-reperfusion (MI/R) induced elevated advanced glycation end product (AGE)/receptor for advanced glycation end product (RAGE) expression and increased nitrative thioredoxin (Trx) inactivation to a greater extent in diabetic mice. **A:** Nε-(carboxymethyl)lysine (CML) by enzyme-linked immunosorbent assay (ELISA). **B:** RAGE expression by Western blot. **C:** nitrotyrosine content by ELISA. **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. *P < 0.05 and **P < 0.01 vs. control sham. ###P < 0.001 vs. DM sham; n = 5–10 hearts/group.
Fig. 2. RAGE small-interfering RNA (siRNA) attenuates 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.

D: Trx-1 activity by insulin disulfide reduction assay.

E: Trx-1 nitration by immunoprecipitation. Representative immunoblot graphs are shown.

RLU, relative light units; nTrx, nitrated Trx.

## P = 0.01 vs. DM + vehicle. * P < 0.05 and ** P < 0.01 vs. DM + MI/R + vehicle; n = 5–10 hearts/group.
RESULTS

MI/R induced elevated AGE/RAGE expression and increased nitrative Trx inactivation to greater extent in diabetic mice vs. control. RAGE expression is upregulated in diabetic tissues subjected to MI/R injury (4). Our recent study demonstrated that nitrative inactivation of Trx-1 is increased significantly 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 Trx inactivation (evidenced by Trx activity and nitrated Trx content). Compared with control, diabetic mice harbored significantly elevated CML (the pre-existing AGE subtype, 266.02 ± 28.74 vs. 139.57 ± 18.45 pg/mg protein, P < 0.05; Fig. 1A) and RAGE (0.37 ± 0.048 vs. 0.16 ± 0.03, P < 0.05; Fig. 1B) in the sham group. After MI/R, both CML content (473.9 ± 51.3 vs. 289.04 ± 39.93 pg/mg protein, P < 0.01; Fig. 1A) and RAGE expression (0.76 ± 0.087 vs. 0.49 ± 0.07, P < 0.01; Fig. 1B) were amplified in diabetic mice compared with control in the MI/R group. The diabetic condition increased nitrosative content (the well-accepted footprint of protein nitration compared with control sham conditions, P < 0.05; Fig. 1C) and Trx nitration (compared with control sham conditions, P < 0.01; Fig. 1E) and attenuated Trx activity (compared with control sham conditions, P < 0.05; Fig. 1D). Compared with control, MI/R further increased nitrosative content (P < 0.001; Fig. 1C) and Trx nitration levels (P < 0.001; Fig. 1E) and decreased Trx activity (P < 0.001; Fig. 1D) to a greater extent in diabetic mice. However, compared with control, MI/R has no effect on the Trx expression (Fig. 3) in diabetic mice.

RAGE siRNA attenuated MI/R-induced oxidative/nitrative stress and nitrative Trx inactivation. To further investigate the downstream mechanisms by which RAGE enhances MI/R injury, siRNA gene silencing techniques were used to knock down RAGE. Our method of intramyocardial siRNA delivery was highly successful, markedly inhibiting basal RAGE expression (vs. vehicle, P < 0.01; Fig. 2A). RAGE siRNA significantly decreased oxidative/nitrative stress, evidenced by decreased O$_2^-$ production (vs. vehicle, P < 0.01; Fig. 2B) and decreased nitrotyrosine content (vs. vehicle, P < 0.05; Fig. 2C). Most importantly, we demonstrated for the first time that RAGE siRNA decreased the MI/R-induced Trx nitration (vs. vehicle, P < 0.01; Fig. 2E), restored I/R-induced diminished Trx activity (vs. vehicle, P < 0.05; Fig. 2D) but had no effect on the Trx expression (Fig. 3). These data also importantly demonstrate for the first time that MI/R injury mediated by RAGE is closely involved with nitrative Trx inactivation, supporting RAGE as a modulator of nitrative Trx inactivation.

MI/R-induced nitrative Trx inactivation was attenuated by iNOS or NADPH oxidase inhibitor in diabetic mice. It is well known that NO reacts with O$_2^-$, resulting in enhanced toxic ONOO$^-$ formation, which is the critical contributor of protein nitrosative modification (34), and the NADPH oxidase is the most important cytosolic source for O$_2^-$ production (12). To further determine the molecular mechanism by which RAGE causes Trx nitration, two additional experiments were performed. First, a selective NADPH oxidase inhibitor (apocynin) or a selective iNOS inhibitor (1400W) was administered 10 min before reperfusion. Apocynin or 1400W significantly decreased MI/R nitrosative production (vs. vehicle, all P < 0.05; Fig. 4A), attenuated Trx nitration (vs. vehicle, P < 0.01 and P < 0.05, respectively; Fig. 4C), and restored Trx activity (vs. vehicle, all P < 0.05; Fig. 4B) but had no effect on Trx expression (Fig. 4D). Second, the effect of RAGE siRNA and siRAGE on the iNOS and gp91phox (a major component of NADPH oxidase) was determined. We demonstrated that the MI/R-induced iNOS (vs. vehicle, P < 0.05; Fig. 4E) and gp91phox (a major component of NADPH oxidase) expression (vs. vehicle, P < 0.05; Fig. 4D) were attenuated significantly by RAGE knockdown or siRAGE. These results demonstrate for the first time that NADPH oxidase and iNOS are important mediators by which RAGE result in the nitrative Trx 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 Trx inactivation and exacerbated MI/R injury in the diabetic heart, three different compounds (the ONOO$^-$ decomposition catalyst EUK134, reduced hTrx, and nitrated hTrx) were administered via intraperitoneal injection 10 min before reperfusion. Reduced hTrx and EUK134 both attenuated I/R-induced myocardial apoptosis (vs. vehicle, P < 0.05; Fig. 5, A and B) and caspase-3 activity (Fig. 5C). Administration of nitratively modified hTrx had no effect on myocardial apoptosis (vs. vehicle, P > 0.05; Fig. 5, A-C). 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 O$_2^-$ production and AGEs/RAGE expression in diabetic mice. Evidence exists that ROS could increase the RAGE expression (38), and Trx act as an antioxidant in the living body. Therefore, we postulate that Trx may have effect on the expression of the AGEs/RAGE expression. Administration of reduced
Fig. 4. MI/R-induced nitrative Trx inactivation was attenuated by inducible nitric oxide synthase (iNOS) or NADPH oxidase inhibitor in diabetic mice. 

A: nitrotyrosine content by ELISA. 
B: Trx-1 activity by insulin disulfide reduction assay. 
C: Trx-1 nitration by immunoprecipitation. Representative immunoblot graphs are shown. 
D: gp91phox expression by Western blot. 

E: iNOS expression by Western blot. sRAGE, soluble RAGE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. DM + MI/R + vehicle; n = 5–10 hearts/group.
hTrx and EUK134 dramatically attenuated MI/R-induced O$_2^-$ production (vs. vehicle, $P < 0.05$; Fig. 6C). More importantly, reduced hTrx and EUK134 significantly decreased MI/R-induced CML production (vs. vehicle, all $P < 0.05$; Fig. 6A) and RAGE expression (vs. vehicle, all $P < 0.05$; Fig. 6B). However, supplementation of nitrated hTrx had no effect on I/R-induced O$_2^-$ production, CML production, and RAGE expression in the diabetic heart (Fig. 6, A–C). 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.

**DISCUSSION**

Several novel observations were made in the present study. First, we demonstrate for the first time that RAGE exacerbates myocardial ischemic injury via nitrative Trx inactivation. Second, 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 cross talk 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. AGEs, a heterogeneous class of compounds, are nonenzymatically 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 RAGE playing a central role in signal-transduction mechanisms (3). RAGE is a multiligand 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 and 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 modulates MI/R injury (1, 6). Presently, we demonstrated that MI/R-induced infarct size is exacerbated in diabetic mice (Fig. 7 and Table 1), and MI/R-induced RAGE expression and CML (the most predominant in vivo AGE) were increased significantly 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 (Fig. 8 and 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 (NO) reactive nitrogen species such as 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. Trx, a 12-kDa 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 ONOO− 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 that 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 (Fig. 3). 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 remain to be elucidated. It is well known that NO reacts with O2−, 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 ROS production, such as O2− (10, 12, 21). Evidence exists that iNOS-null diabetic mice manifest attenuated I/R injury compared with 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 atten-

Table 1. Echocardiography of control and diabetic mice after MI/R

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Values are means ± SE; n = 10 hearts/group. MI/R, myocardial ischemia-reperfusion; DM, diabetic mice; FS, fractional shortening; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter. *P < 0.05 vs. control + MI/R.
uated significantly by RAGE knockdown, which indicates that the RAGE-induced nitrotyrosine production possibly results from the upregulation of iNOS and NADPH oxidase. To further determine the causative link between O2⁻/H2O2 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 significantly mitigated MI/R-induced nitrotyrosine levels. Furthermore, the NADPH oxidase or iNOS inhibitor significantly restored MI/R-attenuated Trx activity.

From these 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 AGE 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 O2⁻ 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. First, RAGE modulates MI/R injury via nitrative Trx inactivation. Second, reduced, but not nitrated, hTrx supplementation directly inhibits MI/R-induced RAGE expression. Taken together, we offer the first direct evidence of existent alternative cross talk between RAGE overexpression and nitrative Trx inactivation. Interventions capable of blocking this cross talk may mitigate diabetic MI/R injury with broad clinical application potential.

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