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Advanced glycation end products accelerate ischemia/reperfusion injury through receptor of advanced end product/nitrative thioredoxin inactivation in cardiac microvascular endothelial cells.

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FORUM ORIGINAL RESEARCH COMMUNICATION

Advanced Glycation End Products Accelerate Ischemia/Reperfusion Injury Through Receptor of Advanced End Product/Nitrative Thioredoxin Inactivation in Cardiac Microvascular Endothelial Cells

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Abstract

The advanced glycation end products (AGEs) are associated with increased cardiac endothelial injury. However, no causative link has been established between increased AGEs and enhanced endothelial injury after ischemia/reperfusion. More importantly, the molecular mechanisms by which AGEs may increase endothelial injury remain unknown. Adult rat cardiac microvascular endothelial cells (CMECs) were isolated and incubated with AGE-modified bovine serum albumin (BSA) or BSA. After AGE-BSA or BSA preculture, CMECs were subjected to simulated ischemia (SI)/reperfusion (R). AGE-BSA increased SI/R injury as evidenced by enhanced lactate dehydrogenase release and caspase-3 activity. Moreover, AGE-BSA significantly increased SI/R-induced oxidative/nitrative stress in CMECs (as measured by increased inducible nitric oxide synthase expression, total nitric oxide production, superoxide generation, and peroxynitrite formation) and increased SI/R-induced nitrative inactivation of thioredoxin-1 (Trx-1), an essential cytoprotective molecule. Supplementation of EUK134 (peroxynitrite decomposition catalyst), human Trx-1, or soluble receptor of advanced end product (sRAGE) (a RAGE decoy) in AGE-BSA precultured cells attenuated SI/R-induced oxidative/nitrative stress, reduced SI/R-induced Trx-1 nitration, preserved Trx-1 activity, and reduced SI/R injury. Our results demonstrated that AGEs may increase SI/R-induced endothelial injury by increasing oxidative/nitrative injury and subsequent nitrative inactivation of Trx-1. Interventions blocking RAGE signaling or restoring Trx activity may be novel therapies to mitigate endothelial ischemia/reperfusion injury in the diabetic population. *Antioxid. Redox Signal.* 15, 1769–1778.

Introduction

Diabetes mellitus is a major risk factor for cardiovascular disease, with vascular complications as the leading etiology of morbidity and mortality in the diabetic population (13). Despite interventional technique advances, the diabetic condition portends an adverse outcome after revascularization (21). Further, diabetic rats subjected to ischemia/reperfusion (I/R) injury manifest increased apoptosis of cardiac microvascular endothelial cells (CMECs) (33). However, the molecular mechanisms by which the diabetic state sensitizes CMECs to I/R injury are unclear.

Many hyperglycemia-induced metabolic derangements and abnormalities have been identified as being responsible for endothelial cell dysfunction. Among them, the advanced glycation end products (AGEs), and their receptor (RAGE), have been strongly implicated in the pathogenesis of diabetic vascular complications (24). It is well known that the interaction of AGEs with RAGE increases the intracellular reactive oxygen species (ROS) generation, subsequently inducing apoptotic cell death and injury in endothelial cells (3, 7, 19). Recent evidence demonstrates that nitric oxide (NO) reactive nitrogen species such as peroxynitrite ($ONOO^-$), a critical contributor of protein nitrative modification and cell injury, play a crucial role in I/R-induced cardiomyocyte injury (26). However, whether AGEs could cause cardiac cell injury by nitrative stress and induce subsequent protein nitrative modification remains incompletely understood. More

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importantly, specific intracellular molecules nitratively modified and thereby contributive to increased endothelial damage in the diabetic patient is completely unknown.

Ubiquitously expressed in living cells, thioredoxin-1 (Trx-1) is a small protein with many protective biological functions. Trx-1 not only exerts cytoprotective functions against oxidative stress but also regulates cell survival signaling pathways (15, 25, 38). In addition to its upregulated or downregulated expression at the gene level, Trx activity is regulated by posttranslational modification (25). Previously, we demonstrated for the first time that Trx-1 can be modified at the tyrosine residue by nitration, resulting in loss of its cardioprotective action (28). In a recent study (37), we demonstrated that nitrative inactivation of Trx-1 increases vulnerability of diabetic hearts to I/R injury. However, the upstream molecules and mechanisms causing increased nitrative Trx inactivation in diabetic endothelial cells remain unidentified.

Therefore, the aims of the present study were (i) to determine whether AGEs could exacerbate CMECs I/R injury; (ii) to examine whether AGEs increase nitrative stress and subsequent nitrative Trx-1 inactivation; and (iii) to determine any cause-effect relationship between AGE-RAGE-induced nitrative Trx inactivation and increased I/R injury in CMECs.

Materials and Methods

Preparation of AGE proteins

AGE-bovine serum albumin (BSA) was prepared as previously described (35). Briefly, BSA (50 mg/ml) was incubated under sterile conditions with 0.5 *M* D-glucose in 100 m*M* sodium phosphate buffer (phosphate-buffered saline [PBS], pH 7.4) at 37°C for 9 weeks. Unincorporated sugars were removed by dialysis against PBS. Control BSA was incubated under the same conditions, in the absence of reducing sugars. AGE content was determined spectrofluorometrically (360 nm excitation, and 450 nm emission) and expressed as the percentage of relative fluorescence compared with control BSA. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co.); no endotoxin was detectable.

CMECs culture and identification

CMECs were isolated as previously described (17), with minor modifications. Briefly, male Wistar rats (200–250 g) were anesthetized with ether, and the heart was rapidly excised and rinsed with PBS supplemented with heparin. After rinsing, the right ventricle, atria, and valvular tissues were removed, and the remaining left ventricle was immersed in 75% ethanol for 20–30 s to devitalize epicardial mesothelial cells and endocardial endothelial cells. About one-third of the outer free ventricular wall was dissected to remove epicardial arteries. The remaining tissue was then minced in PBS and incubated in 0.2% collagenase (type II; Sigma Aldrich) for 10 min, followed by 0.2% trypsin (Sigma Aldrich) for another 6 min at 37°C in a water bath. Dissociated cells were filtered through a 100 mm mesh filter. After centrifugation of the dissociated cells at 1000 rpm for 10 min, cells were resuspended in Dulbecco's minimum essential medium (DMEM) (Invitrogen Gibco) supplemented with 20% (v/v) fetal calf serum and heparin (20 U/ml) and plated on laminin (10 μg/ml)-coated dishes. Primary cultures of CMECs were positively identified by two endothelial cell markers: factor VIII-related antigen and uptake of acetylated low-density lipoprotein (Kalen Biomed). Differential uptake of acetylated low-density lipoprotein, determined by fluorescence-activated cell sorting, indicated that the cultures contained >90% endothelial cells (Fig. 1).

Experimental protocol and simulated ischemia/reperfusion

Passage 2 CMECs were used in the study. After 24 h synchronization, cells were washed with PBS; and nonadherent

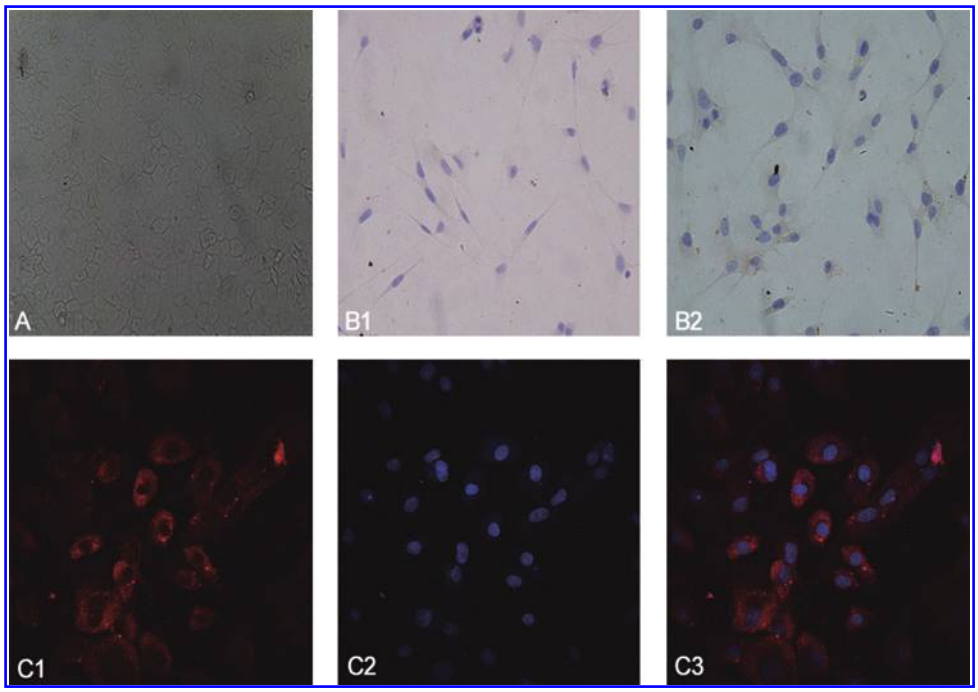

FIG. 1. Characterization of cardiac microvascular endothelial cells (CMECs). (A) CMECs monolayer presents cobble stone appearance by phase-contrast microscopy; expression of factor VIII by immunohistochemistry **(B1:** negative control, **B2:** factor VIII positive**)**; uptake of acetylated low-density lipoprotein by immunofluorescence: **(C1:** accumulation of acetylated low-density lipoprotein, **C2:** 4, 6-diamidino-2-phenylindole staining indicates nuclei, **C3:** merge of **C1** and **C2)**. Original magnification: ×400 **(A, B)**, ×1000 **(C)**.

cells were removed from the culturing system and were randomly assigned to one of the following treatments: BSA (100 μg/ml as control), AGE-BSA (100 μg/ml), AGE-BSA + EUK134 (7 μM, a peroxynitrite decomposition catalyst; Cayman Chemical), AGE-BSA + human Trx-1 (hTrx-1) (1 μg/ml; Sigma), or AGE-BSA + sRAGE (4 μg/ml, a RAGE decoy; Adipobioscience). After 48 h incubation, cells were subjected to either sham simulated ischemia/reperfusion (SI/R, 10 h of normoxia/normal-glucose environment) or SI/R (4 h hypoxia-hypoglycemic environment plus 6 h normoxia/normal glucose environment) as previously described (36). Briefly, the oxygen-glucose deprivation injury occurred by placing cells in a hypoxic environment (1% O_2/5% CO_2/94% N_2) maintained by an incubator in the presence of glucose-free DMEM for 4 h, at which time the medium was exchanged with oxygenated and normal glucose DMEM in an incubator at 37°C to simulate the reperfusion condition for 6 h.

Assessment of SI/R-induced CMECs injury

To determine CMECs death, lactate dehydrogenase (LDH) release was determined by an enzyme activity assay kit (Nanjing Institute of Jiancheng Bioengineering). Caspase-3 activity was determined by caspase-3 activity assay kit (Chemicon). Caspase-3 activity was expressed as nmol pNA/h/mg protein.

Quantification of superoxide production, cellular nitrotyrosine content

Superoxide production, an index of oxidative stress, in viable CMECs was measured by lucigenin-enhanced chemiluminescence as previously described (17) and expressed as relative light units per second per milligram protein. CMECs nitrotyrosine content, an index of protein nitration and nitrative stress, was determined as described in our previous study (27).

Total NO assay

The supernatant fluid of CMECs was harvested, and NO concentrations were measured with Griess reagent using an assay kit (Beyotime Company). The amount of total cellular protein in the respective wells was determined by Lowry's method after lysis with a buffer containing 0.1% of sodium dodecyl sulfate in 10 m*M* Tris, pH 7.4. Total nitrite accumulated in each well was defined as μM/mg of protein in the corresponding well.

Western blot analysis for Trx-1, inducible nitric oxide synthase, RAGE, and gp91phox

CMECs were lysed in lysis buffer and centrifuged; the supernatant was utilized to determine Trx expression. Equal protein amounts were electrophoresed on a 14% sodium dodecyl sulfate–polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 at room temperature for 1 h, the membrane was incubated with a monoclonal anti-murine Trx antibody (Redox Bioscience), an anti-murine RAGE antibody (Santa Cruz), an anti-murine gp91phox (Santa Cruz) antibody, or an anti-murine inducible nitric oxide synthase (iNOS) antibody (Cell Signaling) and then with the HRP linked lgG (Cell Signaling). The blot was developed with an ECL-Plus chemiluminescence reagent kit (Amersham) and visualized with UVP Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS Acquisition and Analysis Software.

Trx activity assay

Trx activity was determined *via* the insulin disulfide reduction assay (11). Briefly, 40 μg of cellular protein extracts were preincubated at 37°C for 15 min with 2 ml activation buffer (100 m*M* HEPES, 2 m*M* ethylenediaminetetraacetic acid, 1 mg/ml BSA, and 2 m*M* DL-Dithiothreitol) to reduce Trx. After addition of 20 μL reaction buffer (100 m*M* HEPES, 2.0 m*M* ethylenediaminetetraacetic acid, 0.2 m*M* NADPH, and 140 m*M* insulin), the reaction was initiated by addition of mammalian Trx reductase (1 ml, 15 mU; Sigma) or water to controls. After incubation for 30 min at 37°C, the reaction was terminated by 125 μL stopping solution (0.2M Tris–CL, 10 *M* guanidine–HCl, and 1.7 m*M* 3-carboxy-4-nitrophenyl disulfide, DTNB), followed by absorption measurement (412 nm). Trx-1 activity was expressed as oxidized NADPH μmol/min/mg of protein.

Detection of Trx-1 nitration

CMECs were homogenized with lysis buffer. Endogenous Trx-1 was immunoprecipitated with a monoclonal anti-murine Trx-1 antibody (Redox Bioscience). After sample separation, Trx-1 nitration was detected with a monoclonal antibody (Upstate) against nitrotyrosine. The blot was developed with an ECL-Plus chemiluminescence reagent kit (Amersham) and visualized with 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 means ± standard error. All data (except Western blot density) were subjected to analysis of variance followed by Bonferroni correction for *post hoc* t test. Western blot densities were analyzed with the Kruskal–Wallis test followed by Dunn's *post hoc* test. Probabilities of 0.05 or less were considered to be statistically significant.

Results

AGE-BSA increases the SI/R injury in CMECs

To investigate the role of AGE-BSA on SI/R-induced injury in CMECs, we examined the effects of AGE-BSA on the SI/R-induced caspase-3 activity and LDH release in CMECs. SI/R induced a significant LDH release (Fig. 2A) and caspase-3 activation (Fig. 2B). Compared with cells precultured in BSA, cells precultured in AGE-BSA had increased SI/R-induced LDH release (Fig. 2A) and caspase-3 activity (Fig. 2B).

AGE-BSA promotes the SI/R-induced oxidative/nitrative stress in CMECs

To determine whether AGE-BSA exacerbates SI/R-induced oxidative/nitrative stress, we examined iNOS protein

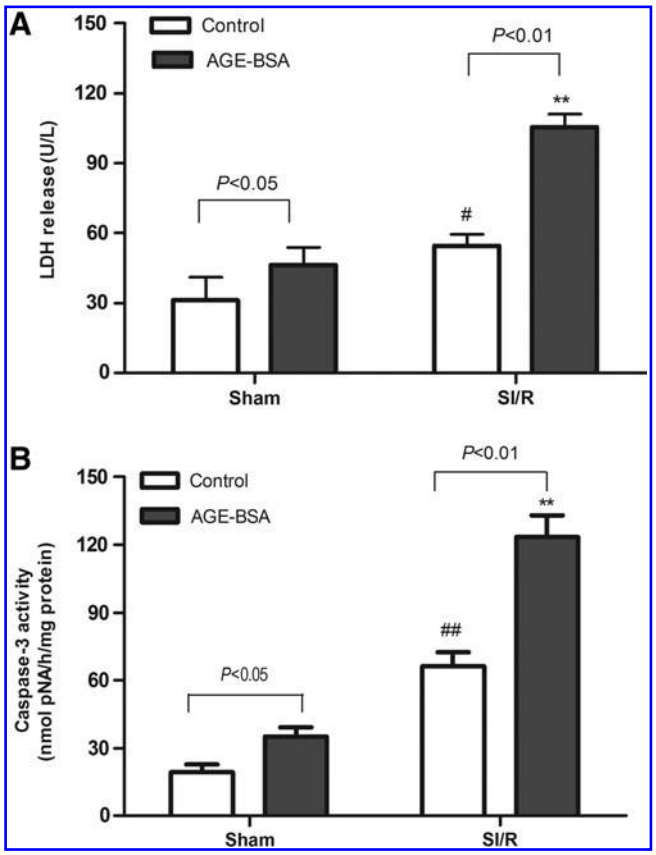

FIG. 2. Effects of AGE-BSA preculture on subsequent simulated ischemia/reperfusion-induced LDH release and caspase-3 activity in CMECs. Advanced glycation end products–modified bovine serum albumin (AGE-BSA) increases the simulated ischemia/reperfusion (SI/R)-induced lactate dehydrogenase (LDH) release **(A)** and caspase-3 activity **(B)** in CMECs. $n = 8$–12 wells/group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* Control + Sham group, $^{**}p < 0.05$ *versus* AGE-BSA + Sham group.

expression and total NO production. It has been documented that iNOS activation can result in excessive reactive nitrogen species production (31). Elevated production of peroxynitrite increases protein modification at the tyrosine residue, increasing nitrotyrosine formation (19). Not only did SI/R increase oxidative stress in CMECs, evidenced by enhanced superoxide generation (Fig. 3A), but also it increased nitrative stress as well, evidenced by greater iNOS expression (Fig. 3B), total NO production (Fig. 3C), and nitrotyrosine production (Fig. 3D). Further, AGE-BSA additionally amplified SI/R-induced superoxide generation (Fig. 3A), iNOS expression (Fig. 3B), total NO production (Fig. 3C), and nitrotyrosine production (Fig. 3D) in CMECs.

AGE-BSA promotes SI/R-induced Trx-1 inactivation and nitration

Recently, we demonstrated that nitrative Trx-1 inactivation plays a causative role in myocardial I/R injury (12). Having demonstrated that AGE-BSA promoted SI/R-induced nitrative stress in CMECs, we tested a hypothesis that AGE-BSA promotes SI/R-injury *via* nitrative Trx-1 inactivation. As shown in Figure 4A, SI/R decreased Trx-1 activity in both control and AGE-BSA group, compared with sham. This observed decrease in Trx-1 activity occurred despite increased expression of Trx-1 protein in both groups (Fig. 4A, B). Further, AGE-BSA additionally amplified SI/R-induced Trx-1 inactivation (but had no effect on Trx-1 expression in sham or SI/R conditions). Moreover, AGE-BSA further enhanced SI/R-induced Trx nitration (Fig. 4C). These studies demonstrated the promotion of Trx-1 inactivation by AGE-BSA possibly *via* posttranslational modification, without alteration of Trx-1 expression.

Preventing Trx-1 nitration or treatment with exogenous Trx-1 attenuates SI/R injury and RAGE expression in cells precultured with AGE-BSA

In the present study, we demonstrated that AGE-BSA exacerbated SI/R injury, increasing SI/R-induced nitrative stress and nitrative Trx-1 inactivation in CMECs. However, whether AGE-BSA-induced increased Trx nitrative inactivation is causatively related to increased SI/R-injury in CMECs remains unknown. We performed the following study to gain more insight. During the 48 h AGE-BSA incubation period, CMECs were treated with EUK134 (a peroxynitrite decomposition catalyst) or recombinant hTrx-1 and then subjected to SI/R. As summarized in Figure 5, treatment with EUK134 or hTrx-1 significantly attenuated SI/R-induced injury, as evidenced by mitigated LDH release (Fig. 5A) and caspase-3 activity (Fig. 5B). EUK134 or hTrx-1 dramatically attenuated both nitrotyrosine content (Fig. 5C) and Trx nitration (Fig. 5D) and recovered Trx-1 activity (Fig. 5E). Interestingly, we unexpectedly found that, compared with vehicle, EUK134 or hTrx-1 significantly decreased RAGE expression (Fig. 5F), suggesting that nitrative Trx-1 inactivation promotes RAGE expression.

Blockade of RAGE attenuated SI/R injury in cells cultured with AGE-BSA

We performed an additional experiment to provide more evidence supporting the central hypothesis that AGE-RAGE stimulated superoxide/NO/peroxynitrite overproduction is the upstream mechanism inducing increased nitrative Trx-1 inactivation in the diabetic condition. During the 48 h AGE-BSA incubation period, CMECs were treated with sRAGE (a RAGE decoy) and then subjected to SI/R. Caspase-3 activity, LDH release, Trx-1 nitration, Trx-1 activity, and gp91phox (the major component of NADPH oxidase) were assessed. sRAGE recovered Trx-1 activity (Fig. 6D), while attenuating CMECs LDH release (Fig. 6A), caspase-3 activity (Fig. 6B), Trx-1 nitration (Fig. 6C), and gp91phox expression (Fig. 6E).

Discussion

We have made several important observations in the present investigation. First, we demonstrated for the first time that AGE-BSA promotes SI/R-induced injury in CMECs. Second, we further demonstrated nitrative Trx inactivation as an exacerbating factor to SI/R-induced injury in AGE-BSA pretreated CMECs, a novel mechanism by which AGEs cause endothelial injury. Third, we provided the first evidence that RAGE acts as a modulator of both nitrative stress and sub-

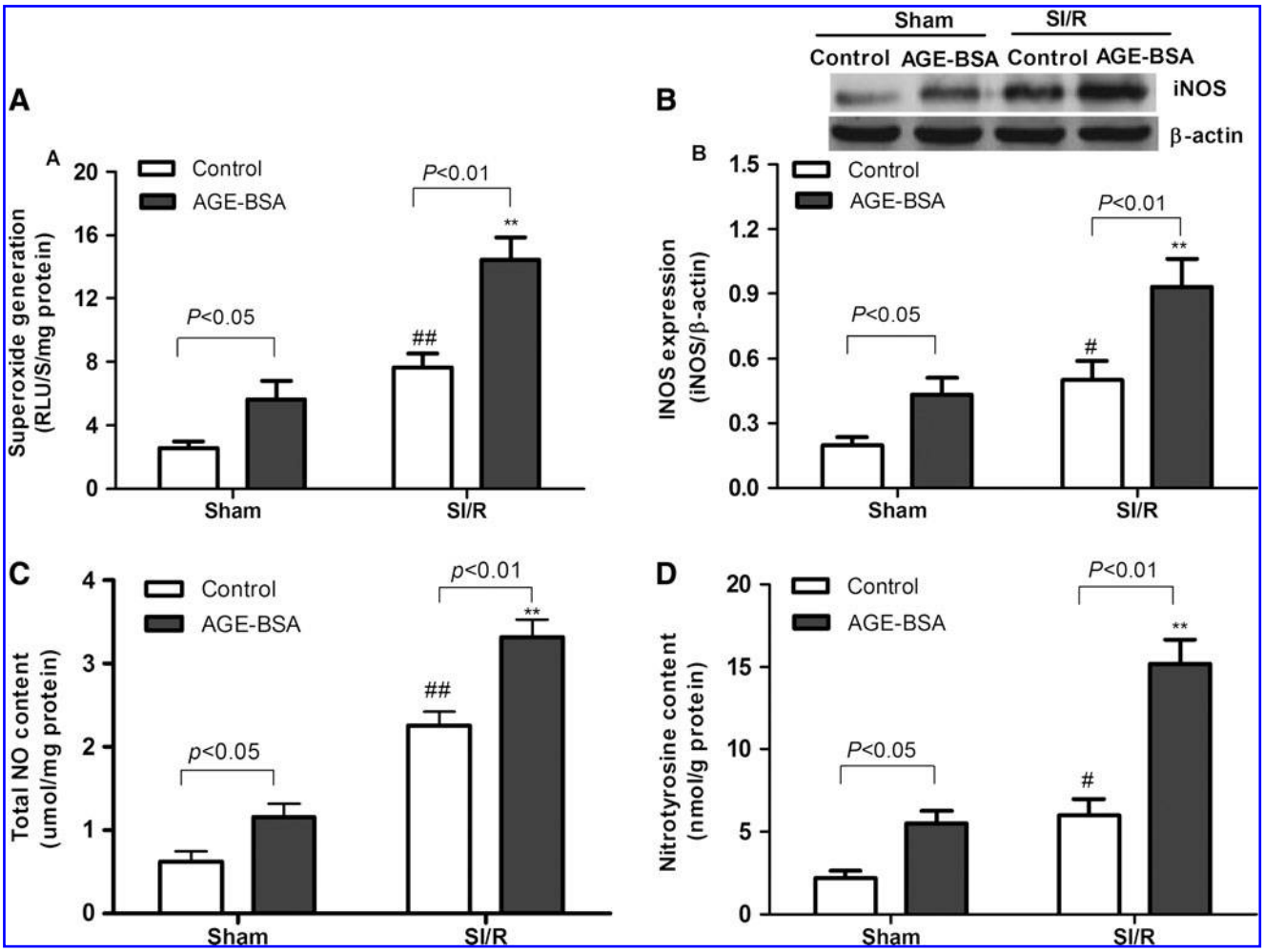

FIG. 3. Effects of AGE-BSA preculture or subsequent simulated ischemia/reperfusion-induced oxidative/nitrative stress in CMECs. AGE-BSA promotes the SI/R-induced superoxide generation **(A)**, inducible nitric oxide synthase (iNOS) expression **(B:** *upper panel*, representative iNOS expression by western blot; *lower panel*, statistic analysis of western results standardized by β-actin**)**, total nitric oxide (NO) content **(C)**, and nitrotyrosine content **(D)** in CMECs. $n = 8$–12 wells/group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* Control + Sham group, respectively. $^{**}p < 0.05$ *versus* AGE-BSA + Sham group.

sequent Trx-1 nitrative inactivation in the setting of AGE-BSA-induced amplification of SI/R-induced CMECs injury. Finally, we have identified effective interventions capable of attenuating diabetic cardiac microvascular endothelial I/R injury.

It has been previously demonstrated that cardiac microvascular endothelial injury precedes cardiomyocyte injury in I/R situations (22). Attenuating endothelial injury can reduce cardiomyocyte cell death after ischemia and reperfusion ultimately. Patients with diabetes with acute myocardial infarction are more likely to suffer from "no reflow" after interventional therapy (20), suggesting the enhanced vulnerability of diabetic endothelial cells to I/R damage. However, the precise mechanism by which this susceptibility occurs requires elucidation. AGEs are nonenzymatically modified proteins or lipids that become glycated and oxidized after contact with sugars (24). AGEs form *in vivo* during aging, or in hyperglycemic environments, and are contributive to the pathophysiology of vascular disease in diabetes (2). Previous studies demonstrate that AGEs alter properties of the large matrix proteins collagen, vitronectin, and laminin *via* formation of intermolecular AGE-AGE covalent or crosslinking bonds that destroy the extracellular structure (10, 12). AGEs can also interact with its receptor RAGE to alter intracellular endothelial function, increasing intercellular adhesion molecule-1, interleukin-6, and vascular cellular adhesion molecule-1 expression, thereby amplifying the inflammatory process (1, 16). As I/R injury is closely related to inflammation pathways; these studies indicate the possible association of AGEs to cardiac microvascular endothelial I/R injury. Presently, we demonstrated that incubation of CMECs with a pathologically relevant concentration of AGEs (34) increased cellular susceptibility to I/R injury evidenced by increased LDH release and caspase-3 activity. These results suggest that interventions which decrease AGE concentration or block AGE signaling can mitigate cardiac microvascular endothelial I/R injury in patients with diabetes.

Considerable evidence demonstrates increased oxidative stress in both patients with diabetes and animal diabetic models (8). It has been documented that AGEs, *via* binding

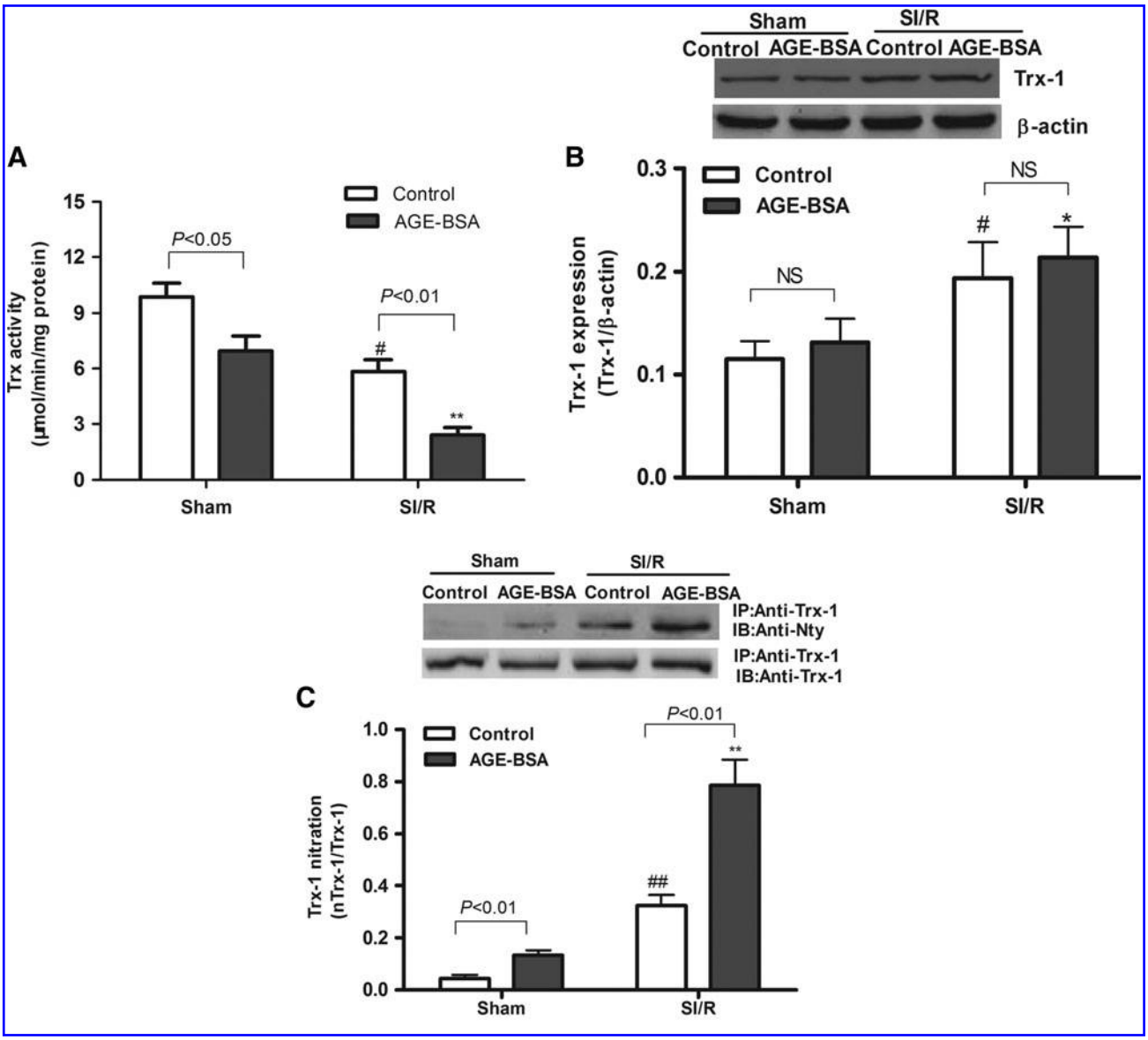

FIG. 4. Effects of AGE-BSA preculture on subsequent simulated ischemia/reperfusion-induced Trx-1 inactivation and nitration in CMECs. AGE-BSA further decreased already SI/R-attenuated thioredoxin (Trx) activity **(A)** and had no effect of the SI/R-induced Trx-1 expression **(B:** *upper panel*, representative Trx-1 expression by western blot; *lower panel*, statistic analysis of western results standardized by *β*-actin**)** and increased the SI/R induced the Trx-1 nitration **(C:** *upper panel*, representative nitrated and total Trx by immunoprecipitation; *lower panel*, statistic analysis of Trx nitration standardized by total Trx**)** in CMECs. $n = 8$–12 wells/group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* Control + Sham group, $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* AGE-BSA + Sham group, respectively.

with RAGE, activate NADPH oxidase and increase production of ROS in endothelial cells (32). The increase of ROS induced by AGEs result in the activation of NF-κB, followed by increased intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1 expression, causing endothelial cell damage (16). Recent studies report that nitrative stress might be instrumental in the pathogenesis of diabetes (14). In patients with diabetes and animals, iNOS expression and activity is elevated (4). The increased nitric oxide produced by iNOS reacts with increased superoxide generated by NADPH oxidase, resulting in peroxynitrite overproduction, leading to subsequent protein modification and cellular injury in diabetes (18, 29). In the present study, we demonstrated that AGE-BSA not only increased oxidative stress in CMECs (evidenced by increased superoxide anion formation) but also increased total iNOS expression, NO content, and nitrotyrosine production. These data indicate that AGEs promote SI/R injury not only by enhancing oxidative stress but also by nitrative stress. Our results revealing the beneficial effects of a peroxynitrite decomposition catalyst provided direct evidence that AGEs increase SI/R injury in CMECs *via* nitrative stress augmentation.

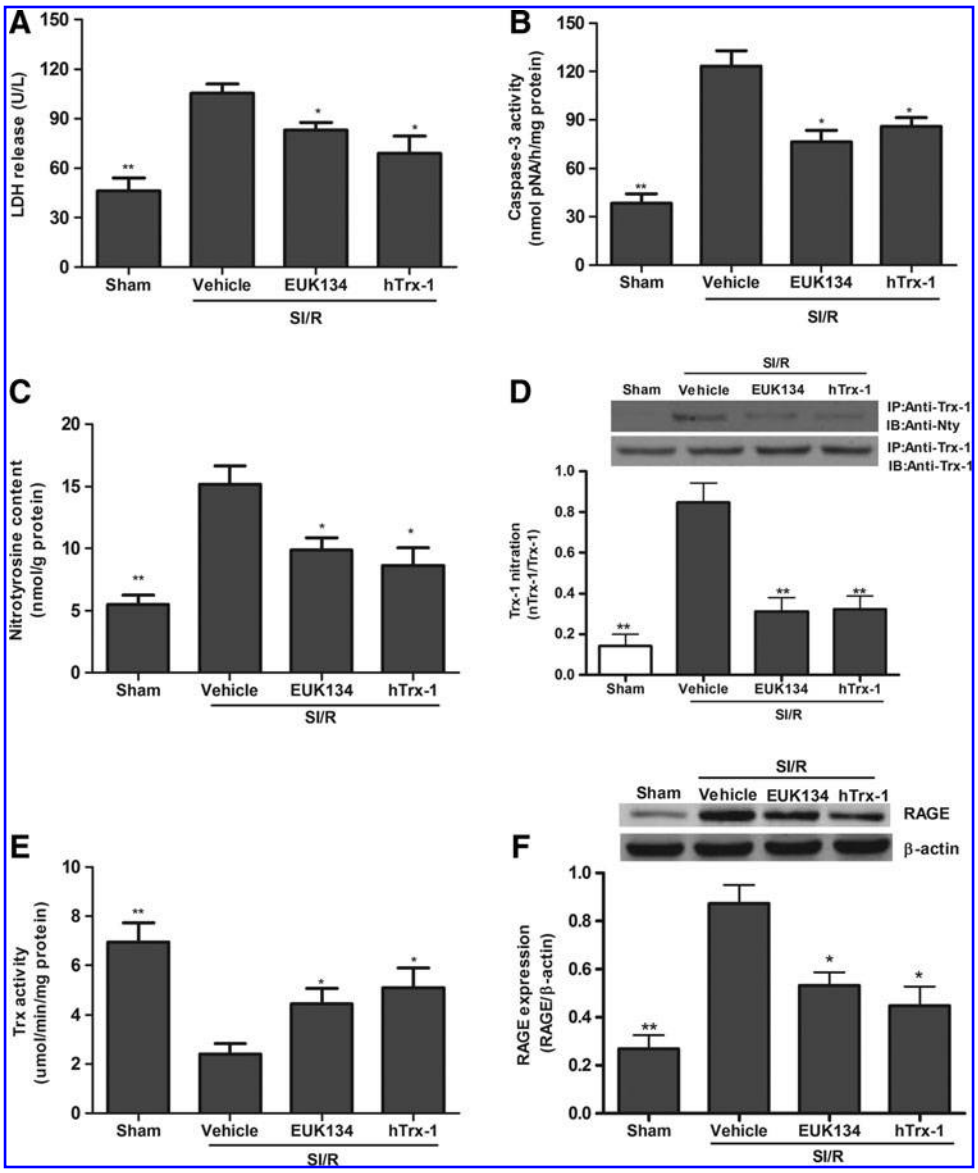

FIG. 5. Effects of preventing Trx-1 nitration or treatment with exogenous Trx-1 on subsequent simulated ischemia/reperfusion-induced injury and RAGE expression in AGE-BSA precultured CM ECs. EUK134 or hTrx-1 attenuates SI/R-induced LDH release **(A)** and caspase-3 activity **(B)** and nitrotyrosine content **(C)** and decreased the Trx nitration **(D:** *upper panel* representative nitrated and total Trx by immunoprecipitation, *lower panel* statistic analysis of Trx nitration standardized by total Trx.**)** and receptor of AGE (RAGE) expression **(F:** *upper panel*, representative RAGE expression by western blot; *lower panel*, statistic analysis of western results standardized by *β*-actin**)** and recovered Trx activity **(E)** in the cells precultured with AGE-BSA. Nty, nitrotyrosine. $n = 8$–12 wells/group. $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* SI/R + vehicle group, respectively.

Trx is a 12-kDa protein ubiquitously expressed in all living cells, performing a variety of biological functions related to cell proliferation and apoptosis (15, 38). Studies have demonstrated that in addition to upregulation or downregulation of Trx expression at the gene level, Trx activity is regulated by posttranslational modification, such as oxidation, glutathionylation, and S-nitrosylation (6, 9). Recently, it has been demonstrated that Trx can be modified at its tyrosine residue, in a process known as protein nitration, resulting in irreversible inactivation *via* a peroxynitrite-dependent fashion (37). Moreover, this nitrative Trx inactivation plays a key pathologic role in situations such as I/R injury in diabetes (37). In the present study, we found that AGE-BSA exacerbated Trx inactivation after SI/R without altering Trx expression. These results indicate that AGE-BSA decreases Trx activity *via* post-translational modification. Indeed, we found that AGE-BSA amplified SI/R-induced Trx nitration and that recombinant hTrx-1 supplementation effectively attenuated SI/R-induced injury.

Several different receptors for AGEs have been discovered. These include RAGE, AGE-R1 (oligosaccharyl transferase-48), AGE-R2 (80K-H phosphoprotein), AGE-R3 (galectin-3), and the class A macrophage scavenger receptor types I and II (30). Among the receptors for AGEs, RAGE is recognized to initiate the intracellular signaling disruptive of cellular function *via* recognition and binding of AGEs (5). In the present study, we found that AGE-BSA promoted nitrative stress and subsequent Trx-1 nitration in CMECs. More importantly, we demonstrated that treatment with sRAGE (a decoy of RAGE) attenuated AGE-BSA exacerbated nitrative stress and Trx nitration and decreased SI/R-induced injury. These results suggest that RAGE signaling is responsible for AGE-BSA-induced nitrative stress and Trx nitration post-SI/R. It has been shown that RAGE is upregulated when AGE ligands accumulate, resulting in positive-feedback activation (23). We also currently demonstrate that RAGE expression is decreased in the setting of nitrative stress and Trx nitration inhibition in CMECs, suggestive of a vicious cycle involving

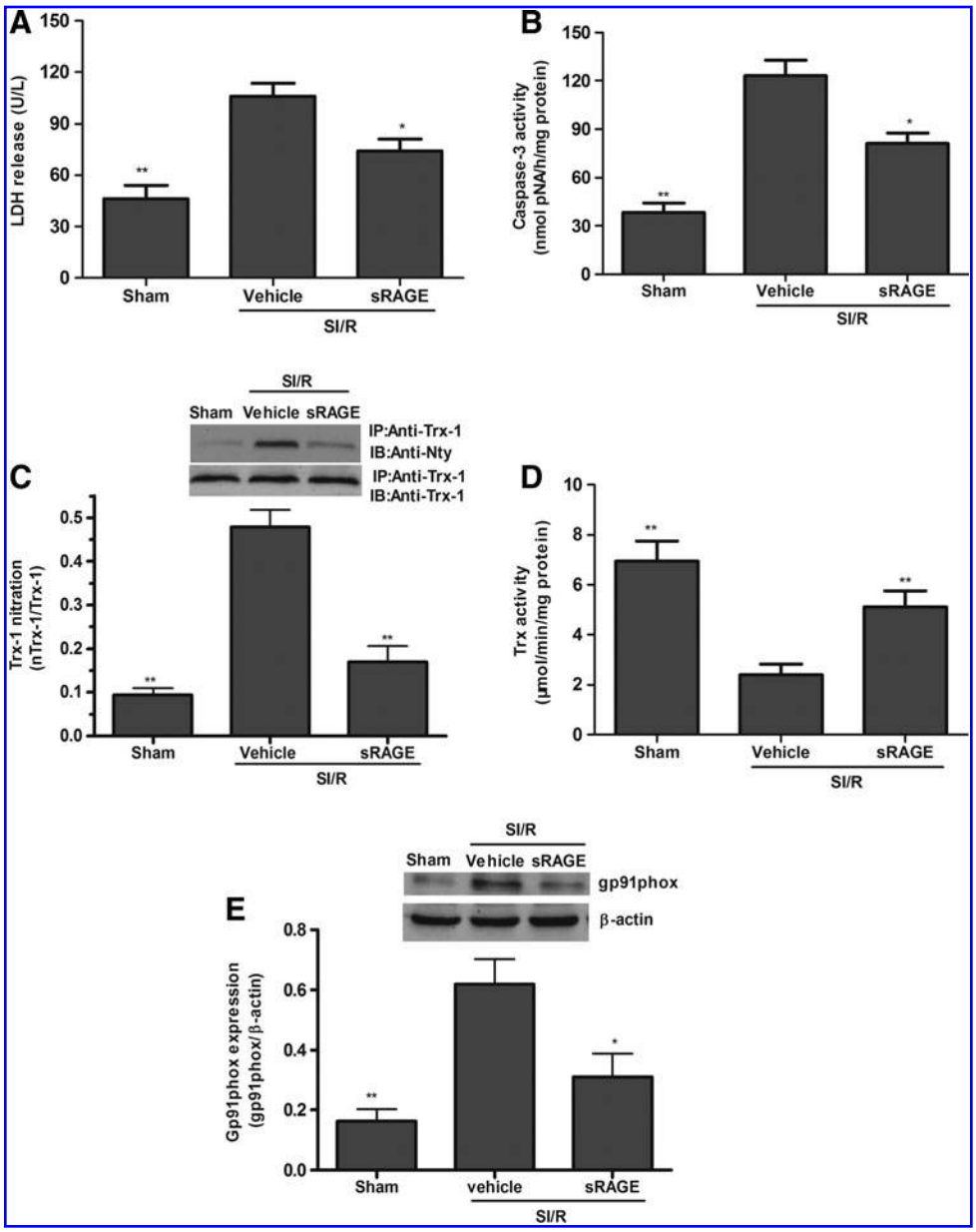

FIG. 6. Effects of blocking RAGE on subsequent simulated ischemia/reperfusion-induced injury and Trx-1 inactivation and nitration in AGE-BSA precultured CMECs. Soluble RAGE (sRAGE) attenuates SI/R-induced LDH release **(A)** and caspase-3 activity **(B)** and Trx nitration **(C:** *upper panel*, representative nitrated and total Trx by imunoprecipitation; *lower panel*, statistic analysis of Trx nitration standardized by total Trx**)** and gp91phox expression **(E:** *upper panel*, representative gp91phox expression by western blot; *lower panel*, statistic analysis of western results standardized by β-actin**)** and recovered SI/R reduced Trx activity **(D)** in the cells precultured with AGE-BSA. Nty, nitrotyrosine. $n = 8$–12 wells/group. $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* SI/R + vehicle group, respectively.

AGE-RAGE signaling and Trx nitration. Experiments directed toward identification of the detailed signaling network regulating AGE-RAGE/Trx inactivation are currently ongoing.

In summary, our results demonstrated that AGEs promote I/R injury in CMECs through the RAGE/Trx nitration pathway. Blocking peroxynitrite formation and RAGE signaling by sRAGE or exogenous Trx-1 supplementation significantly protected AGE-BSA-induced CMECs from SI/R-induced injury. These results suggest that therapeutic interventions preserving Trx-1 activity in the patient with diabetes may further help in improving patient outcomes after myocardial I/R injury.

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Author Disclosure Statement

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Abbreviations Used

AGEs = advanced glycation end products
BSA = bovine serum albumin
CMECs = cardiac microvascular endothelial cells
DMEM = Dulbecco's minimum essential medium
I/R = ischemia/reperfusion
LDH = lactate dehydrogenase
NO = nitric oxide
PBS = phosphate-buffered saline
RAGE = receptor of advanced end product
ROS = reactive oxygen species
SI/R = simulated ischemia/reperfusion
sRAGE = soluble receptor of advanced end product
Trx = thioredoxin

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