RAGE and Modulation of Ischemic Injury in the Diabetic Myocardium

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OBJECTIVE—Subjects with diabetes experience an increased risk of myocardial infarction and cardiac failure compared with nondiabetic age-matched individuals. The receptor for advanced glycation end products (RAGE) is upregulated in diabetic tissues. In this study, we tested the hypothesis that RAGE affected ischemia/reperfusion (I/R) injury in the diabetic myocardium. In diabetic rat hearts, expression of RAGE and its ligands was enhanced and localized particularly to both endothelial cells and mononuclear phagocytes.

RESEARCH DESIGN AND METHODS—To specifically dissect the impact of RAGE, homozygous RAGE-null mice and transgenic (Tg) mice expressing cytoplasmic domain-deleted RAGE (DN RAGE), in which RAGE-dependent signal transduction was deficient in endothelial cells or mononuclear phagocytes, were rendered diabetic with streptozotocin. Isolated perfused hearts were subjected to I/R.

RESULTS—Diabetic RAGE-null mice were significantly protected from the adverse impact of I/R injury in the heart, as indicated by decreased release of LDH and lower glycoxidation products carboxymethyl-lysine (CML) and pentosidine, improved functional recovery, and increased ATP. In diabetic Tg mice expressing DN RAGE in endothelial cells or mononuclear phagocytes, markers of ischemic injury and CML were significantly reduced, and levels of ATP were increased in heart tissue compared with littermate diabetic controls. Furthermore, key markers of apoptosis, caspase-3 activity and cytochrome c release, were reduced in the hearts of diabetic RAGE-modified mice compared with wild-type diabetic littermates in I/R.

CONCLUSIONS—These findings demonstrate novel and key roles for RAGE in I/R injury in the diabetic heart. ***Diabetes* 57: 1941–1951, 2008**

Cardiac complications remain a leading cause of morbidity and mortality in subjects with diabetes (1–3). Although many factors contribute to depressed cardiac function in diabetes, innate disturbances within the diabetic heart contribute importantly to progressive dysfunction, which often leads to irreversible failure and death (3). Alterations in substrate metabolism and increased levels of oxygen free radicals have been observed in diabetic tissues. Inflammatory cytokines may exert direct negative inotropic effects on cardiac myocytes and contribute to aberrant remodeling in the failed heart (4–8). The pathophysiology of diabetes-associated cardiac complications is complex and involves a host of factors linked to metabolic and immune/inflammatory cell activation.

The accumulation of late-stage glycoxidation adducts of proteins, termed advanced glycation end products (AGEs), occurs in diabetic tissues. AGEs modify long-lived molecules in the blood vessel wall and structural tissues of the heart considerably earlier than symptomatic cardiac dysfunction occurs (9). A major way in which AGEs exert their cellular effects is by ligation of the multiligand receptor for AGE (RAGE) (10–13).

We tested the role of RAGE in rodent models of type 1 diabetes, and we show that pharmacological blockade of ligand-RAGE interaction or genetic modulation of RAGE suppresses ischemia/reperfusion (I/R) injury in the isolated perfused heart, at least in part secondary to critical contributions evoked from RAGE-expressing endothelial cells and mononuclear phagocytes in the diabetic heart.

RESEARCH DESIGN AND METHODS

All animal studies were performed with the approval of the Institutional Animal Care and Use Committee of Columbia University and conform with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Male diabetic Bio Bred (BB/W) rats, a model of type 1 diabetes (Biomedical Models, Worcester, MA) (14,15), were used. Rats became diabetic between 69 and 80 days of age and were entered into study at diagnosis of hyperglycemia. Age-matched nondiabetic BB/W rats were controls. Diabetic rats were maintained on human insulin (3–5 units/dose) (Humulin; Eli Lilly, Indianapolis, IN) twice daily. Insulin was discontinued 12 h before isolated heart perfusion studies. Rats were killed after 2 weeks of established diabetes. Murine soluble RAGE (sRAGE; 500 μg/day) or equal volumes of its diluent, PBS, were administered by intraperitoneal route beginning immediately at the diagnosis of hyperglycemia (serum glucose ≥250 mg/dl) and continued for 14 days. sRAGE was prepared in a baculovirus expression system, and the material was purified and devoid of contaminating lipopolysaccharide (16).

Male BALB/c or C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME) were rendered diabetic by 55 mg/kg i.p. streptozotocin (STZ) per day in fresh citrate buffer (0.05 mol/l; pH 4.5) for 5 consecutive days. Mice displaying serum glucose ≥250 mg/dl were considered diabetic. Control (nondiabetic) animals received citrate buffer (16).

Homozygous RAGE-null mice and transgenic mice. Homozygous RAGE-null mice (RAGE-KO) (17–19) were backcrossed 10 generations into C57BL/6 before study. Male RAGE-KO and littermate mice were used. In other studies, two sets of transgenic mice were prepared to express signal transduction–deficient RAGE, or dominant-negative (DN) RAGE (11,12,18) in endothelial cells (driven by the preproendothelin-1 [PPET] promoter; 20) or cells of mononuclear phagocyte lineage (driven by the macrophage scavenger receptor type A [MSR] promoter; 21,22). Transgenic (Tg) DN MSR RAGE and Tg DN PPET RAGE mice were prepared and characterized as previously described

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Received 19 March 2007 and accepted 9 April 2008.
Published ahead of print at http://diabetes.diabetesjournals.org on 16 April 2008. DOI: 10.2337/db07-0326.
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(20,22). Mice were backcrossed >10 generations into C57BL/6 before the study. Littermates not expressing the transgene were used as controls.

Western blotting. Left ventricles were retrieved and subjected to SDS-PAGE/Western blotting using 1 μg/ml anti-RAGE IgG (16); 0.25 μg/ml anti-inducible nitric oxide (NO) synthase (iNOS) IgG (Santa Cruz Biotechnology); anti–cytochrome c IgG (Santa Cruz Biotechnology); or anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH) IgG (Cayman). After probing with the primary antibodies, membranes were stripped of bound immunoglobulins and reprobed with anti–β-actin IgG (Sigma-Aldrich, St. Louis, MO). All antibodies were used according to the manufacturer's instructions. Analysis of band density was performed using Image Quant/Molecular Dynamics software (Foster City, CA). Results are reported as relative units based on the ratio of test antibody densitometry units/β-actin densitometry units.

Immunohistochemistry. Hearts were retrieved, and sections were prepared for immunohistochemistry. The following antibodies were used: anti-RAGE IgG (16), anti-AGE IgG (16), anti-S100/calgranulin IgG (12), and anti-nitrotyrosine IgG (Upstate, Lake Placid, NY). To localize cell-specific expression of RAGE, additional antibodies were used: anti-Mac 3 IgG (Pharmingen, San Diego, CA) and anti-CD31 IgG (Pharmingen). Immunostaining with nonimmune IgGs of the relevant species were negative.

Levels of total nitrite and nitrate and cGMP. Hearts were retrieved and rapidly snap-frozen in liquid nitrogen after I/R. Lysates were prepared and levels of total nitrite and nitrate and cGMP were measured using a kit from Oxis Research Products (Portland, OR).

Isolated perfused heart and measurement of cardiac function. Experiments were performed using an isovolumic isolated heart preparation and modified, as indicated, for mice hearts (23,24). Mice and rats were anesthetized using a mixture of ketamine and xylazine (80 and 10 mg/kg, respectively). After deep anesthesia was achieved, hearts were rapidly excised, placed in iced saline, and retrogradely perfused at 37°C in a nonrecirculating mode through the aorta. In mice, the perfusion rate was 2.5 ml/min, and in rats, the perfusion rate was 12.5 ml/min. Rat and mice hearts were perfused with modified Krebs-Henseleit buffer containing 118 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 25 mmol/l $NaHCO_3$, 5 mmol/l glucose, 0.4 mmol/l palmitate, 0.4 mmol/l BSA, and 70 mU/l insulin. The perfusate was equilibrated with a mixture of 95% O_2–5% CO_2, which maintained perfusate PO_2 >600 mmHg. Left ventricular developed pressure (LVDP) and left ventricular end diastolic pressure were measured using a latex balloon in the left ventricle. LVDP, heart rate, and coronary perfusion pressure were monitored continuously on a four-channel Powerlab (AD Instruments, Grand Junction, CO).

The ischemia/perfusion protocol was as follows: Hemodynamic function was monitored throughout the protocol. Mice hearts were paced at 420 beats/min with the use of pacing electrodes placed on the right atrium. Rat hearts were paced at 300 beats/min. Hearts were subjected to 30 min of global (zero-flow) ischemia and 60 min of reperfusion. Perfusate temperature was maintained at 37°C at all times during the protocol (i.e., during baseline, ischemia, and reperfusion). After an equilibration period of 30 min, both groups of hearts were perfused with modified Krebs-Henseleit buffer throughout ischemia and reperfusion.

LDH release, a marker of I/R injury, was measured in the effluents for the entire 60 min of reperfusion in each heart. The levels of LDH were assayed using commercially available kits from Sigma-Aldrich. LDH release is expressed as international unit per gram dry weight of tissue. Caspase-3 activity was assayed in hearts using reagents provided in the caspase-3 activity kit from R&D Systems. Briefly, caspase-3 activity is based on cleavage of the synthetic caspase substrate linked to the color reporter molecule *p*-nitroaniline. The amount of hydrolyzed substrate was measured as absorbance at 405 nm. Recombinant human caspase-3 was used as a positive control and to generate a standard curve. The results are expressed as caspase activity relative to that of wild-type control animals.

Measurement of ATP. ATP was measured in the neutralized perchloric acid extracts of hearts according to previously published procedures (23,24).

Determination of AGEs in heart tissue. Heart tissue (25–100 mg wet wt) was extracted with 10 ml 2:1 chloroform-methanol for 24 h, washed with 10 ml methanol, and rehydrated in water. Samples were homogenized for 60 s with a Brinkmann Polytron in 2 ml Chelex-treated PBS (pH 7.4) containing 1 mmol/l diethylenetriaminepentaacetic acid (DTPA) and protease inhibitor cocktail (Roche) using one tablet per 10 ml buffer. Homogenates were centrifuged at 1,600 rpm for 20 min using a Sorvall RT 6000D centrifuge. Pellets were washed three times with water while supernatants were exhaustively dialyzed versus water. All samples were freeze-dried and stored at −80°C until further processing. Three milligrams of lyophilized tissue was weighed and placed into Eppendorf tubes on ice. Samples were solubilized with 100 μl PBS and 900 μl 1% HCl/Acetone kept at −20°C to remove heme, vortexed, and centrifuged at 15,000 rpm for 15 min at 4°C. This procedure was repeated twice. Pellets were then washed with ice-cold 1 mmol/l DTPA, freeze-dried, and acid hydrolyzed with 6 N HCl for 19 h. AGE analysis (carboxymethyl-lysine [CML], carboxyethyl-lysine [CEL], fructose-lysine [furosine], and 2-aminoadipic acid) was by gas chromatography–mass spectrometry as described previously (25). Pentosidine was determined by high-performance liquid chromatography according to Sell et al. (26)

Statistical analysis. Significant differences between experimental groups were detected using ANOVA for unpaired variables. Post hoc comparisons were performed using Tukey's or Dunnett's procedures as indicated. SAS package software was used in the statistical analyses, and statistical significance was ascribed to the data when $P < 0.05$.

RESULTS

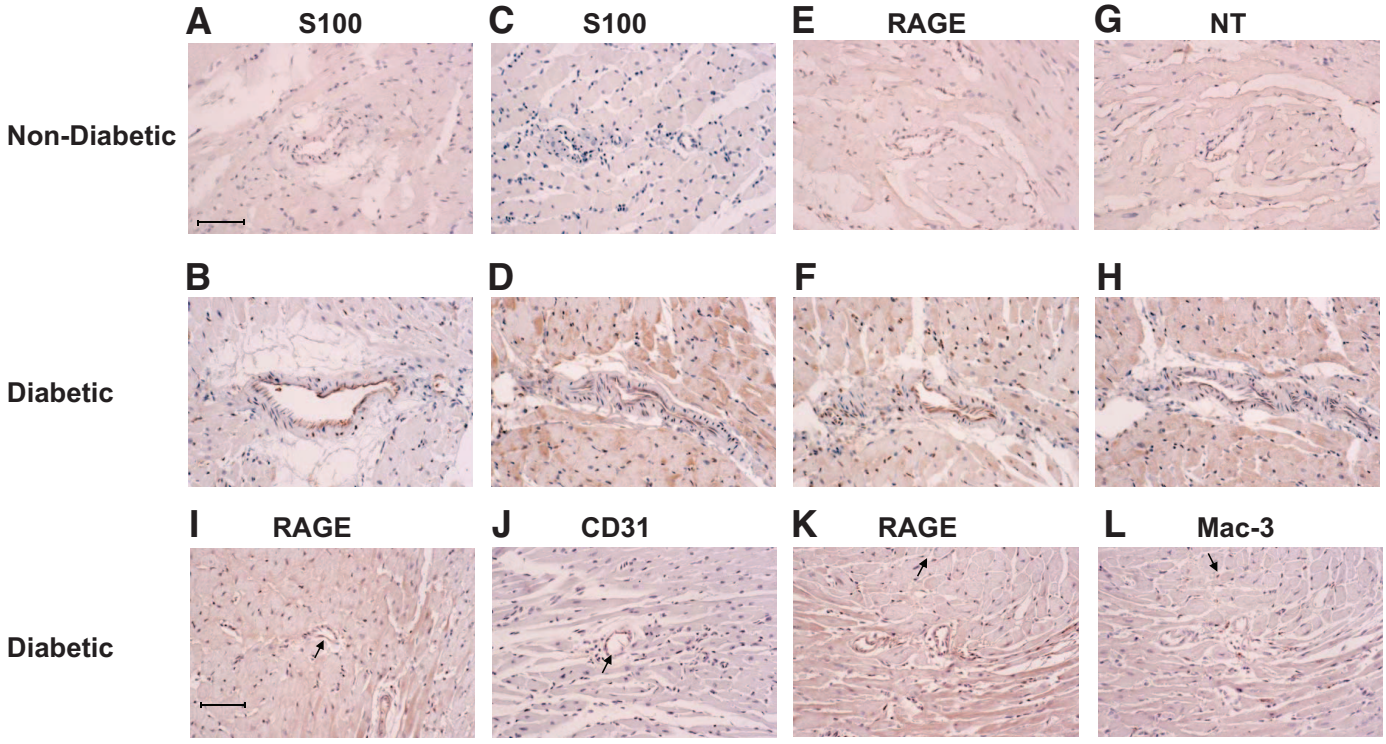
Ligand/RAGE axis in the diabetic rat heart. We used the Biobred/Worcester (BB/W) rat, a model of autoimmune type 1 diabetes, to dissect the potential role of RAGE in the pathogenesis of cardiac dysfunction in diabetes (14,15) and explored the expression/distribution of RAGE and its ligands in the diabetic rat heart at baseline, in the absence of I/R. First, we examined epitopes for CML AGEs (27,28). CML AGEs are specific AGE ligands of RAGE (11). In nondiabetic hearts, CML epitopes were not detected (Fig. 1*A*). In contrast, heart tissue retrieved from hyperglycemic rats displayed immunoreactive epitopes for CML AGE (Fig. 1*B*). In addition to AGEs, although expression of RAGE ligand S100/calgranulin (12) was not demonstrated in the hearts of nondiabetic BB/W rats (Fig. 1*C*), S100/calgranulin epitopes were evident in diabetic BB/W rat heart (Fig. 1*D*), in both vascular cells and cardiomyocytes.

Diabetic BB/W hearts displayed increased RAGE antigen compared with nondiabetic hearts by immunohistochemistry (Fig. 1*F* and *E*, respectively) and Western blotting ($P < 0.05$; Fig. 1*M*). We examined the hearts to detect nitrotyrosine epitopes, markers of oxidative injury. Compared with nondiabetic hearts in which virtually no nitrotyrosine epitopes were evident, immunohistochemistry demonstrated extensive nitrotyrosine epitopes in the hearts of diabetic BB/W rats (Fig. 1*G* and *H*, respectively).

To specifically localize the cells expressing RAGE in the diabetic heart, additional studies were performed. Immunohistochemistry using anti-RAGE IgG and anti-CD31 IgG showed that RAGE was expressed in endothelial cells in the diabetic heart (Fig. 1*I* and *J*, respectively). Immunohistochemistry using anti-RAGE IgG and anti–Mac-3 IgG demonstrated that RAGE was expressed in mononuclear phagocytes in the diabetic heart (Fig. 1*K* and *L*, respectively). Together, these studies indicated that RAGE expression was increased in the type 1 diabetic heart, particularly in endothelial cells and mononuclear phagocytes.

To test the impact of diabetes on expression of RAGE and the effects of RAGE blockade, we first administered sRAGE, the extracellular ligand-binding domain of RAGE. sRAGE was administered to diabetic rats commencing at the diagnosis of hyperglycemia once daily for 14 days. In diabetic rats treated with sRAGE, significantly lower levels of RAGE antigen in the heart were observed compared with vehicle-treated diabetic rats ($P < 0.05$; Fig. 1*M*). This observation is consistent with the concept that ligand-RAGE interaction sustains enhanced expression of RAGE; once interrupted, cellular expression of RAGE was significantly lower than that observed in the hearts of vehicle-treated diabetic rats (29).

Diabetes increases expression of iNOS in the rat heart: impact of RAGE. To explore the potential impact of the ligand-RAGE axis in modulating perturbation and oxidant stress in the heart, we assessed levels of iNOS in



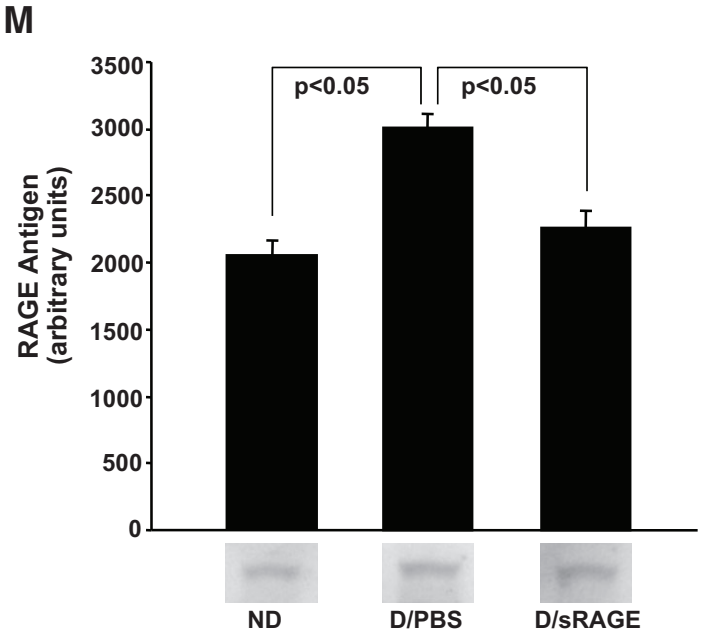


FIG. 1. RAGE, its ligands, and the diabetic rat heart. Hearts of type 1 diabetic BB/W rats were retrieved. Immunostaining of CML/AGEs (*A* and *B*), S100/calgranulin (*C* and *D*), RAGE (*E* and *F*), and nitrotyrosine epitopes (*G* and *H*) in nondiabetic and diabetic hearts is shown. RAGE was expressed in endothelial cells and mononuclear phagocytes, evident using anti-RAGE IgG (*I* and *K*), anti-CD31 IgG (to detect endothelial cells) (*J*), and anti–Mac-3 IgG (to detect mononuclear phagocyte) (*L*). Scale bar = 100 μm. *n* = 3 rats/group. Western blotting using anti-RAGE IgG was performed to detect RAGE (*M*). *n* = 6 rats/group.

the heart in the basal state. Compared with nondiabetic controls, hearts from diabetic rats displayed increased iNOS antigen by Western blotting; in the presence of treatment with sRAGE, levels of iNOS antigen were significantly lower ($P < 0.05$; Fig. 2*A*).

In parallel, levels of total nitrite and nitrate and cGMP were increased in diabetes ($P < 0.05$); in the presence of sRAGE, levels of total nitrite and nitrate and cGMP in diabetic hearts were significantly lower ($P < 0.05$) and similar to those observed in nondiabetic age-matched hearts (Fig. 2*B* and *C*, respectively).

Pharmacological blockade of ligand-RAGE axis attenuates I/R injury in the diabetic rat heart. We assessed the potential impact of RAGE on cardiac dysfunction consequent to I/R in the diabetic rat heart. Two indexes of cardiac recovery were assessed: LVDP and release of LDH on reperfusion, the latter an index of myocardial injury. Diabetic rats displayed a marked decrease in LVDP recovery on reperfusion compared with nondiabetic rats subjected to the same degree of I/R ($P < 0.05$; Fig. 3*A*). LDH release was significantly higher in diabetic hearts compared with nondiabetic hearts ($P < 0.05$; Fig. 3*B*). Treat-

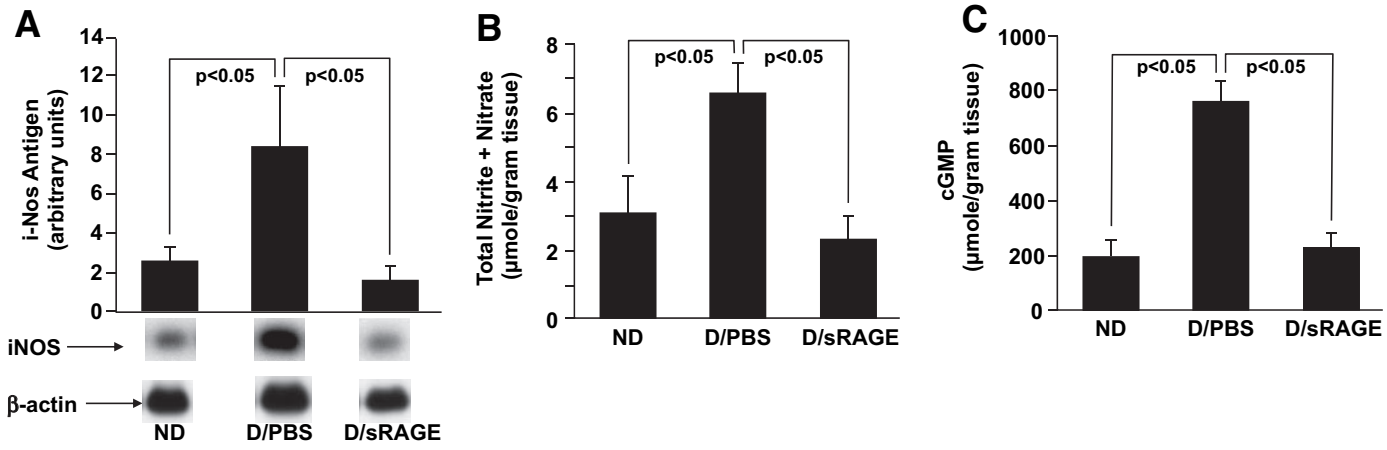


FIG. 2. Diabetes upregulates iNOS in the rat heart: effect of RAGE. Hearts of type 1 BB/W diabetic rats treated with sRAGE or PBS were retrieved, and Western blotting was performed for detection of iNOS (*A*) antigen. Levels of total nitrite and nitrate (*B*) and cGMP (*C*) were measured. n = 6 rats/group. D, diabetic; ND, nondiabetic.

ment for 14 days with sRAGE resulted in improvement in LVDP recovery and significantly lower LDH release ($P < 0.05$; Fig. 3*A* and *B*).

Role of RAGE in diabetes-associated cardiac ischemic injury: studies in mice. We extended our studies to murine models, wherein genetic modification of RAGE would complement pharmacological blockade of ligand-RAGE interaction (sRAGE). We rendered wild-type BALB/c mice diabetic with STZ and examined the impact of hyperglycemia on expression of RAGE and its ligands in the heart. After 14 days of diabetes, we examined levels of specific AGE species, CML, CEL, furosine (glycated lysine), and pentosidine in BALB/c mouse hearts (Table 1). As expected, we found that levels of CML, furosine, and pentosidine were significantly higher in the diabetic BALB/c mice hearts compared with their nondiabetic controls (Table 1). In diabetic mice, treatment with sRAGE prevented the increases in CML and pentosidine levels, but there was no effect on furosine levels, suggesting an effect independent of tissue glucose levels (Table 1). Levels of argpyramidine, CEL, and 2-aminoadipic acid, a marker of metal catalyzed oxidation, were similar in all of the groups studied (data not shown). Consistent with increased AGE ligand levels in the diabetic heart, a significant increase in RAGE antigen by Western blotting was observed in diabetic compared with nondiabetic hearts ($P < 0.05$; Fig. 4*A*). In diabetic mice treated with sRAGE, levels of RAGE antigen were significantly lower ($P < 0.05$) and not different from levels observed in euglycemic controls.

To test the impact of diabetes on oxidant stress in the diabetic heart, we examined levels of iNOS antigen in the basal state. Compared with nondiabetic mice, significant increases in iNOS antigen were demonstrated in diabetic BALB/c mouse hearts by Western blotting ($P < 0.05$; Fig. 4*B*). In the presence of sRAGE, levels of iNOS were significantly lower ($P < 0.05$; Fig. 4*B*). In parallel, levels of total nitrite and nitrate and cGMP were increased in the diabetic versus nondiabetic heart and reduced by sRAGE ($P < 0.05$; Fig. 4*C* and *D*, respectively). When hearts were subjected to I/R, significantly less release of LDH was demonstrated in hearts retrieved from sRAGE-treated diabetic mice versus vehicle-treated diabetic mice ($P < 0.05$; Fig. 4*E*).

Diabetic homozygous RAGE-KO mice. To specifically address the contribution of RAGE, we used homozygous RAGE-KO mice. RAGE-KO mice or wild-type RAGE-expressing littermate animals were rendered diabetic with STZ. We extended the period of diabetes to 12 weeks to test the impact of RAGE in a chronically diabetic environment.

We assessed the impact of injury triggered by I/R on functional recovery of the diabetic heart. LVDP recovery was significantly higher in the diabetic RAGE-KO heart versus RAGE-expressing littermates ($P < 0.05$; Fig. 5*A*). In parallel, levels of LDH measured in the effluent in the I/R-subjected diabetic animals were significantly lower in the diabetic RAGE-KO versus littermate mice ($P < 0.05$; Fig. 5*B*). Levels of ATP in the heart were significantly higher in the diabetic RAGE-KO hearts versus diabetic controls ($P < 0.05$; Fig. 5*C*).

Levels of total nitrite and nitrate after I/R were significantly lower in diabetic RAGE-KO versus wild-type diabetic hearts (2.9 ± 0.9 vs. 6.8 ± 1.1 μmol/g tissue; $P < 0.05$). Furthermore, levels of cGMP in the diabetic heart

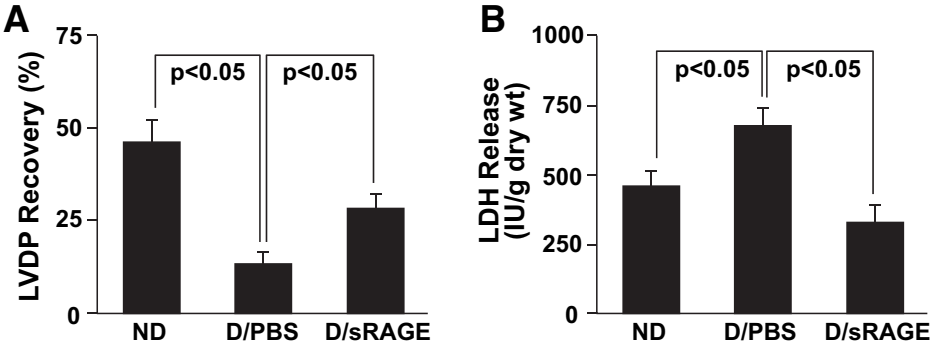


FIG. 3. Diabetes enhances cardiac dysfunction and injury in the rat heart: effect of RAGE. Type 1 BB/W diabetic rats received 500 μg/day sRAGE or PBS. Changes in cardiac function, LVDP (*A*) and release of LDH (*B*) in hearts subjected to I/R, are shown. n = 6 rats/group. D, diabetic; ND, nondiabetic.

TABLE 1
Measurement of AGEs in diabetic BALB/c mice hearts under normoxic perfusion conditions

	BALB/c	BALB/c-DM	BALB/c-DM + sRAGE
AGEs			
CML (μmol/mol lysine)	12.62 ± 3.39	18.49 ± 1.96*	14.52 ± 1.61
CEL	62.13 ± 9.87	71.46 ± 16.67	70.27 ± 10.91
Furosine (μmol/mol lysine)	53.51 ± 7.53	71.08 ± 14.01†	67.57 ± 8.63
Pentosidine (fmol/mg protein)	171.16 ± 27.97	361.40 ± 95.69‡	166.22 ± 16.25

Data are means ± SD. *$P < 0.05$ vs. BALB/c-DM + sRAGE and BALB/c for CML comparison. †$P < 0.05$ vs. BALB/c furosine comparison. ‡$P < 0.05$ vs. BALB/c-DM + sRAGE and BALB/c for pentosidine comparison.

were lower after I/R in RAGE-KO versus wild-type diabetic hearts (206 ± 66 vs. 616 ± 86 pmol/g tissue; $P < 0.05$).

Based on these findings in diabetic mice with global deletion of RAGE, we addressed the specific roles of RAGE signaling in endothelial cells or mononuclear phagocytes on I/R stress in the heart. As endothelial cells and mononuclear phagocytes were principal RAGE-expressing cells in the diabetic heart, we used two sets of Tg mice in which RAGE signal transduction was deficient based on introduction of a transgene encoding cytoplasmic DN RAGE in these cell types.

Tg mice expressing DN RAGE in endothelial cells. To dissect the role of RAGE signaling in endothelial cell in the diabetic heart, we generated Tg mice in which DN RAGE expression was directed to endothelial cells by the PPET (20). Immunostaining with anti-RAGE IgG revealed that RAGE expression in the Tg mouse heart was localized to endothelial cells and not to cardiomyocytes (Fig. 6*A*).

Mice were rendered diabetic with STZ. Hearts were excised after 12 weeks of hyperglycemia and subjected to the isolated perfused heart protocol. On induction of ischemia or I/R, levels of iNOS antigen in the heart were significantly higher in wild-type mice versus Tg DN PPET RAGE mice with diabetes ($P < 0.05$; Fig. 6*B* and *C*). In parallel, levels of total nitrite and nitrate were significantly lower in the diabetic Tg DN PPET RAGE mice hearts versus littermate hearts after I/R ($P < 0.05$; Fig. 6*D* and *E*, respectively).

To assess the impact of DN RAGE in endothelial cells in I/R injury in the diabetic heart, levels of LDH were measured in the effluent. LDH release was significantly less in the diabetic Tg DN PPET RAGE hearts versus wild-type hearts after I/R ($P < 0.05$; Fig. 6*F*). Levels of ATP in the heart were significantly higher in the diabetic Tg mice versus wild-type hearts after I/R (Fig. 6*G*). In contrast, LVDP recovery was not significantly different between the diabetic Tg DN PPET RAGE versus diabetic wild-type littermate hearts after I/R (LVDP recovery on reperfusion was 39 ± 14% in diabetic Tg DN PPET RAGE vs. 46 ± 11% in diabetic nontransgenic littermate hearts). These findings suggest that the impact of RAGE signaling in endothelial cells influences injury but not functional recovery.

Tg mice expressing DN RAGE in cells of mononuclear phagocyte lineage. To dissect the potential role of mononuclear phagocyte RAGE signaling in modulating the response to diabetes and I/R in the heart, we previously prepared Tg mice expressing DN RAGE selectively in cells of mononuclear phagocyte lineage using the scavenger receptor type A promoter (referred to as MSR DN RAGE)

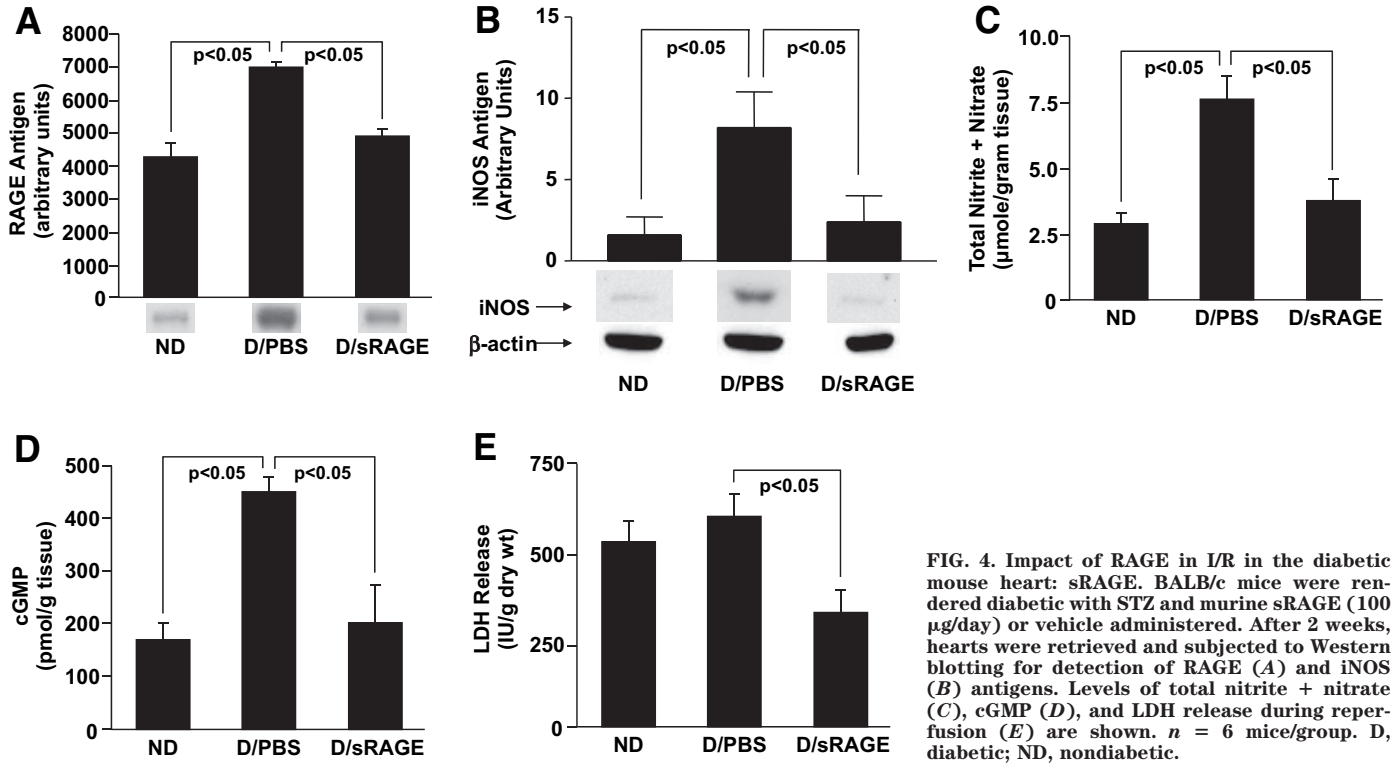


FIG. 4. Impact of RAGE in I/R in the diabetic mouse heart: sRAGE. BALB/c mice were rendered diabetic with STZ and murine sRAGE (100 μg/day) or vehicle administered. After 2 weeks, hearts were retrieved and subjected to Western blotting for detection of RAGE (*A*) and iNOS (*B*) antigens. Levels of total nitrite + nitrate (*C*), cGMP (*D*), and LDH release during reperfusion (*E*) are shown. *n* = 6 mice/group. D, diabetic; ND, nondiabetic.

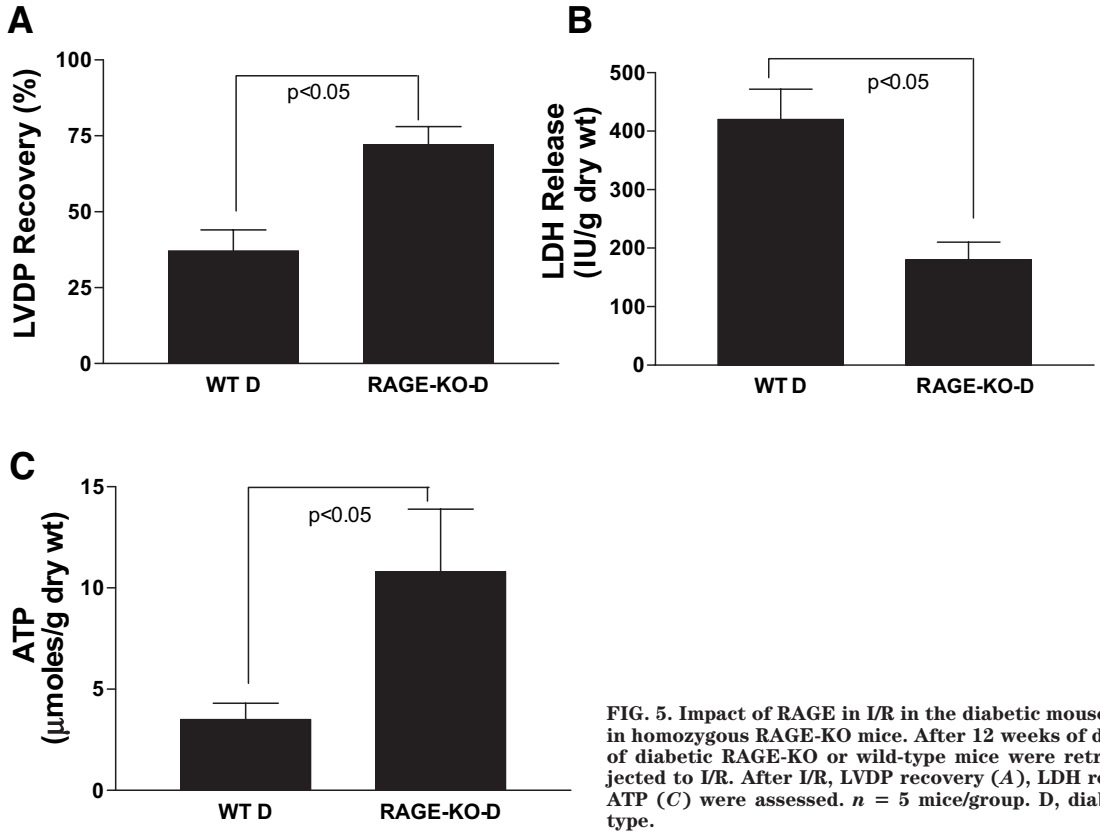


FIG. 5. Impact of RAGE in I/R in the diabetic mouse heart: studies in homozygous RAGE-KO mice. After 12 weeks of diabetes, hearts of diabetic RAGE-KO or wild-type mice were retrieved and subjected to I/R. After I/R, LVDP recovery (*A*), LDH release (*B*), and ATP (*C*) were assessed. *n* = 5 mice/group. D, diabetic; WT, wild type.

(22). DN RAGE was selectively expressed in mononuclear phagocyte, and there was no evidence that transgene expression was extended outside of monocytes/macrophages (22).

Consequent to ischemia, significantly higher iNOS antigen was expressed in the diabetic wild-type heart versus Tg DN MSR Tg DN MSR RAGE heart ($P < 0.05$; Fig. 7*A*). Similar findings were observed after I/R (Fig. 7*B*). In parallel, diabetic Tg DN MSR Tg DN MSR RAGE mice hearts displayed significantly decreased levels of total

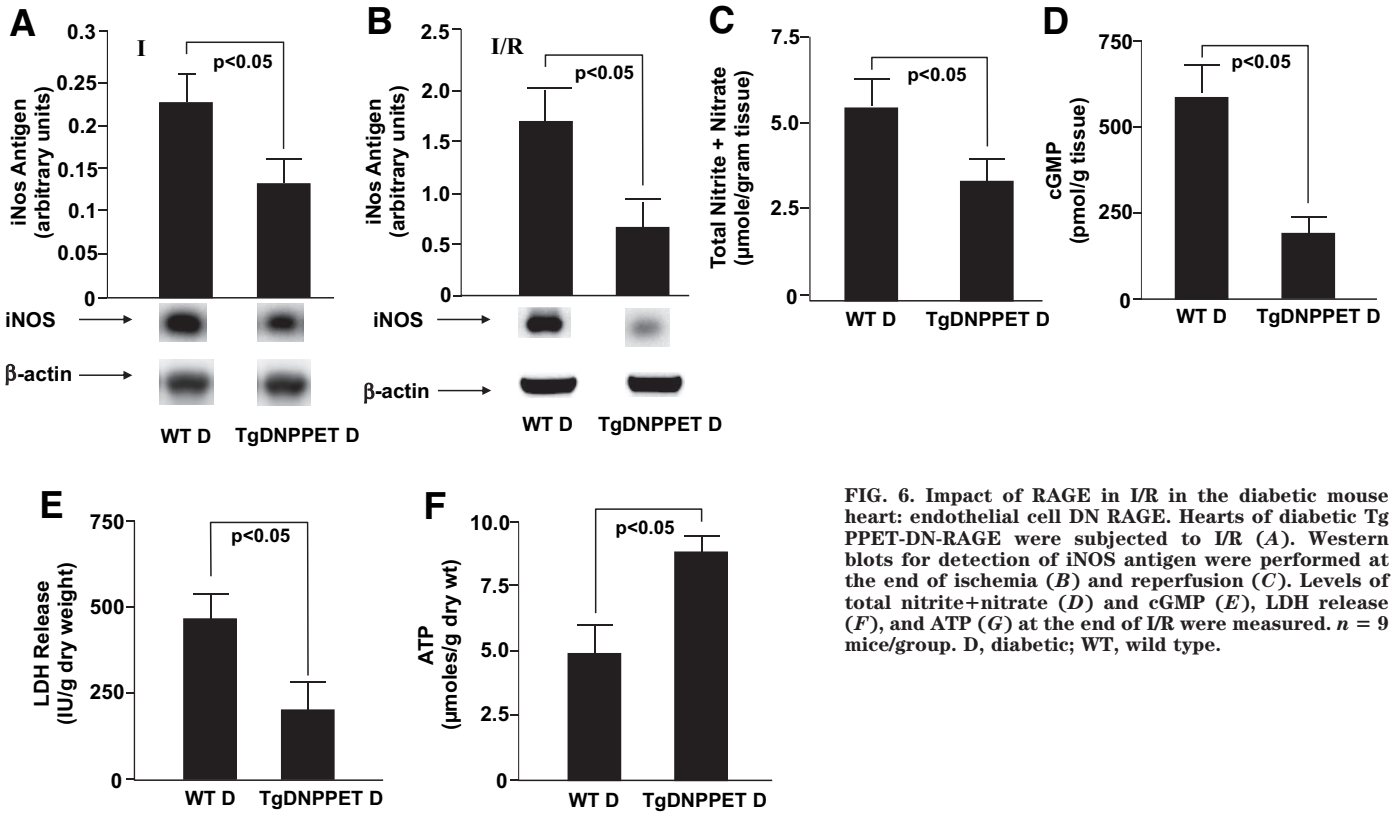


FIG. 6. Impact of RAGE in I/R in the diabetic mouse heart: endothelial cell DN RAGE. Hearts of diabetic Tg PPET-DN-RAGE were subjected to I/R (*A*). Western blots for detection of iNOS antigen were performed at the end of ischemia (*B*) and reperfusion (*C*). Levels of total nitrite+nitrate (*D*) and cGMP (*E*), LDH release (*F*), and ATP (*G*) at the end of I/R were measured. *n* = 9 mice/group. D, diabetic; WT, wild type.

TABLE 2
Measurement of AGEs in diabetic RAGE KO mice hearts under baseline normoxic and I/R conditions

		Wild-type-DM	RAGE-KO-D	Tg DN PPET-D	Tg DN MSR-D
CML	B	34.88 ± 4.46	21.73 ± 1.25*	31.92 ± 4.67	39.44 ± 7.87
	I/R	41.83 ± 5.48†	24.63 ± 3.44	22.73 ± 1.25	27.86 ± 4.88
Pentosidine	B	191.16 ± 27.97	137.67 ± 20.61‡	197.37 ± 38.02	209 ± 49.57
	I/R	190.65 ± 44.83	105.66 ± 20.55§	180.46 ± 34.71	116.52 ± 29.07§
Furosine	B	135.51 ± 7.79	98.07 ± 2.40¶	126.76 ± 9.31	121.58 ± 11.32
	I/R	123.30 ± 4.76	101.81 ± 8.05‖	107.87 ± 5.09‖	129.77 ± 9.94

Data are means ± SD. *$P < 0.05$ RAGE-KO-D vs. all other groups for CML comparison. †$P < 0.05$ wild-type-DM vs. all other groups for CML comparison. ‡$P < 0.05$ RAGE-KO-D vs. all other groups for pentosidine comparison. §$P < 0.05$ RAGE-KO-D, DNMSR vs. all other groups for pentosidine comparison. ¶$P < 0.05$ RAGE-KO-D vs. all other groups for furosine comparison. ‖$P < 0.05$ RAGE-KO-D, DNPPET vs. all other groups for furosine comparison. CML and furosine are expressed as μmol/mol lysine, whereas pentosidine is expressed as fmol/mg protein.

nitrate/nitrite and cGMP after I/R ($P < 0.05$; Fig. 7*C* and *D*, respectively).

We examined the impact of the DN RAGE transgene in mononuclear phagocyte on I/R injury in the diabetic heart. Significantly less LDH release was observed after I/R in the diabetic Tg DN MSR Tg DN MSR RAGE heart versus diabetic littermate hearts ($P < 0.05$; Fig. 7*E*). Furthermore, levels of ATP in the hearts of diabetic Tg mice were significantly higher than those observed in diabetic littermate hearts ($P < 0.05$; Fig. 7*F*). LVDP recovery on reperfusion was similar in both the diabetic Tg and littermate hearts (LVDP recovery on reperfusion was 47 ± 11% in diabetic Tg DN MSR RAGE vs. 41 ± 9% in diabetic nontransgenic littermate hearts).

AGEs in diabetic mice hearts subjected to I/R. We examined the levels of specific AGEs, CML, pentosidine, and furosine (glycated lysine) in diabetic wild-type, RAGE-KO, Tg DN PPET RAGE, and Tg DN MSR RAGE heart under baseline conditions and after I/R (Table 2). In the baseline condition, CML, furosine, and pentosidine levels were significantly lower in diabetic RAGE-KO versus diabetic wild-type hearts. In diabetic Tg DN PPET RAGE, and Tg DN MSR RAGE mice hearts, concentrations of CML, furosine, and pentosidine were similar to those in diabetic wild-type hearts (Table 2). CEL, argpyrimidine, and 2-aminoadipic acid were similar in all the groups of hearts studied here (data not shown).

Interestingly, after I/R, CML levels were significantly greater in diabetic WT hearts in comparison with diabetic RAGE-KO, Tg DN PPET RAGE, and Tg DN MSR RAGE hearts (Table 2). Furosine was significantly reduced in diabetic RAGE-KO and Tg DN PPET RAGE hearts versus diabetic wild-type or Tg DN MSR RAGE hearts after I/R. Pentosidine was significantly attenuated in diabetic RAGE-KO and Tg DN MSR RAGE versus diabetic wild-type or Tg DN PPET RAGE hearts after I/R. I/R increased CML by 20% in wild-type diabetic hearts but not in RAGE-KO D and Tg DN MSR D. These data indicate that CML, pentosidine, and furosine are significantly reduced in diabetic RAGE-KO hearts under all perfusion conditions, whereas in Tg DN PPET RAGE and Tg DN MSR RAGE hearts, only CML levels are attenuated under all perfusion conditions. Thus, these data indicate that suppressive effect of DN RAGE on pentosidine is cell specific, i.e., expression of DN RAGE reduced pentosidine in Tg DN MSR but not in Tg DN PPET mice hearts. Furthermore, data indicate that most of CML effects appear to be associated with the endothelial cells, whereas pentosidine effects are pronounced in the mononuclear phagocytes.

Effect of RAGE deletion on apoptosis in diabetic mice hearts after I/R. To determine whether the RAGE-ligand interaction mediates cell death in the diabetic heart consequent to I/R, we performed analyses to probe apoptotic signals, such as cytochrome c release and caspase-3 activation. Caspase-3 activation (Fig. 8*A*) was significantly reduced in diabetic RAGE RAGE-KO, Tg DN PPET RAGE, and Tg DN MSR RAGE hearts versus diabetic wild-type hearts after I/R ($P < 0.05$). Similarly, cytochrome c release (Fig. 8*B*) was also reduced in diabetic RAGE RAGE-KO, Tg DN PPET RAGE, and Tg DN MSR RAGE hearts versus diabetic wild-type hearts after I/R ($P < 0.05$).

Of note, pharmacological blockade of RAGE, by administration of sRAGE, or genetic deletion or modulation of RAGE signaling in endothelial cells or mononuclear phagocyte had no impact on the levels of glucose in diabetic rats or mice (Table 3).

DISCUSSION

RAGE and NOS. In diabetes, cells within the vascular milieu produce NO and superoxide, at least in part, via the direct action of glucose itself and by its downstream effector species, such as AGEs (30). The generation of a potent longer-lived oxidant peroxynitrite, due to interaction of NO and superoxide, has deleterious consequences, such as inhibition of mitochondrial electron transport, oxidation of sulfhydryl groups in proteins, initiation of lipid peroxidation, nitration of amino acids (31), and uncoupling of endothelial NO synthase by peroxynitrite leading to amplification of oxidant stress (32). Peroxynitrite induces formation of CML-modified adducts by cleavage of Amadori products and the generation of glucosone and glyoxal from glucose, thus providing a "refueling mechanism" by which diabetes-mediated generation of CML leads to oxidant stress and, in part, regeneration of CML epitopes (33).

It is likely that inhibition of the synthesis of NO by iNOS plays an important role. Our results are consistent with previous studies of acute ischemia and reperfusion in which administration of semi-selective inhibitors of iNOS or iNOS-null mice enhanced cardiac performance and/or reduced myocardial infarct size (34–37). Previous studies showed that iNOS played critical roles in diabetes and I/R in the heart, because iNOS-null mice displayed protection from the adverse effect of this injury, especially in diabetes (38). Our findings definitively link RAGE and RAGE signaling in endothelial cells and mononuclear phagocyte to generation of NO and pathogenic oxidant stress species in the diabetic heart. The impact of DN RAGE in both

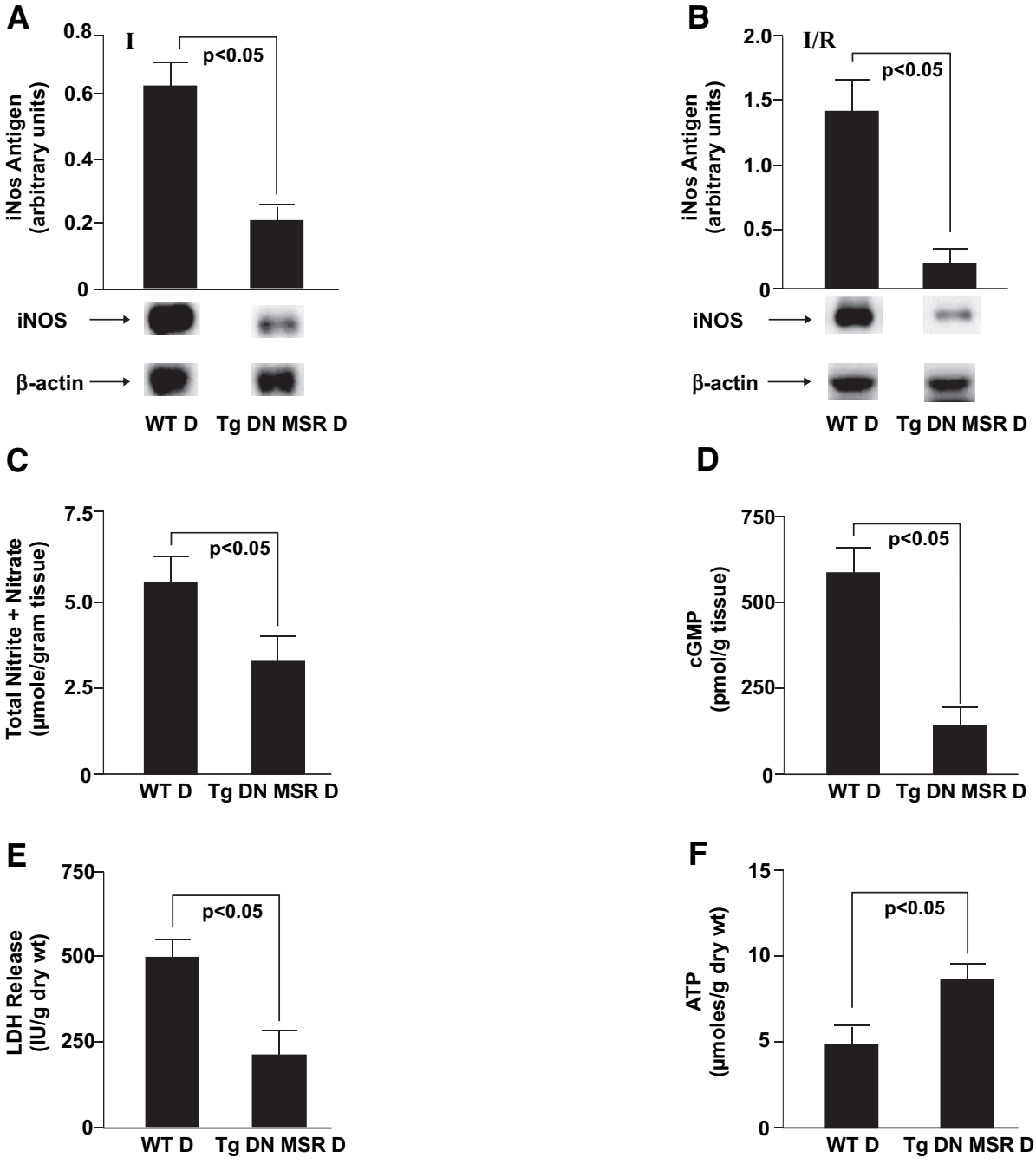


FIG. 7. Impact of RAGE in I/R in the diabetic mouse heart: mononuclear phagocyte DN RAGE. Tg mice expressing DN RAGE in mononuclear phagocytes were rendered diabetic with STZ. After 12 weeks, hearts were subjected to I/R. Western blots for detection of iNOS antigen were performed at the end of ischemia (*A*) or reperfusion (*B*). Levels of total nitrite+nitrate (*C*) and cGMP (*D*), LDH release (*E*), and ATP (*F*) were determined at the end of I/R. *n* = 9 mice/group. D, diabetic; WT, wild type.

endothelial cells and mononuclear phagocyte is likely explained, in part, by the fundamental interplay and cross-talk between the molecular and biochemical consequences of endothelial cells and mononuclear phagocyte activation.

RAGE and myocardial energy metabolism. Our studies indicate that RAGE influenced myocardial energy metabolism. At the end of global ischemia, levels of cardiac ATP were reduced significantly in all hearts; however, higher levels of ATP were observed in diabetic RAGE signaling mutant Tg mice or RAGE-KO mice compared with diabetic littermates. In global ischemia, ATP production results primarily from anaerobic glycolysis. Nitrosylation of GAPDH is a potential mechanism by which NO is likely to mediate inhibition of anaerobic glycolysis during global ischemia (39,40). Recent findings from our laboratory demonstrated reduced myocardial GAPDH activity and levels of glyceraldehyde-3-phosphate at the end of the ischemic period and normalization of these changes by inhibition of iNOS (41). Higher iNOS expression and NO production have been shown, in part, to impair glycolysis and to reduce ATP levels in ischemic hearts. Our data demonstrating reduced iNOS expression, total nitrite and nitrate levels, and improved ATP levels in RAGE-inhibited ischemic hearts are consistent, in part, with RAGE blockade improving anaerobic glycolysis.

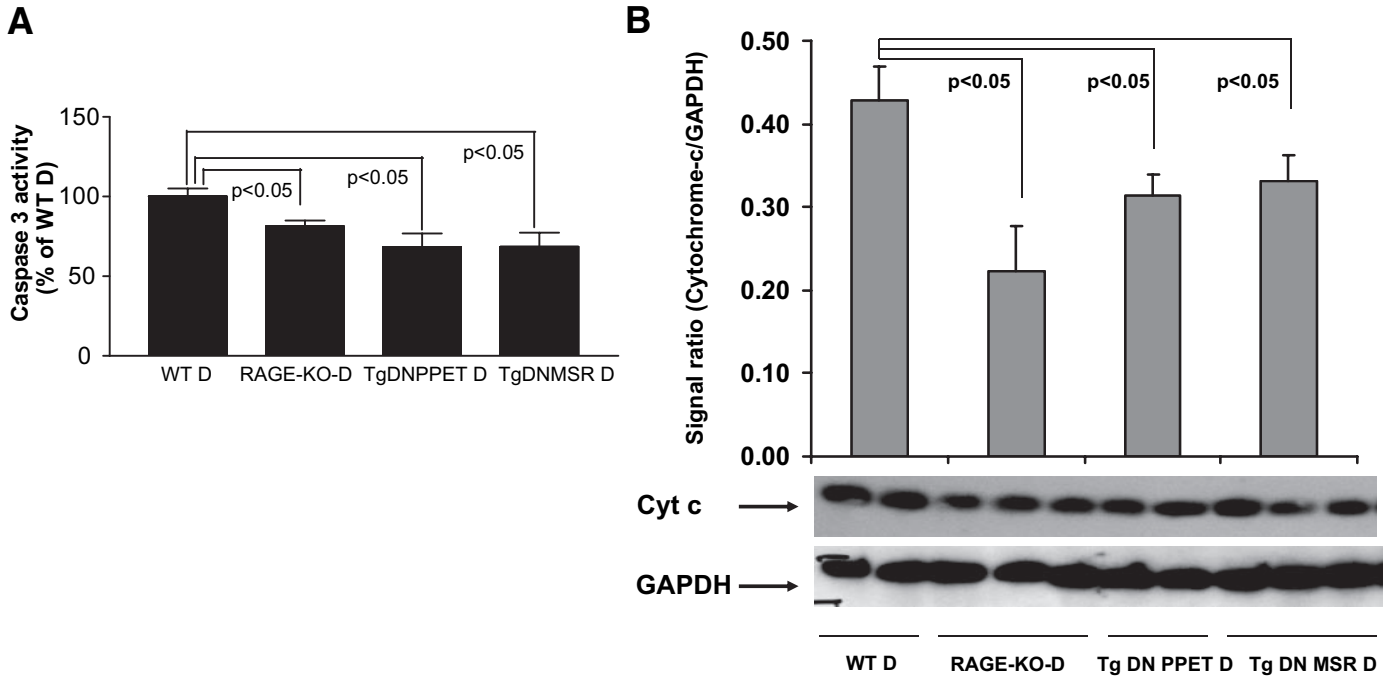


FIG. 8. Impact of RAGE on apoptosis in diabetic hearts after I/R. Diabetic wild-type, RAGE KO, Tg DN PPET RAGE, and Tg DN MSR RAGE mice were subjected to I/R, and the hearts were assessed for caspase-3 activity (*A*) and cytochrome c release (*B*) at reperfusion. For cytochrome c (Cyt c) release, hearts were retrieved at reperfusion and subjected to Western blotting for detection of cytochrome c; blots were then stripped and re-probed with antibody for detection of GAPDH. Densitometry was performed and relative units reported from *n* = at least 5 mice per group. D, diabetic; WT, wild type.

RAGE and cardiac ischemic injury. Our findings stress the impact of RAGE in generation of iNOS and nitrite and nitrate and cGMP, especially consequent to I/R. In rats rendered diabetic with STZ, the impact of diabetes and NOS in the heart was demonstrated by the beneficial effects of L-NAME (L-N^G-nitro-L-arginine methyl ester) in improvement of ventricular performance (42). Our findings extend these concepts to link RAGE in the diabetic rat heart and RAGE signaling in either mononuclear phagocyte or endothelial cells in the diabetic mouse heart to iNOS expression and enhanced injury in I/R, as measured by LDH release. The finding that levels of ATP are restored in diabetic RAGE-KO mice or Tg mice expressing signaling-deficient mutant RAGE mice in I/R provides a direct link between oxidant stress, NOS, and energy metabolism.

TABLE 3
Glucose levels

	Serum glucose (mg/dl)
Type 1 BB/W rats	
Control/(−) diabetes	70.3 ± 10.5
Diabetes/PBS	353.4 ± 53.6*
Diabetes/sRAGE	365.0 ± 87.2*
BALB/c mice	
Control/(−) diabetes	74.9 ± 11.9
Diabetes/PBS	312.8 ± 40.2*
Diabetes/sRAGE	306.3 ± 26.8*
Homozygous RAGE-KO mice and littermate controls	
Wild-type (−) diabetes	72 ± 10.2
Wild-type (+) diabetes	437 ± 56*
RAGE-KO (−) diabetes	86 ± 9.4
RAGE-KO (+) diabetes	388 ± 99*
Transgenic PPET DN RAGE mice	
Wild-type (−) diabetes	66.9 ± 8.5
Wild-type (+) diabetes	300.2 ± 13.3*
Tg DN PPET RAGE (−) diabetes	79.1 ± 5.6
Tg DN PPET RAGE (+) diabetes	318.4 ± 44.6*
Transgenic MSR DN RAGE mice	
Wild-type (−) diabetes	69.4 ± 7.4
Wild-type (+) diabetes	309.7 ± 18.7*
Tg DN MSR RAGE (−) diabetes	73.7 ± 9.4
Tg DN MSR RAGE (+) diabetes	331.2 ± 45.6*

Data are means ± SD. Glucose levels were determined in BB/W rats, BALB/c, homozygous RAGE-KO, and DN RAGE transgenic mice at the time of death. *$P < 0.05$ vs. comparing diabetes with the absence of diabetes in respective controls.

Our studies in diabetic rats definitively showed that pharmacological blockade of RAGE protected the isolated perfused heart from damage and dysfunction, the latter measured by LVDP. In parallel, release of LDH was attenuated by RAGE blockade. In mice, pharmacological blockade of RAGE or genetic manipulation (RAGE-KO) attenuated the release of LDH and improved LVDP and ATP recovery. Although RAGE signaling significantly affected I/R injury and iNOS expression in diabetes, there was no effect on functional recovery in DN RAGE Tg mice. However, studies in mice globally deficient in RAGE displayed improvement in functional recovery consequent to I/R. These findings suggest a number of possibilities. First, it is plausible that distinct cell types expressing basal levels of RAGE, such as cardiomyocytes or T lymphocytes, may contribute to the findings on functional recovery observed in RAGE-KO mice. Second, the interplay between inflammatory cell and endothelial RAGE signaling may be a critical component of the biochemical and molecular events that, taken together, augur impairment in functional recovery in the I/R diabetic heart. Thus, future studies must address distinct signal transduction mutants of RAGE to broadly probe the potential effects of RAGE signaling in alternative cell types.

These studies support that the ligands of RAGE, upregu-

lated in the diabetic heart, play important roles in transducing the long-term impact of hyperglycemia. Certainly, basally increased levels of AGEs in the hyperglycemic heart may set the stage for amplification of injury, via RAGE, on superimposed I/R. Distinct RAGE ligands, such as S100/calgranulins may also play important roles in the I/R heart. In the isolated perfused heart, ischemic rat hearts release S100b (43). Studies have linked S100b to maladaptive remodeling after experimental infarction in mouse models (44). Our recent studies, performed in the absence of diabetes, demonstrated that a primary stimulus to release and recruitment of the RAGE axis ensues primarily from the ischemic insult (45). Ischemic insult induces increases in AGE-RAGE expression and leads to increased injury (45).

In this study, we show that diabetes increases AGEs such as CML, pentosidine, and furosine under baseline and I/R conditions. Levels of CML, pentosidine, and furosine were significantly reduced in diabetic RAGE KO mice hearts. Targeted expression of DN RAGE in endothelial cells or mononuclear phagocytes attenuated CML accumulation in these diabetic hearts under all perfusion conditions, whereas pentosidine was attenuated in diabetic Tg DN MSR RAGE after I/R and furosine was attenuated in diabetic Tg DN PEET RAGE hearts after I/R. These data indicate that CML is a key AGE that is linked to RAGE-dependent injury after I/R in diabetic hearts. Taken together, our findings highlight potential roles for the RAGE axis at multiple stages of injury in the diabetic, ischemic heart.

We observed that in diabetic RAGE KO, Tg DN PPET RAGE, and Tg DN MSR RAGE mice hearts, protection from injury due to I/R was also associated with reductions in caspase-3 activation and cytochrome c release. These experiments are consistent with our and other earlier studies that have linked protection of ischemic hearts to reduced apoptosis in mice models of I/R including in the setting of diabetes (46–50)

In conclusion, these studies definitively link RAGE to myocardial ischemic injury and dysfunction in the diabetic heart. We propose that blockade of RAGE may provide a novel means to exert cardioprotection in the vulnerable diabetic heart, particularly consequent to I/R injury.

ACKNOWLEDGMENTS

A.M.S. is a recipient of a Juvenile Diabetes Research Foundation Scholar Award. R.R. is an Established Investigator of the American Heart Association. This work was supported by grants from the U.S. Public Health Service (HL61783, HL68954, HL60901, and AG18436), the Juvenile Diabetes Research Foundation International, and the LeDucq Foundation.

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