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. 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 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.
RAGE, additional antibodies were used: anti-Mac 3 IgG (Pharmingen, San 
IgG (16), anti-AGE IgG (16), anti-S100/calgranulin IgG (12), and anti-nitroty-
for immunohistochemistry. The following antibodies were used: anti-RAGE 
Immunohistochemistry.

KCl, 2.5 mmol/l CaCl2, 1.2 mmol/l MgCl2, 25 mmol/l NaHCO3, 5 mmol/l glucose,
with modified Krebs-Henseleit buffer containing 118 mmol/l NaCl, 4.7 mmol/l
placed in iced saline, and retrogradely perfused at 37°C in a nonrecirculating
respectively). After deep anesthesia was achieved, hearts were rapidly excised,
mastectomy as indicated, for mice hearts (23,24). Mice and rats were anesthe-
isolated perfused heart and measurement of cardiac function. 

RAGE expression was increased in the type 1 diabetic 

In nondiabetic hearts, CML AGEs were not detected 

(14,15) and explored the expression/distribution of RAGE 

Ligand/RAGE axis in the diabetic rat heart.

RESULTS 


cance was ascribed to the data when 

were performed using Tukey’s or Dunnett’s procedures as indicated. SAS 

Statistical analysis. 


cells and cardiomyocytes.

Diabetic BB/W hearts displayed increased RAGE anti-
gen compared with nondiabetic hearts by immunohisto-
chemistry (Fig. 1F and E, respectively) and Western 

LDH release, a marker of I/R injury, was measured in the effluents for the 

entire 60 min 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-nitroani-
line. 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 chloride-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 
diethylenetriaminepentacetic acid (DTPA) and protease inhibitor cocktail 
(Roche) using one tablet per 10 ml buffer. Homogenates were centrifuged at 
1,600 rpm for 20 min with 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 
processes. Milligram-illl of lyophilized tissue was weighted 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-
lysinine [CML], carboxyethyl-lysine [CEL], fructose-lysine [furosine], and 2-amid-
noacidic 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 signifi-
cance 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 autoim-
mune 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. 1A). In contrast, heart tissue retrieved from hyper-
glycemic rats displayed immunoreactive epitopes for CML 
AGE (Fig. 1B). In addition to AGEs, although expression of 
RAGE ligand S100/calgranulin (12) was not demon-
strated in the hearts of nondiabetic BB/W rats (Fig. 1C), 
S100/calgranulin epitopes were evident in diabetic 
BB/W rat heart (Fig. 1D), in both vascular and 
cardiomyocytes.

Diabetic BB/W hearts displayed increased RAGE anti-
gen compared with nondiabetic hearts by immunohisto-
chemistry (Fig. 1F and E, respectively) and Western 
blotting (P < 0.05; Fig. 1M). We examined the hearts to 

LDH and GSH were measured in parallel from an 

injury, coronary perfusion pressure were 

vessel. The levels of LDH were assayed 

this with 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
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. 2A).

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. 2B 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. 3A). LDH release was significantly higher in diabetic hearts compared with nondiabetic hearts ($P < 0.05$; Fig. 3B). Treat-
ment for 14 days with sRAGE resulted in improvement in LVDP recovery and significantly lower LDH release (P < 0.05; Fig. 3A 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 mouse 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 argipyrimidine, CEL, and 2-aminoacidic 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. 4A). 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. 4B). In the presence of sRAGE, levels of iNOS were significantly lower (P < 0.05; Fig. 4B). 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. 4C 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. 4E).

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. 5A). 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. 5B). Levels of ATP in the heart were significantly higher in the diabetic RAGE-KO hearts versus diabetic controls (P < 0.05; Fig. 5C).

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 μmole/g tissue; P < 0.05). Furthermore, levels of cGMP in the diabetic heart
were lower after I/R in RAGE-KO versus wild-type diabetic hearts (206 ± 66 vs. 616 ± 86 pmol/g tissue; *P < 0.05).

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. 6F). Levels of ATP in the heart were significantly higher in the diabetic Tg mice versus wild-type hearts after I/R (Fig. 6G). 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).

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

| AGEs                        | BALB/c       | BALB/c-DM    | BALB/c-DM + sRAGE |
|-----------------------------|--------------|--------------|-------------------|
| CML (µmol/mol lysine)       | 12.62 ± 3.39 | 18.49 ± 1.96*| 14.52 ± 1.61      |
| CEL (µmol/mol lysine)       | 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.
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. 7A). Similar findings were observed after I/R (Fig. 7B). In parallel, diabetic Tg DN MSR Tg DN MSR RAGE mice hearts displayed significantly decreased levels of total nitrite/nitrate (Fig. 7D) and cGMP (Fig. 7E), LDH release (Fig. 7F), and ATP (Fig. 7G) at the end of I/R were measured. $n = 9$ mice/group. D, diabetic; WT, wild type.

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.

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.
nitrate/nitrite and cGMP after I/R ($P < 0.05$; Fig. 7C 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. 7E). 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. 7F). 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. 8A) was significantly reduced in diabetic 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. 8B) was also reduced in diabetic 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 sulphhydryl groups in proteins, initiation of lipid peroxidation, nitrization 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 glyxal 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

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**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   | 34.88 ± 4.46 | 21.73 ± 1.25 | 31.92 ± 4.67 | 39.44 ± 7.87 |
| Pentosidine | 41.83 ± 5.48 | 24.63 ± 3.44 | 22.73 ± 1.25 | 27.86 ± 4.88 |
| Furosine | 191.16 ± 27.97 | 137.67 ± 20.61 | 197.37 ± 38.02 | 209 ± 49.57 |
| Furosine | 190.65 ± 44.83 | 105.66 ± 20.55 | 180.46 ± 34.71 | 116.52 ± 29.07 |
| Furosine | 135.51 ± 7.79 | 98.07 ± 2.40 | 126.76 ± 9.31 | 121.58 ± 11.32 |
| Furosine | 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 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.
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. 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.
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-Nω-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.

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 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 myocardic 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, and the Le-Ducq Foundation.

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