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## **Glycation, Inflammation, and RAGE: A Scaffold for the Macrovascular Complications of Diabetes and Beyond**

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This Review is part of a thematic series on **Cardiovascular Role of Sugar Modifications**, which includes the following articles:

Glycation, Inflammation, and RAGE: A Scaffold for the Macrovascular Complications of Diabetes and Beyond

Protein Glycation and Endothelial Dysfunction

David A. Kass, Editor

## Glycation, Inflammation, and RAGE

### A Scaffold for the Macrovascular Complications of Diabetes and Beyond

Shi Fang Yan, Ravichandran Ramasamy, Yoshifumi Naka, Ann Marie Schmidt

**Abstract**—The cardiovascular complications of diabetes represent the leading cause of morbidity and mortality in affected subjects. The impact of hyperglycemia may be both direct and indirect: indirect consequences of elevated blood glucose lead to generation of advanced glycation endproducts, the products of nonenzymatic glycation/oxidation of proteins/lipids that accumulate in the vessel wall, and are signal transduction ligands for Receptor for AGE (RAGE). Although enhanced in diabetes, AGE accumulation also occurs in euglycemia and aging, albeit to lower degrees, driven by oxidant stress and inflammation. In hyperglycemia, production of 3-deoxyglucosone, at least in part via the polyol pathway, provides an amplification loop to sustain AGE generation, oxidant stress, and vascular activation. Furthermore, recruitment of inflammatory cells bearing S100/calgranulins, also ligands for RAGE, augments vascular dysfunction. We hypothesize that activation of RAGE is a final common pathway that transduces signals from these diverse biochemical and molecular species, leading to cardiovascular perturbation. Ultimately, these pathways synergize to construct a scaffold on which the complications of diabetes in the vasculature and heart may be built. We propose that antagonism of RAGE will provide a unique means to dismantle this scaffold and, thereby, suppress initiation/progression of vascular disease and cardiac dysfunction that accompany diabetes and aging. (*Circ Res.* 2003;93:1159-1169.)

**Key Words:** receptors ■ glycoxidation ■ hyperglycemia ■ polyol pathway ■ vascular disease

#### The Problem of Diabetic Vascular Complications: The Blood Vessel Never Forgets

Diabetes is associated with aggressive vascular dysfunction in human subjects; atherosclerosis represents the leading cause of morbidity and mortality in diabetic subjects.<sup>1</sup> Chronic perturbation of the vasculature, such as that caused by diabetes, leads to increased incidence, size, and complexity of atherosclerotic plaques. Furthermore, molecular mechanisms associated with lesion instability are enhanced in diabetes, and mediate increased incidence and severity of clinical events, such as heart attacks and strokes.<sup>2-6</sup> It is increasingly postulated that events portending accelerated

atherosclerosis in subjects with diabetes, especially hyperglycemia mediated by insulin resistance, are underway well before the formal diagnosis of diabetes.<sup>7</sup> In 1993, the Diabetes Control and Complications Trial (DCCT) Research Group did not report that intensive treatment of hyperglycemia was sufficient to significantly reduce excess risk for macrovascular disease. A recently published pivotal study, however, definitively showed decreased progression of intima-media thickness 6 years after the end of the trial among patients who received intensive therapy during the DCCT.<sup>8,9</sup> These considerations support the concept that elevated levels of glucose impart long-term “memory” in the vessel wall that augments vascular perturbation. Is it possible that DCCT subjects managed with strict glucose control displayed diminished

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vascular production and accumulation of AGEs, and that this phenomenon contributed, at least in part, to diminished atherosclerosis years later? These considerations have led us to hypothesize that accumulation of advanced glycation endproducts (AGEs) and S100/calgranulins, and their interaction with receptor for AGE (RAGE), provides a plausible mechanism for induction of vessel wall memory and sustained perturbation, processes that if left unchecked, lead to progression of atherosclerosis, plaque instability, and the emergence of clinical events. In this review, we will focus on the role of the ligand-RAGE axis in vascular dysfunction.

### **RAGE: A Multiligand Receptor**

RAGE is a multiligand member of the immunoglobulin superfamily of cell surface molecules.<sup>10,11</sup> The extracellular domain of the receptor, consisting of one V-type immunoglobulin domain followed by two C-type immunoglobulin domains, is the site of ligand engagement, specifically within the V-type domain.<sup>11–13</sup> RAGE interacts with a diverse class of ligands, including advanced glycation endproducts (AGEs), S100/calgranulins, amphoterin, and amyloid- $\beta$  peptide (and the class of  $\beta$ -sheet fibrils). In cardiovascular disease and atherosclerosis, data suggest roles for AGEs and proinflammatory ligands in lesion initiation and progression.

### **Advanced Glycation Endproducts (AGEs)**

RAGE was first identified as a signal transduction receptor for AGEs. AGEs, the products of nonenzymatic glycation and oxidation of proteins and lipids, accumulate in the vessel wall especially in diabetes, and in euglycemia as well; in the latter case, driven by oxidant stress.<sup>14,15</sup> These structures accumulate in the vasculature, thus highlighting the likelihood that AGEs may participate in the vascular memory of diabetes. In addition to hyperglycemia and oxidant stress, AGEs, an heterogeneous class of species, may form in multiple milieux, such as inflammation and renal failure; in settings beyond the vasculature, AGEs have been reported to accumulate in neurodegenerative disorders, such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS).<sup>16–21</sup> Although there are a wide range of AGE-related chemical structures likely to be present in the vasculature and other tissues, specific AGEs commonly found in diabetic tissues include carboxymethyl-lysine (CML)-protein adducts (the predominant AGEs present *in vivo*<sup>22–24</sup>), carboxyethyl-lysine (CEL)-protein adducts, pentosidine-adducts (a major AGE crosslink found in diabetic tissues linked to destabilization of collagen and basement membranes<sup>25–28</sup>), pyrallines, imidazolones, methylglyoxal (a precursor to formation of a range of other AGEs), and crosslines.<sup>29–34</sup>

The effects of AGEs on vascular memory are likely to be diverse. AGEs may exert their biologic effects by receptor-independent or receptor-dependent pathways. By receptor-independent means, AGEs may directly impact on the structural integrity of the vessel wall and underlying basement membrane. In particular, excessive cross-linking of matrix molecules such as collagen may disrupt matrix-matrix and matrix-cell interactions.<sup>35,36</sup> Inside the cell, nonenzymatic glycation of intracellular molecules such as basic fibroblast growth factor may impair its function.<sup>37</sup> In addition, other

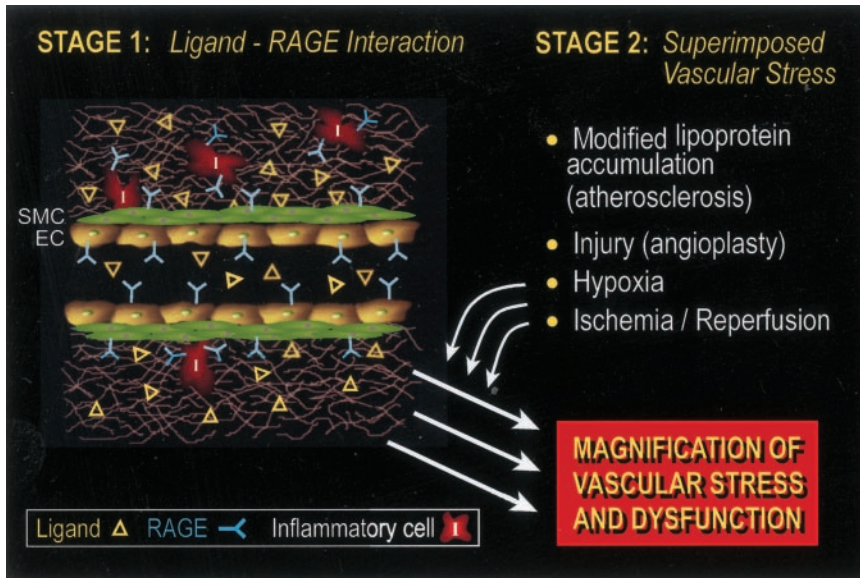
studies have shown that AGEs quench nitric oxide, thereby potentially impacting on vascular relaxation and function.<sup>38</sup> The impaired ability of diabetic vasculature to respond appropriately to stimuli such as acetylcholine both in human subjects and experimental models suggests that such endothelial dysfunction may provide a window into the extent of vascular disease and atherosclerosis.<sup>38–42</sup>

AGEs may also exert their pathogenic effects by engagement of cellular binding sites/receptors. To date, a number of cell surface interaction sites for AGEs have been identified, such as macrophage scavenger receptor (MSR) type II, OST-48, 80K-H, galectin-3, CD36, and RAGE.<sup>43–46</sup> These receptors have been ascribed a range of functions in the diabetic tissues, including removal and detoxification of AGEs, as well as modulation of cellular properties by receptor-triggered signal transduction on AGE engagement. RAGE does not appear to contribute to removal/detoxification of AGEs. Rather, RAGE is a signal transduction receptor for AGEs. RAGE mediates the effects of CML-adducts, the most prevalent AGE identified thus far *in vivo*, via signal transduction.<sup>12</sup> Both *in vitro* and *in vivo*, physiologically relevant concentrations of CML-adducts activate endothelial cells (ECs), vascular smooth muscle cells (SMCs), and mononuclear phagocytes (MPs); these events cause expression of a range of proinflammatory molecules and activation of nuclear factor (NF)- $\kappa$ B.<sup>12</sup> Introduction of a RAGE transgene in which the cytosolic domain was deleted into wild-type RAGE-bearing cells imparted a dominant-negative (DN) effect. However, although transfected RAGE was firmly embedded in the cell membrane and was capable of binding ligand, CML-adduct engagement failed to stimulate signaling pathways or trigger increased expression of proinflammatory molecules.<sup>12</sup> It is highly likely that AGEs beyond CML-modified species interact with RAGE; such studies are the focus of investigation.

An emerging view in diabetes complications is that mitochondrial-derived reactive oxygen species, generated by excess concentrations of glucose, make an important contribution to the pathogenesis of diabetic complications.<sup>47,48</sup> We propose that one such consequence of hyperglycemia, AGE interaction with RAGE, is a key component initiating and/or accelerating macrovascular complications. Because AGEs may form by oxidant stress and inflammatory pathways, their impact is likely to extend to euglycemic vascular disease.

### **S100/Calgranulins**

In addition to AGEs, RAGE is a signal transduction receptor for S100/calgranulins, a family of multiple members, which have important intracellular properties, where their roles are linked to homeostatic properties, such as calcium binding.<sup>49–51</sup> These molecules, such as S100A12 and S100B, have been shown to activate ECs, MPs, SMCs, and peripheral blood mononuclear cells (PBMCs), including T cells via RAGE, thus triggering activation of signaling cascades and generation of cytokines and proinflammatory adhesion molecules.<sup>13,52,53</sup> Consistent with a role for RAGE in amplification of inflammation pathways, at least in part via interaction with S100/calgranulins, blockade of RAGE in euglycemic mice suppressed the challenge phase of delayed type hyper-



**Figure 1.** Ligand/RAGE interaction: a scaffold for the development of diabetic complications and beyond. Glycation, oxidant stress, and inflammatory mechanisms synergize in the vessel wall, particularly in diabetes, to generate a scaffold to amplify the impact of superimposed stresses, such as modified lipoprotein accumulation, physical injury, hypoxia, or ischemia/reperfusion. Once set in motion, these RAGE-dependent processes lead to sustained vascular perturbation and failure of reparative mechanisms.

sensitivity in response to methylated BSA, diminished colonic inflammation in mice deficient in interleukin (IL)-10, decreased phenotypic and molecular indices of arthritis in DBA/1 mice subjected to sensitization/challenge with bovine type II collagen, and suppressed inflammatory cell infiltration and spinal cord damage in a murine model of experimental autoimmune encephalomyelitis.<sup>13,52,53</sup>

Much remains to be learned about the precise biochemical and molecular signals that regulate transcription/translation of S100s. An emerging body of evidence, however, suggests that these molecules may be released by activated cells, such as monocytes.<sup>54,55</sup> Based on these considerations, it is, thus, our hypothesis that the biological impact of these molecules may be highly relevant in atherosclerosis. Indeed, in our studies, we have used pathophysiologically relevant concentrations of S100/calgranulins,<sup>55</sup> thus supporting the premise that interaction of these species with RAGE is a plausible mechanism amplifying vascular inflammation and tissue injury in the vascular wall.

### Amphoterin

Amphoterin is also a signal transduction ligand of RAGE. Amphoterin is a member of the HMG (high mobility group)-1 family of DNA binding proteins that, in addition to functions within the cell, also may exist extracellularly and on the surface of cells, especially migrating cells in neuronal development and tumors.<sup>56,57</sup> Engagement of RAGE on the surface of embryonic neurons is one axis linked to their ability to migrate within the developing nervous system, because, at least in vitro, blockade of RAGE, using either soluble(s) RAGE, the extracellular ligand binding domain of the receptor, or blocking F(ab')<sub>2</sub> fragments of anti-RAGE IgG, suppressed neurite outgrowth selectively on amphoterin, but not poly-L-lysine-coated matrices.<sup>58</sup> In addition, amphoterin is also expressed on the surface of transformed cells, thereby implying its potential role in tumor cell migration.<sup>59</sup> Engagement of tumor cell RAGE by amphoterin enhances cellular migration, invasion, proliferation, and generation of matrix

metalloproteinases; processes linked, at least in part, to local tumor growth and distant invasion.<sup>59</sup>

Recent observations have expanded the potential biological roles of amphoterin. Like S100/calgranulins, amphoterin may be released from activated MPs, thereby leading to propagation of inflammatory responses.<sup>60,61</sup> In vivo, administration of blocking antibodies to amphoterin led to enhanced survival in rodents subjected to conditions mimicking that of overwhelming septic shock.<sup>60</sup> Recent observations have suggested important roles for amphoterin in animal models of arthritis.<sup>62</sup>

A resounding theme in our studies is that the ligands of RAGE are involved in the inflammatory response. In the vasculature, especially that affected by atherosclerosis, AGEs, generated by the consequences of hyperglycemia and oxidant stress, recruit a second round of invading species such as S100/calgranulins and amphoterin, transported into sites of vascular injury by inflammatory cells such as MP and T cells.

These considerations form the basis of our work on RAGE in the vasculature. We hypothesize that in diabetic tissues, smoldering interaction of accumulating AGEs, S100/calgranulins and amphoterin interacting with multiple RAGE-bearing cell types linked to atherosclerosis, such as ECs, SMCs, and MPs, alters the vascular landscape and provides a scaffold for augmentation of superimposed vascular stresses. Specifically, on addition of stresses such as accumulation of modified lipoproteins within the vessel wall, arterial injury, hypoxia, and ischemia/reperfusion, we propose that ligand-RAGE interaction sustains the host-response eventuating in chronic injury (Figure 1). Efforts to test the concept that blockade of these pathways may interrupt vascular perturbation and restore homeostasis within the vessel wall in diabetes and euglycemia are being tested.

Additional ligands for RAGE, specifically amyloid- $\beta$  peptide and  $\beta$ -sheet fibrils interact with the receptor and have implications for the pathogenesis of chronic degenerative diseases such as Alzheimer's disease and amyloidoses.<sup>63,64</sup>



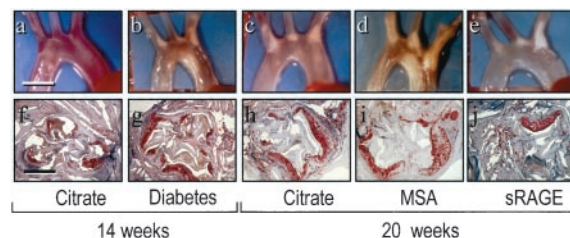
## Recruitment of RAGE and Activation of Diverse Signaling Pathways

AGEs, S100/calgranulins, and amphotericin may activate a range of cells with important links to atherosclerosis initiation and progression, such as ECs, MPs, and SMCs; a key consequence of ligand engagement of RAGE is activation of multiple signaling pathways, including p21ras, erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, rho GTPases, phosphoinositol-3 kinase and the JAK/STAT pathway, and downstream consequences such as activation of NF- $\kappa$ B and CREB.<sup>12,13,65–71</sup> Ligand-engagement of RAGE triggers generation of reactive oxygen species (ROS) linked to triggering of cell signaling via RAGE, at least in part via activation of NADPH oxidase. Monocytes retrieved from NADPH oxidase (0) mice, when compared with wild-type monocytes, failed to display increased generation of tissue factor on incubation with AGEs.<sup>72</sup>

RAGE-dependent signaling depends on the specific cell type and the state of activation/stress. It is for these reasons that blockade of specific downstream signaling pathways in atherosclerosis and vascular stress may not be a fruitful approach. Given the diversity of RAGE-dependent signal transduction pathways recruited on response to ligand, a more effective approach is likely to involve blockade at the level of the receptor itself. In the sections to follow, we will present evidence suggesting the role of RAGE in vascular stresses and insights into the impact of blockade of RAGE signaling in vascular disease.

## RAGE and Atherosclerosis

The role of the ligand-RAGE axis in atherosclerosis was first tested in a murine model of accelerated atherosclerosis in mice. Mice are inherently resistant to the development of complications as their lipid profile, high in functional high-density lipoprotein (HDL), largely protects them from atherogenesis. Thus, to test the role of RAGE in atherosclerosis, we examined the impact of RAGE in mice susceptible to the development of atherosclerosis due to genetic modification of lipid profile. First, we used apoE null mice as these mice develop spontaneous hypercholesterolemia on a normal chow diet.<sup>73,74</sup> ApoE null mice were rendered diabetic with streptozotocin and maintained on a normal rodent chow. Induction of diabetes was associated with a significant increase in mean atherosclerotic lesion area at the aortic sinus after 6 weeks of diabetes compared with euglycemic apoE null mice of the same age.<sup>75</sup> Diabetes-associated atherosclerotic lesions displayed increased accumulation of AGEs and S100/calgranulins and enhanced expression of RAGE.<sup>76</sup> In addition to lesion area, lesion complexity was accelerated. Complex lesions, defined as cholesterol clefts, necrosis, or fibrous caps were much more commonly observed in diabetic mice at age 14 weeks versus controls.<sup>76</sup> To test the impact of RAGE, diabetic apoE null mice were treated once daily with murine soluble RAGE immediately on documentation of diabetes.<sup>75</sup> Administration of sRAGE suppressed accelerated lesion area and complexity in a dose-dependent manner.<sup>75</sup> In parallel, levels of tissue factor, VCAM-1, AGEs/S100/calgranulins, and nuclear translocation of NF- $\kappa$ B were decreased in the aortae of sRAGE-treated mice compared with vehicle-treated



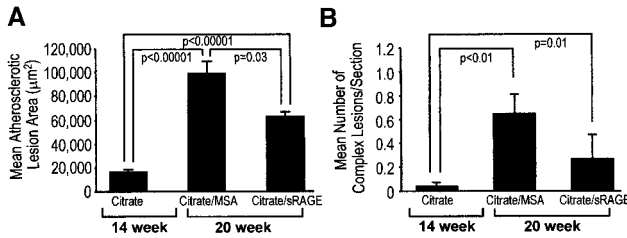
**Figure 2.** RAGE blockade halts the progression of established atherosclerosis in apoE null mice: impact in diabetes. ApoE null mice were rendered hyperglycemic using streptozotocin or were treated with citrate buffer vehicle at age 6 weeks. At age 14 weeks, mice were treated with sRAGE, 100  $\mu$ g per day, or murine serum albumin (MSA). Mice were euthanized at age 20 weeks and the aortae dissected and photographed (a through e). In other studies, sections at the aortic root were prepared and stained with Oil Red O (f through j). Scale bar, a through e=0.3 cm; f through j=125  $\mu$ m.

diabetic animals.<sup>75,76</sup> Blockade of RAGE did not affect lipid or glycemic profile. Thus, these findings defined ligand-RAGE interaction as a pathway important in the development of accelerated atherosclerosis in diabetes.

These findings were not limited to apoE null mice rendered diabetic with streptozotocin, as similar results were observed in other murine models of hyperlipidemia. For example, induction of diabetes in LDL receptor null mice resulted in accelerated atherosclerosis; a process prevented by administration of sRAGE.<sup>77</sup> Furthermore, these concepts are applicable in murine models of insulin-resistant (type 2) diabetes. In recent experiments, we bred apoE null mice into the db/db background. ApoE null/db/db mice displayed markedly accelerated atherosclerosis at the aortic root, along with increased vascular inflammation and expression of prothrombotic molecules, including VCAM-1, tissue factor, and matrix metalloproteinase (MMP)-9 antigen/activity. These effects were prevented by administration of sRAGE.<sup>78</sup>

In these settings, sRAGE was begun immediately at the time of diagnosis of hyperglycemia, thus addressing the impact of RAGE on early atherogenesis. To further study the role of this receptor in vascular stress, it was necessary to test the effects of blockade of RAGE on established atherosclerotic plaques. ApoE null mice were rendered diabetic at age 6 weeks. Diabetes was associated with accelerated atherosclerosis at both 14 and 20 weeks of age compared with nondiabetic counterparts. Mice were untreated until age 14 weeks; at that time, treatment was begun for an additional 6 weeks with either sRAGE or vehicle, murine serum albumin (MSA). Administration of sRAGE suppressed progression of atherosclerotic lesion area and complexity (Figure 2).<sup>79</sup> In parallel, migration/proliferation of SMCs and MPs were suppressed in sRAGE-treated mice, along with decreased vascular expression of cox-2 and nitrotyrosine epitopes, VCAM-1, JE-MCP-1, MMP-9 activity, tissue factor, and phosphorylation of p38 MAP kinase.<sup>79</sup>

How do these concepts extend to human atherosclerosis? To address this key question, Cipollone and colleagues<sup>80</sup> demonstrated upregulation of RAGE in human diabetic atherosclerotic plaques. Importantly, expression of RAGE, cox-2/type 1/type 2 microsomal Prostaglandin E<sub>2</sub> and matrix



**Figure 3.** RAGE blockade halts the progression of established atherosclerosis in apoE null mice: impact in euglycemia. From age 14 to 20 weeks, nondiabetic apoE null mice were treated with sRAGE, 100 µg/d, or vehicle, murine serum albumin (MSA). At age 20 weeks, animals were euthanized and sections prepared from the aortic root were stained with Oil Red O. Quantification of mean atherosclerotic lesion area (A) and mean number of complex lesions per section (B) were determined.

metalloproteinases was enhanced and colocalized particularly in macrophages at the vulnerable regions of the atherosclerotic plaques. Further, expression of RAGE in the plaques linearly correlated with the level of glycosylated hemoglobin.<sup>80</sup> These findings provide further support for the likely relevance of the RAGE axis in human diabetic vascular disease.

As AGEs and inflammatory mechanisms are also important in euglycemic atherosclerosis, it was important to test the potential role of RAGE in nondiabetic atherosclerosis in apoE null mice. Administration of sRAGE stabilized atherosclerotic lesion area and complexity in nondiabetic apoE null mice subjected to this therapy between 14 and 20 weeks of age (Figure 3).<sup>79</sup> These findings highlighted for the first time the potential impact of RAGE blockade on atherosclerosis independent of diabetes.

Despite the fact that in both diabetes and nondiabetes we did not observe frank lesion regression, the striking decrease in vascular inflammation triggered by RAGE blockade strongly suggests that the atherosclerotic plaques were less vulnerable to progression and, perhaps, the development of lesion instability. Such facets are now being studied in the innominate arteries of these animals both in the presence/absence of diabetes.<sup>81,82</sup>

### RAGE and Neointimal Expansion Triggered by Acute Arterial Injury

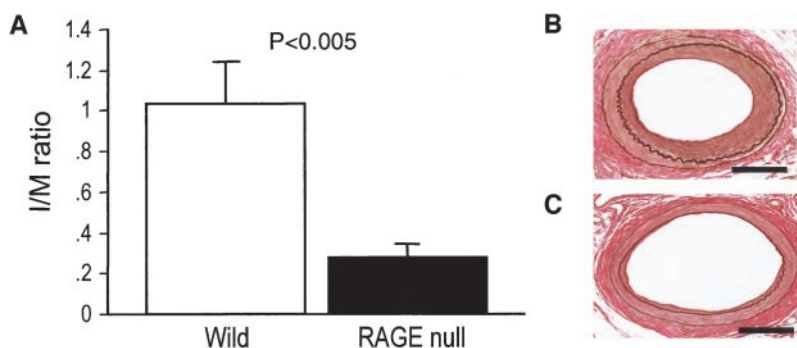
In addition to the dramatic acceleration of chronic atherosclerosis, diabetic subjects demonstrate exaggerated responses to arterial injury, such as that induced by therapeutic angioplasty. Particularly in diabetes, arterial injury triggers processes leading to rapid neointimal expansion, typified by enhanced vascular SMC proliferation and migration, and production of extracellular matrix. Indeed, in human diabetic subjects, stenting of the treated vessel may not offer full protection from restenosis and coronary events.<sup>83,84</sup> These considerations underscore the concept that SMC properties are altered in hyperglycemia. SMC proliferation, migration, and generation of extracellular matrix are triggered in acute injury and are augmented in chronic atherosclerosis.<sup>85–90</sup>

We tested these concepts in diabetic animals. In hyperglycemic fatty Zucker rats subjected to acute balloon injury of the carotid artery, administration of sRAGE caused decreased

neointimal expansion, in parallel with decreased incorporation of 5'-bromo-2'-deoxyuridine (BrdU) in the expanding neointima.<sup>91</sup> To test these concepts and the role of SMC RAGE in neointimal expansion in euglycemia and to use genetically modified RAGE animals to test our concepts, we induced femoral artery endothelial denudation injury in C57BL/6 mice. First, we examined the expression of RAGE and its ligands in these settings. By RT-PCR, RAGE transcripts were increased by day 3 after arterial endothelial denudation, compared with control vessels, and remained elevated through day 28.<sup>92</sup> Immunostaining of the injured arterial segment demonstrated enhanced RAGE antigen in neointimal and medial cells by day 4 in a distribution overlapping with the SMC marker  $\alpha$ -actin. These studies suggested that upregulation of RAGE accompanied vascular injury. RT-PCR demonstrated induction of S100 transcripts by day 3 after injury, which persisted through day 28.<sup>92</sup> Polyclonal antibody reactive with S100b demonstrated S100 antigen throughout the intima and media of the damaged vessel.<sup>92</sup> Generation of AGEs also occurred at the site of arterial injury as demonstrated by the presence of immunoreactive AGEs; the antibody largely reacts with CML-modified adducts.<sup>92</sup> AGEs were observed in the neointima within 4 days of arterial injury and persisted until day 21.<sup>92</sup> Thus, in the context of acute arterial injury in euglycemia, these findings placed RAGE and two of its ligands, S100 proteins and AGE adducts, at the site of arterial injury, especially within SMC. The enhanced activity of myeloperoxidase in the injured vessel wall suggested at least one potential means, generation of oxidant stress, by which AGE upregulation would occur in the euglycemic vessel wall on acute denudation of the endothelium.<sup>16,92</sup>

The co-localization of RAGE and its ligands led us to consider that upregulation of this axis provided a scaffold in the vessel wall to augment the response to vascular injury. We thus tested the premise that interception of RAGE interaction with its ligands might impact on neointimal expansion. RAGE blockers were given daily from the day before injury (day 0) to day 7 after injury, and animals were evaluated up to day 28. Mice treated with vehicle, MSA, displayed progressively increasing intimal/medial (I/M) ratios over 1 to 3 weeks, whereas animals receiving sRAGE, 100 µg per day, showed significantly decreased I/M ratios; the impact of sRAGE was dose-dependent.<sup>92</sup>

Because sRAGE exerts its effects indirectly, by binding ligands and preventing their interaction with cell surface RAGE, we directly blocked the receptor as well. Administration of anti-RAGE F(ab')<sub>2</sub> from days 0 to 7 (injury on day 1) suppressed neointimal expansion, in contrast to the lack of beneficial effect with nonimmune F(ab')<sub>2</sub>.<sup>92</sup> In addition to pharmacological blockade of RAGE, we tested the impact of acute arterial injury in homozygous RAGE null mice. Compared with wild-type littermates, RAGE null mice displayed a striking decrease in neointimal expansion on acute femoral artery injury (Figure 4).<sup>92</sup> Further, transgenic mice expressing DN RAGE selectively in SMCs (driven by the SM-22 $\alpha$  promoter) displayed significantly decreased neointimal expansion compared with wild-type littermates, indicating the



**Figure 4.** Homozygous RAGE null mice display decreased neointimal expansion on acute femoral artery endothelial denudation. Homozygous RAGE null mice and wild-type littermates were subjected to femoral artery guide wire injury. Intima/media ratio was determined on day 28 after injury (A). Van Gieson's elastic stain was performed on a representative femoral artery section from a wild-type mouse (B) and a RAGE null mouse (C). Scale bar=50  $\mu\text{m}$ .

critical requirement for RAGE signaling in mediating the impact of smooth muscle perturbation in acute injury.<sup>92</sup>

These findings suggested that RAGE/RAGE signaling were importantly involved in neointimal expansion on acute arterial injury. To test this further, we prepared extracts from the injured vessel segments to examine the signaling pathways linked to cellular proliferation after arterial injury impacted on by blockade of RAGE. First, we studied phosphorylation of Erk1/2 and protein kinase B (PKB), a downstream target of PI3K, as these pathways have been implicated in SMC proliferation/migration after injury.<sup>93,94</sup> Immunoblotting of homogenates of the damaged artery harvested 30 minutes after injury demonstrated increased levels of phosphoErk 1/2 and phosphoPKB compared with controls; however, vessels harvested from animals treated with sRAGE showed no suppression of the phosphorylation of Erk1/2 or PKB.<sup>92</sup> Other studies have demonstrated activation of Janus kinase (Jak)2 and signal transducer and activator of transcription Stat3 after arterial injury.<sup>95</sup> Because other work has shown RAGE-mediated activation of the Jak/stat pathway in a line of cultured cells,<sup>96</sup> we analyzed phosphorylation of Jak2 and Stat3 in injured femoral artery segments from C57BL/6 mice. On day 7, we observed increased phospho-Jak2 and phosphoStat3, compared with untreated controls.<sup>92</sup> Arterial segments retrieved on day 7 from animals treated with sRAGE displayed prominent suppression of Jak2 and Stat3 phosphorylation.<sup>92</sup> In isolated SMC, S100b stimulation enhanced phosphorylation of Jak2/Stat3, but not in RAGE null or transgenic SM22 $\alpha$  DN RAGE SMCs.<sup>92</sup> These findings suggest that RAGE activation, in part, by phosphorylation of Jak2 and Stat3, contributes to enhanced SMC proliferation within the injured vessel wall.

Taken together, these findings, together with those in chronic atherosclerosis, importantly link ligand-RAGE interaction to the pathogenesis of exaggerated neointimal expansion and suggest the plausibility of RAGE blockade as a therapeutic target in vascular injury, both in euglycemia and diabetes.

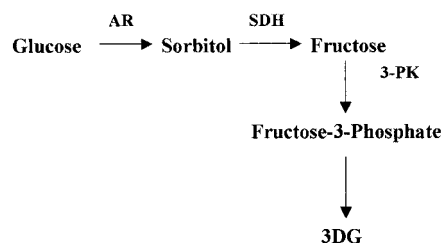
### Aldose Reductase and RAGE: The Heart of the Matter

Cardiac dysfunction emanating from long-standing diabetes emerges from macrovascular disease and innate disturbances of the myocardium resulting from long-standing disease. Additional factors predisposing to cardiac complications in

diabetes include disturbed autonomic balance and impaired fibrinolytic activity.<sup>97–103</sup>

What are the pathways linked to perturbation in the diabetic heart? One possible contributor is activation of the polyol pathway (Figure 5). In this pathway, glucose is reduced to sorbitol by aldose reductase (AR); fructose generated by this pathway is converted into fructose-3-phosphate by the action of 3-phosphokinase (3-PK). This leads to the generation of 3-deoxyglucosone, a central precursor in the generation of an array of AGEs, in particular, CML-adducts and others.<sup>104,105</sup> Plasma levels of 3-DG have been shown to increase, along with increased AR levels in erythrocytes in the presence of renal failure.<sup>105</sup> Other studies have found that administration of epalrestat (an inhibitor of AR) reduced the levels of CML adducts and their precursors in erythrocytes, as well as resulting in lowered plasma levels of thiobarbituric acid reactive substances (TBARS), a measure of oxidant stress, in diabetic patients.<sup>106</sup> Thus, although these AGEs were measured intracellularly, the effect of 3-DG on general AGE formation resulted in increased levels of plasma TBARS. The ability of AR-dependent mechanisms to generate and sustain production of AGEs provides an amplification loop to fuel AGE-RAGE interaction in the myocardium.

In this context, the role of AR in myocardial injury has been tested in experimental systems. Inhibition of AR protects hearts from ischemic injury.<sup>107–110</sup> Maintenance of high-energy phosphates by substrate metabolism is critical to managing normal sodium and calcium homeostasis. It has



**Figure 5.** Polyol pathway and generation of 3-deoxyglucosone: precursor to AGE formation. In the polyol pathway, glucose is metabolized to sorbitol via the action of aldose reductase (AR); subsequently, sorbitol is metabolized to fructose via sorbitol dehydrogenase (SDH). The action of 3-phosphokinase (3-PK) leads to the generation of fructose-3-phosphate, a precursor in the generation of 3-deoxyglucosone (3-DG), a reactive intermediate that leads to the formation of a range of AGEs, including CML-modified adducts, signal transduction ligands of RAGE.



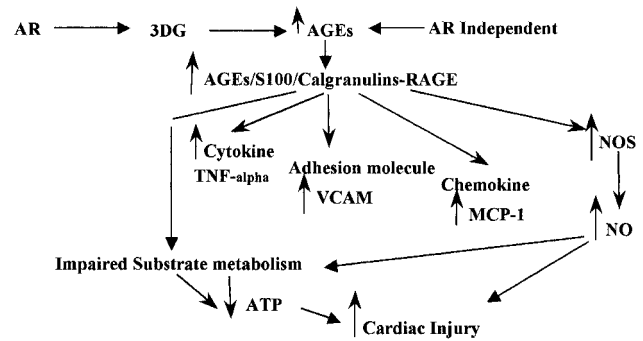
been shown that regulation of intracellular sodium and calcium changes are important downstream determinants of the severity of ischemic injury. Moreover, studies have demonstrated the complex interplay between glucose metabolism, altered intracellular sodium and calcium, and ischemic injury in diabetes. Most notably, interventions that inhibit any of the above steps, and especially those preventing the rise in intracellular sodium, reduce injury to the myocardium during ischemia.<sup>111–117</sup>

The role of AR-dependent pathways in generation of AGEs led us to test the concept that RAGE transduces at least in part, the biological impact of AR activation in the injured heart. Recent pilot studies from our laboratory have shown that CML-AGEs and S100/calgranulins are increased in the diabetic mouse and rat heart after 3 months diabetes; in parallel, RAGE expression was enhanced particularly in EC and infiltrating MP.<sup>118</sup> When diabetic rats or mice were treated with daily sRAGE, expression of inducible nitric oxide synthase (iNOS) was reduced in the diabetic heart. In addition, decreased levels of NO and cGMP were observed in sRAGE-treated diabetic hearts.<sup>118</sup>

One disadvantage of studying cardiac function and the response to ischemia in murine systems is the markedly lower levels of AR in mouse hearts compared with human or rat hearts.<sup>119</sup> The recent development of transgenic mice expressing human AR to physiologically relevant degrees (to human) in a broad manner driven by the major histocompatibility complex class I promoter provides an ideal means to best test the role of RAGE in mediating the downstream biochemical and molecular impact of AR, especially in the diabetic heart.<sup>120</sup> We propose that ischemia augments generation of AGEs by AR-dependent and independent mechanisms. Moreover, these processes are exaggerated in diabetes, leading to further generation of AGEs (by 3-DG, in part) and S100/calgranulin ligands for RAGE, causing generation of cytokines, chemokines, and adhesion molecules; a mechanism to augment inflammation and perturbation. Increased levels of NOS generate reactive oxygen species (ROS). AGEs impair substrate metabolism, leading to decreased ATP; together with ROS, these processes synergize to augment cardiac injury (Figure 6). The recent development of RAGE null mice and transgenic mice expressing physiologically relevant (to human) levels of AR provides an ideal set of tools with which to test the role of RAGE as a downstream effector pathway for the impact of AR on biochemical and metabolic disturbances in diabetes and euglycemia initiated by ischemic injury.

### Conclusions and Perspectives: Dismantling the Scaffold of Vascular Disease and Diabetic Complications

The generation of AGEs and augmentation of proinflammatory mechanisms in the vessel, at least in part via accumulation of S100/calgranulins and amphoterin released from activated inflammatory cells, provides a potent feedback loop for sustained oxidant stress, ongoing generation of AGEs, and vascular perturbation. The vessel wall and heart, especially in diabetes and to a lesser but quantifiable degree in euglycemia, becomes largely irreversibly altered. On superimposed of



**Figure 6.** Activation of aldose reductase (AR) and RAGE: building a scaffold for the impact of ischemic injury in the cardiovascular complications of diabetes and beyond. We propose that ischemia/reperfusion augments generation of AGEs by AR-dependent and independent-mechanisms. Moreover, these processes are exaggerated in diabetes, leading to further generation of AGEs (by 3-deoxyglucosone, in part) and S100/calgranulin ligands for RAGE, causing generation of cytokines, chemokines, and adhesion molecules, a mechanism to augment inflammation and perturbation. Increased levels of NOS generate reactive oxygen species (ROS). AGEs impair substrate metabolism, leading to decreased ATP; together with ROS, these processes synergize to augment cardiac injury triggered by ischemia.

new stresses, such as elevated levels of lipids, physical injury, or ischemia/reperfusion, dysfunction is magnified, leading to accelerated injury and failure of repair mechanisms.

Indeed, these concepts are relevant beyond the cardiovascular system. Blockade of RAGE in db/db mice, a murine model of insulin-resistant hyperglycemia, has been shown to restore effective wound healing on physical injury and to prevent the structural and functional derangements in the kidney that accompany long-standing diabetes.<sup>121,122</sup> In both settings, AGEs/S100/calgranulins were found in excess in the diabetic target tissue, in parallel with increased numbers and, likely, function of inflammatory cells such as MP. Breaking the cycle of ligand/RAGE interaction in those settings beneficially modulated the course of impaired wound healing and renal dysfunction. Importantly, in those cases, blockade of RAGE in euglycemic mice had no adverse impact on wound healing or renal function.<sup>121,122</sup>

The vulnerability of the vasculature and the cardiovascular system to the deleterious impact of these pathways is accentuated by the lack of a fully directed effective therapies for reducing complications, particularly in diabetes. Our findings suggest that blockade of RAGE may represent a targeted means to dismantle this perturbed scaffold in the blood vessel wall and heart and suppress vascular dysfunction and irreversible injury. The finding that homozygous RAGE null mice are viable and lacking an obvious phenotype in the absence of stress strongly suggests that antagonism of this axis is likely to be feasible and tolerated in the clinic.

We propose that antagonism of RAGE, especially in concert with complementary therapies, such as strict glyce-mic and lipid control, will remodel the landscape of the perturbed vasculature leading to prevention/stabilization of vascular and cardiac dysfunction in diabetes and beyond.



Rigorously controlled clinic trials are required to test these concepts in human subjects and are on the horizon.

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