Receptor-mediated Endothelial Cell Dysfunction in Diabetic Vasculopathy

Soluble Receptor for Advanced Glycation End Products Blocks Hyperpermeability in Diabetic Rats

Jean-Luc Wautier, Clotilde Zoukourian, Olivier Chappey, Marie-Paule Wautier, Pierre-Jean Guillausseau, Rong Cao,* Osamu Hori,* David Stern,* and Ann Marie Schmidt*

*Unite d'Immunohematologie and Department of Internal Medicine, Laboratoire de Recherche en Biologie Vasculaire et Cellulaire, Inserm Unite 294, Universite Paris 7 France; and *Department of Physiology, Medicine and Surgery, Columbia University, College of Physicians and Surgeons, New York 10032*

Abstract

Dysfunctional endothelium is associated with and, likely, predates clinical complications of diabetes mellitus, by promoting increased vascular permeability and thrombogenicity. Irreversible advanced glycation end products (AGEs), resulting from nonenzymatic glycation and oxidation of proteins or lipids, are found in plasma, vessel wall, and tissues and have been linked to the development of diabetic complications. The principal means through which AGEs exert their cellular effects is via specific cellular receptors, one of which, receptor for AGE (RAGE), is expressed by endothelium. We report that blockade of RAGE inhibits AGE-induced impairment of endothelial barrier function, and reverses, in large part, the early vascular hyperpermeability observed in diabetic rats. Inhibition of AGE- and diabetes-mediated hyperpermeability by antioxidants, both in vitro and in vivo, suggested the central role of AGE-RAGE–induced oxidant stress in the development of hyperpermeability. Taken together, these data support the concept that ligation of AGEs by endothelial RAGE induces cellular dysfunction, at least in part by an oxidant-sensitive mechanism, contributing to vascular hyperpermeability in diabetes, and that RAGE is central to this pathologic process. (*J. Clin. Invest.* 1996. 97:238–243.) Key words: diabetes • permeability • glycation • advanced glycation end product • immunoglobulin superfamily receptor

Introduction

Exposure of proteins or lipids to reducing sugars results in nonenzymatic glycation and oxidation. Initially, reversible early glycation adducts, Schiff bases and Amadori products, form on free amino groups (1). Further complex molecular rearrangements produce irreversible advanced glycation end products (AGEs):[1] heterogeneous structures of yellow-brown color, characteristic fluorescence, and a propensity to form cross-links, which generate reactive oxygen intermediates and interact with specific cellular receptors (1–4). The presence of AGEs in tissue has been linked to development of vasculopathy, especially in the setting of diabetes (1, 3). AGE-modified adducts on long-lived proteins in extracellular matrix alter basement membrane structure by trapping plasma macromolecules and by increasing vessel wall rigidity through formation of cross-links (3). The principal means through which AGEs influence cellular properties is by binding to specific receptors (4–6), the best characterized of which is the receptor for AGEs (RAGE), a member of the immunoglobulin superfamily expressed by endothelial cells (ECs), smooth muscle cells, and mononuclear phagocytes (7), cells central to both vascular homeostasis and the pathogenesis of vascular lesions. A potential role for RAGE in vascular dysfunction is suggested by two lines of evidence: (*a*) engagement of AGEs by cellular RAGE affects critical properties of these cells in a manner contributory to vascular dysfunction (4); and (*b*) there is enhanced expression of RAGE in diabetic vasculopathy and in arteriosclerotic and other vascular lesions, such as inflammatory vasculitides (8).

Increased vascular permeability is characteristic of diabetic vasculopathy (9), even at the earliest stages in which microalbuminuria may be the only harbinger of vascular complications yet to come (10). As ECs are the critical guardians of vascular barrier function, we postulated that AGEs in plasma or the subendothelium would promote vascular hyperpermeability by interacting with RAGE. We demonstrate that when diabetic rat red cells bearing AGEs are infused into normal animals, increased vascular permeability results, an effect which is prevented by blockade of RAGE. Hyperpermeability in streptozotocin-induced diabetic rats was largely prevented by infusion of soluble RAGE (sRAGE), the extracellular domain of the membrane-anchored receptor, which blocks binding of AGEs to cell surface RAGE (4). Since one of the principal consequences of AGE–RAGE interaction is the induction of cellular oxidant stress (2, 11), we postulated that hyperpermeability in diabetes might be due, at least in part, to involvement of AGE-RAGE–dependent oxidant-sensitive pathways. Consistent with this hypothesis, hyperpermeability due to the pres-

Address correspondence to Dr. Jean-Luc Wautier, Hopital Lariboisiere, 2 rue Ambroise Pare, 75475 Paris cedex 10 France. Phone: 33-1-49 95 62 03; FAX: 33-1-42 85 21 71.

Received for publication 5 April 1995 and accepted in revised form 21 September 1995.

J. Clin. Invest.

0021-9738/96/01/0238/06 $2.00
Volume 97, Number 1, January 1996, 238–243

1. *Abbreviations used in this paper:* AGE, advanced glycation end product; EC, endothelial cell; RAGE, receptor for AGE; sRAGE, soluble RAGE; TBIR, tissue-blood isotope ratio.

ence of AGEs or diabetes itself, both in vitro and in vivo, was prevented in the presence of antioxidants. These data indicate the potential impact of AGE–RAGE interaction on vascular dysfunction underlying diabetic complications and suggest that inhibition of this interaction might constitute a novel, potentially useful therapeutic target.

Methods

Human diabetic red cells, cultured ECs, and in vitro permeability assays. Blood was obtained from normal subjects and patients in accordance with provisions of the declaration of Helsinki and with the rules of our institution. Normal volunteers were from the Blood Transfusion Center, and diabetic patients were hospitalized in the Department of Internal Medicine for diabetes control. The group of patients (n = 18 each for the normal and diabetic subjects) was comparable in terms of age (mean age 42±4 yr for normal patients and 47.8±6 yr for diabetic patients). The mean duration of diabetes was 6.8±2.2 yr, and fasting blood glucose and hemoglobin A_{1c} levels were 13.9±1.5 mmol/liter and 13.7±0.9% (diabetic patients) and 4.6±0.4 mmol/liter and 5.1±0.6% (normal subjects), respectively. Cultured bovine aortic ECs, prepared as described (12), were used ≈ 7 d after reaching confluence (based on morphologic criteria and baseline permeability measurements; most cultures were used 7 d after confluence was achieved) on nucleopore membranes (12, 13). ECs were incubated with either normal or diabetic RBCs for 24 h, washed with minimal essential medium, and then permeability was studied in minimal essential medium containing fetal calf serum (10%) by adding ^{125}I-albumin (67 μg/ml; 5.8×10^9 cpm/mg; reference 13) or ^{3}H-inulin (4.06 mg/ml; 246 mCi/gram) to the upper chamber, and emergence of radioactivity in the lower chamber was determined over the next 24 h at 37°C (there was no hydrostatic or oncotic gradient between upper and lower chambers). Aliquots of medium (5 μl) were sampled from top and bottom chambers every 10 min over the first hour and at 1, 2, 4, and 24 h (final volume of medium in the chamber varied by < 10% during the experiment) to determine a permeability coefficient (P): $P = J \cdot 1/A \cdot 1/C_T - C_B$; where J is the flux of molecules across the filter; A is the surface area; and C is the concentration of tracer in top (C_T) and bottom (C_B) chambers (13). Postconfluent monolayers displaying a permeability coefficient > 6.5×10^{-7} cm/s (for ^{125}I-albumin) or > 5×10^{-6} cm/s (for ^{3}H-inulin) were excluded. Where indicated, ECs were pretreated for 16 h with vitamin E (100 μg/ml) or probucol (50 μM) at 37°C before and during incubation with diabetic red cells (24 h).

Preparation of RAGE and antisera. RAGE was purified from rat lung by the same procedure described for bovine RAGE and migrated with $M_r \approx 30{,}000$ on reduced and nonreduced SDS-PAGE, corresponding to that expected for the extracellular domain (the latter has been termed sRAGE; analysis of tryptic peptides has shown only extracellular sequences to be present [4]). sRAGE binds AGE ligands and competes with cell surface RAGE for such ligands (4). sRAGE, radioiodinated by the lactoperoxidase method (7.2 μCi/nmol; reference 14), was shown to maintain its AGE-binding activity and to have unchanged migration on SDS-PAGE. Monospecific, blocking polyclonal antibodies to RAGE were prepared in rabbits and IgG was purified from immune serum on protein A–Sepharose (14). Nonimmune IgG was similarly prepared and $F(ab')_2$ fragments were made as described (15).

In vivo permeability studies. In vivo permeability studies were performed by infusing diabetic or normal red cells into syngeneic normal or diabetic rats. Diabetic RBCs for transfusion studies were prepared from male Wistar rats (CERJ, Laval, France) weighing ≈ 200 grams (at the start of the experiment). Rats were treated with streptozotocin (45 mg/kg; Sigma Immunochemicals, St. Louis, MO) intravenously (all invasive procedures were done under appropriate anesthesia), and animals were maintained for 9–11 wk before experiments. Glycemia in diabetic rats was 35–40 mmol/liter, four to six times higher than that of their normal counterparts. Erythrocytes were collected by puncture of the lower abdominal aorta from each group of animals (normal/diabetic) into dextrose (2.4%), citric acid (0.73%), sodium citrate (2.2%) (two parts anticoagulant to eight parts blood). Blood was centrifuged to remove plasma and buffy coat, and packed RBCs were washed and infused (4.2×10^9 cells/animal) into normal rats (vol = 0.5 ml). Normal rats received either diabetic or normal red cells from syngeneic animals alone, or also were administered sRAGE, anti-RAGE IgG, nonimmune IgG (or $F[ab']_2$ derived from the nonimmune IgG), or native albumin. In other studies, diabetic rats received one of the above proteins by a single intravenous infusion. Permeability in normal and diabetic rats was determined using the tissue-blood isotope ratio (TBIR) (16) method with

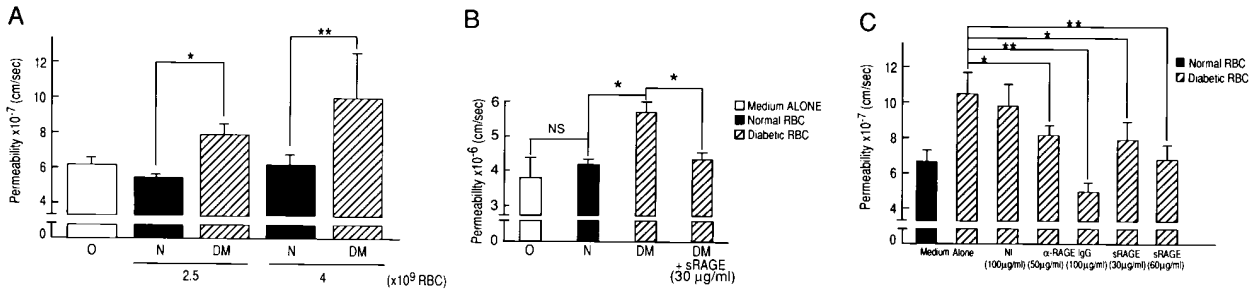

Figure 1. Effect of diabetic red cells on the barrier function of cultured EC monolayers. (*A* and *B*) Effect of diabetic versus normal red cells on diffusional transit of ^{125}I-albumin (*A*) or ^{3}H-inulin (*B*) across EC monolayers. Postconfluent bovine aortic ECs were incubated for 24 h at 37°C with medium alone (*O*) or in the presence of RBCs from normal human volunteers (*N* [RBCs from six patients]) or diabetic patients (*DM* [RBCs from six patients]). Two concentrations of red cells (2.5×10^9 and 4×10^9 cells/ml) were used in experiments with ^{125}I-albumin (*A*), and sRAGE (30 μg/ml) was used in the study with ^{3}H-inulin (*B*). (*C*) Effect of anti-RAGE IgG and sRAGE. EC monolayers were incubated with normal red cells alone (*solid bar*, *Medium*) or medium containing diabetic red cells (*hatched bars*) with either no further addition, or anti-RAGE IgG (*α-RAGE*), nonimmune IgG (*NI*), or sRAGE as indicated in the figure. Permeability of monolayers to ^{125}I-albumin was determined as above. The results are presented as mean±SEM (n = 7). Statistical analysis in *A* was made using two-way ANOVA to compare the permeability of ECs in the presence of normal or diabetic red cells. The difference at 2.5×10^6 and at 4×10^6 RBC between normal and diabetic red cells was statistically significant (*$P < 0.05$ and **$P < 0.01$, respectively). Statistical analysis in *B* and *C* was performed using one-way ANOVA to compare the results of permeability obtained with diabetic RBCs to other experimental conditions followed by the parametric Dunnetts' test. In *B*, sRAGE reduced the permeability significantly (*$P < 0.05$); and in *C*, sRAGE and anti-RAGE IgG (high dose versus low dose) reduced the permeability significantly (**$P < 0.01$ and *$P < 0.05$, respectively). Values obtained with endothelial monolayers exposed to RBCs harvested from normal rats were the same as those with ECs exposed to medium alone. In all cases, red cells from either six normal or six diabetic individuals were used in each experiment.

^{51}Cr-labeled RBCs (specific radioactivity of 1.5×10^6 cpm/ml; 0.33 ml/animal) (16) and ^{125}I-albumin (5.8×10^9 cpm/mg; 5.1×10^7 cpm/kg; radiolabeled as described for AGE albumin) (17) according to the formula $[^{125}I/^{51}Cr\ (tissue)][^{125}I/^{51}Cr\ (blood)]^{-1}$, where $^{125}I/^{51}Cr$ is the ratio of radioactivity in tissue versus that in an arterial blood sample harvested before excising the heart (16). Where indicated, normal animals were pretreated with probucol (25.8 μg/kg body weight dissolved in 0.1 ml ethanol; intravenous) 30 min before the infusion of diabetic red cells, or diabetic animals were treated with probucol (25.8 μg/kg body weight dissolved in 0.1 ml ethanol; intravenous) daily for 5 d before time of killing and determination of TBIR. In both cases, control infusions with 0.1 ml ethanol were without effect on the parameters of hyperpermeability (data not shown).

Results

Diabetic RBC-induced perturbation of cultured EC barrier function. AGE-modified structures, whether present on plasma proteins or diabetic red cells, display increased adhesivity for endothelium because of their binding to cell surface RAGE (4, 17–19). Exposure of postconfluent cultured ECs to RBCs harvested from diabetic patients increased diffusional transit (permeability) of macromolecular tracers (^{125}I-albumin and ^{3}H-inulin in Fig. 1, *A* and *B*, respectively) across cell monolayers compared with normal red cells, which were comparable with untreated controls. This diminution of endothelial barrier function was dependent on the concentration of added diabetic red cells (Fig. 1 *A*) and resulted from their interaction with endothelial RAGE, as it was completely inhibited by anti-RAGE IgG in a dose-dependent manner, but not by nonimmune IgG (Fig. 1 *C*). These concentrations of anti-RAGE IgG were comparable with those shown previously to block binding of diabetic red cells to endothelium (17). Similar studies in which sRAGE was added to mixtures of diabetic RBCs and ECs also demonstrated prevention of diabetic red cell–induced endothelial hyperpermeability in a dose-dependent manner (Fig. 1, *B* and *C*; these concentrations of sRAGE block enhanced binding of diabetic RBCs to endothelium [17]).

Infusion of diabetic RBCs into normal rats induces vascular hyperpermeability: inhibition by blockade of RAGE. Our observations in cell culture led us to predict that diabetic RBCs would increase vascular permeability in vivo. Red cells from rats rendered diabetic by streptozotocin were infused into syngeneic nondiabetic animals. Such diabetic RBCs have been shown previously to display moderately shortened survival after transfusion into normal rats, in large part corrected by blockade of RAGE (17). Animals transfused with diabetic RBCs manifested increased permeability in a range of organs, determined by TBIR, as compared with controls receiving the same number of normal RBCs (Fig. 2 *A*). No increase in hemolysis was observed in rats infused with diabetic red cells, based on release of hemoglobin or ^{51}Cr from radiolabeled red cells (data not shown). Permeability in rats infused with normal RBCs was unchanged from that in untreated animals. A central role for the interaction of diabetic RBCs with RAGE in mediating this perturbation of vascular function was illustrated by the inhibitory effect of pretreatment with anti-RAGE IgG (Fig. 2 *A*), which completely blocked increased permeability in the various organs; the same amount of nonimmune IgG or $F(ab')_2$ prepared from nonimmune IgG had no effect. As an alternate means to affect RAGE blockade, animals receiving diabetic red cells were pretreated with sRAGE, which also completely reversed vascular hyperpermeability (Fig. 2 *B*). Infusion of the same amount of nonglycated albumin or nonimmune IgG in place of sRAGE had no effect on permeability. These data indicate that AGE–RAGE interaction is an important contributing factor to the increased permeability observed after infusion of diabetic red cells.

sRAGE diminishes hyperpermeability in streptozotocin-induced diabetic rats. The critical question was whether blockade of RAGE would modulate the increased permeability observed in diabetic animals (17). The only means to achieve specific RAGE blockade was to use sRAGE, as anti-RAGE IgG had a selectively toxic effect in diabetic animals (this was not anticipated by previous results in normal rats; Fig. 2 *A*). Studies were first performed to assess the plasma half-life and tissue deposition of sRAGE in order to determine its suitability for RAGE blockade in diabetic animals. ^{125}I-Labeled rat sRAGE infused intravenously into the jugular vein resulted in

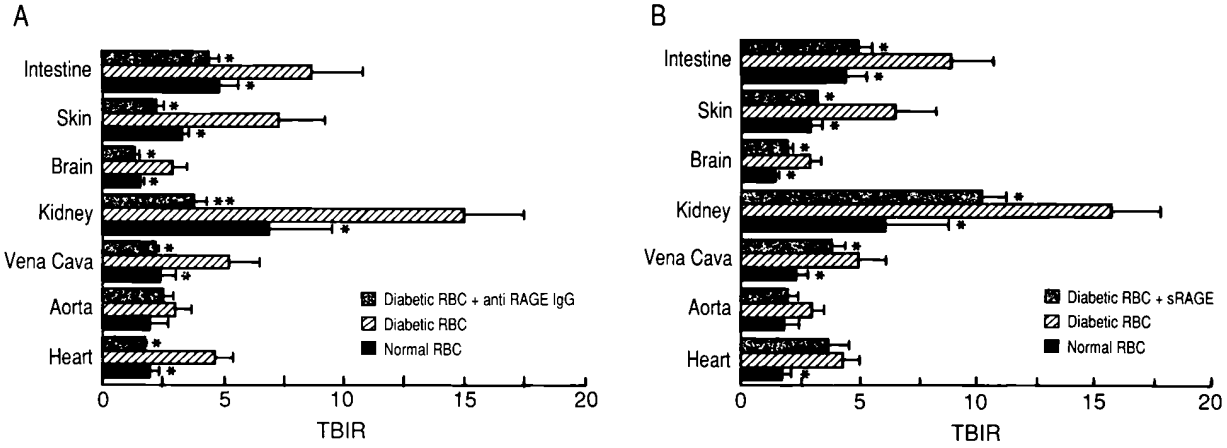

Figure 2. Infusion of diabetic rat red cells into syngeneic rats increases vascular permeability: effect of RAGE blockade. (*A*) Comparison of normal and diabetic red cells: effect of anti-RAGE IgG. Red cells were prepared from streptozotocin-treated rats after 9–11 wk of diabetes and were transfused into normal rats. 1 h later, TBIR was determined by infusing ^{125}I-albumin and ^{51}Cr-labeled normal rat red cells. Where indicated, anti-RAGE IgG (7 mg/kg) was infused 1 h before the transfusion. Infusion of nonimmune IgG (7 mg/kg) was without effect on vascular permeability. (*B*) Effect of sRAGE. Normal rats were infused with diabetic red cells as above (*A*) after pretreatment with sRAGE (5.15 mg/kg) or normal red cells. The results are presented as mean±SEM. In all cases, six rats were used for each experimental condition. One-way ANOVA followed by Dunnett's test was used to analyze the data from each organ. $^*P < 0.05$ and $^{**}P < 0.01$.

half-lives of 21.7±0.43 and 2.55±0.43 h for the elimination and distribution phases, respectively, in diabetic animals (Fig. 3 *A*; half-lives for normal animals are also shown). The longer half-life in diabetic versus normal animals may reflect binding of sRAGE to intravascular AGEs, though this will require further study to prove. Radiolabeled sRAGE accumulated in various organs, especially the kidney (Fig. 3 *B*). sRAGE in plasma and tissues appeared intact, as it was precipitable in trichloroacetic acid and SDS gel electrophoresis/autoradiography revealed a major band at $M_r \approx 30{,}000$ (data not shown). These data suggested that sRAGE could mediate blockade of RAGE for several hours after its intravenous administration, indicating that it is a pharmacologically reasonable and feasible intervention in this model.

Rats rendered diabetic by treatment with streptozotocin were studied 9–11 wk after the development of diabetes (plasma glucose levels of 30–40 mM, compared with nondiabetic controls, plasma glucose level of 5 mM), and displayed increased vascular permeability in a similar manner and distribution to that observed previously (16). Changes in permeability were most marked in diabetic intestine, skin, and kidney, being 2.8-, 3-, and 2.8-fold increased, respectively, compared with nondiabetic controls. Diabetic rats were infused with sRAGE at two different doses (2.25 and 5.15 mg/kg), as this was expected to achieve plasma concentrations in the range of 10–30 and 40–60 μg/ml, respectively, over the first 3 h (Fig. 3 *C*). At the lower dose of sRAGE, increased vascular leakage was completely blocked in diabetic intestine and skin, and largely blocked (≈ 60% inhibited) in the kidney. At the higher dose of sRAGE, hyperpermeability was blocked in intestine and skin, and ≈ 90% in kidney. Reversal of increased permeability in diabetic animals was achieved within 1 h of sRAGE administration (Fig. 3 *C*). Control diabetic rats infused with nonimmune IgG or nonglycated albumin at the same concentrations as sRAGE showed no change in vascular permeability (data not shown).

Diabetic vascular hyperpermeability and oxidant stress. The rapid effect of sRAGE (within 1 h) in inhibiting diabetic hyperpermeability suggested the involvement of short-lived moieties, such as reactive oxygen intermediates, species likely associated with immediate perturbation of endothelial function. To test the hypothesis that AGE-RAGE/diabetes-mediated hyperpermeability was due, at least in part, to oxidant-sensitive mechanisms, we performed in vitro and in vivo experiments of permeability in the presence of antioxidants. Pretreatment of ECs with vitamin E or probucol prevented increased permeability of the monolayers to ^{125}I-albumin in the presence of diabetic red cells (Fig. 4 *A*). Similarly, pretreatment of ECs with probucol prevented increased monolayer permeability to ^{3}H-inulin upon incubation with diabetic red cells (Fig. 4 *B*). Extension of these studies to the in vivo models presented above revealed nearly complete reversal of hyperpermeability in the organs after infusion of diabetic red cells into normal rats in the presence of probucol (Fig. 4 *C*). Furthermore, treatment of diabetic rats with probucol significantly reduced hyperpermeability in a range of organs (Fig. 4 *D*).

Discussion

The formation of irreversible AGEs on plasma proteins or cellular elements is a long-term consequence of hyperglycemia (1–4). Our data suggest a receptor-dependent mechanism through which AGEs perturb central homeostatic properties of endothelium to increase vascular permeability. Although other mechanisms, such as accumulated products of the polyol pathway, may also contribute to perturbation of vascular barrier function in diabetes (16), our results underscore a central role for AGE–RAGE interaction. Both sRAGE and anti-

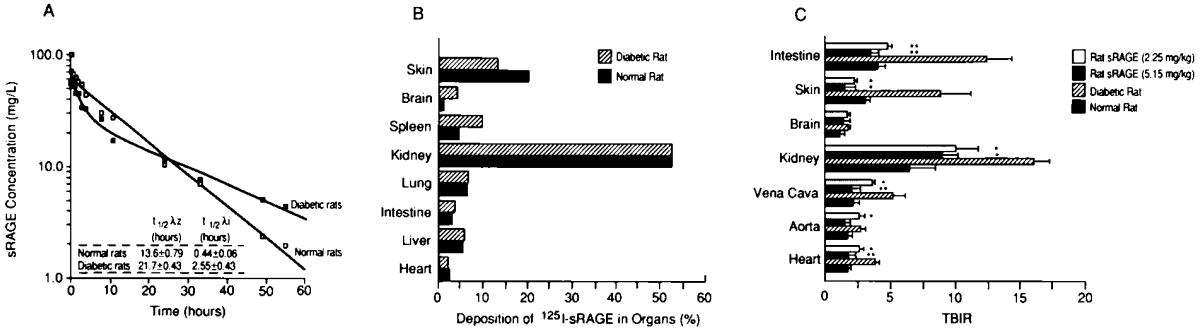

Figure 3. Infusion of sRAGE: effect on vascular permeability in diabetic rats. (*A*) Kinetics of ^{125}I-sRAGE disappearance from plasma of normal and diabetic rats. Normal rats or rats rendered diabetic with streptozotocin (as above; rats were used 9–11 wk after induction of diabetes) were infused with ^{125}I-sRAGE (10^7 cpm; sample volume ≤ 0.1 ml), and blood samples were taken at the indicated times. Plasma sRAGE concentration data were fit to a two-compartment open model using nonlinear regression by extended least-squares analysis (Siphar; SIMED, Creteil, France). To assess the "goodness of fit," residual analysis (an examination of the standard deviation) was performed. In addition to the likelihood test, Akaike, Leonard, and Schwarz criteria were tested to select the most appropriate model (26). $t_{1/2}\lambda z$ and $t_{1/2}\lambda i$ denote half-lives for elimination and distribution, respectively. (*B*) Deposition of ^{125}I-sRAGE in tissues. Normal or diabetic rats were infused with rat ^{125}I-sRAGE as above, and animals were killed after 48 h. Organs were solubilized, radioactivity was determined (cpm/mg dry tissue weight), and total radioactivity in all organs evaluated (brain, kidney, lung, spleen, intestine, aorta, vena cava, heart, liver, and skin) was assigned a value of 100%. Percentage of deposited radioactivity in selected organs is shown in normal and diabetic animals (mean of duplicates). (*C*) Diabetic animals were infused with sRAGE (2.25 or 5.15 mg/kg) and TBIR was determined as described above. The results of permeability measurements in normal animals are shown for comparison (infusion of sRAGE had no effect on TBIR in normal controls). The results are presented as mean±SEM (normal, $n = 8$; diabetic, $n = 11$; diabetic plus sRAGE, $n = 6$). One-way ANOVA followed by Dunnetts' test was used to compare diabetic rats either untreated or treated with sRAGE. Data for each organ were analyzed separately. $^{*}P < 0.05$; $^{**}P < 0.01$. Infusion of nonimmune IgG or nonglycated albumin into diabetic rats had no effect on permeability (data not shown).

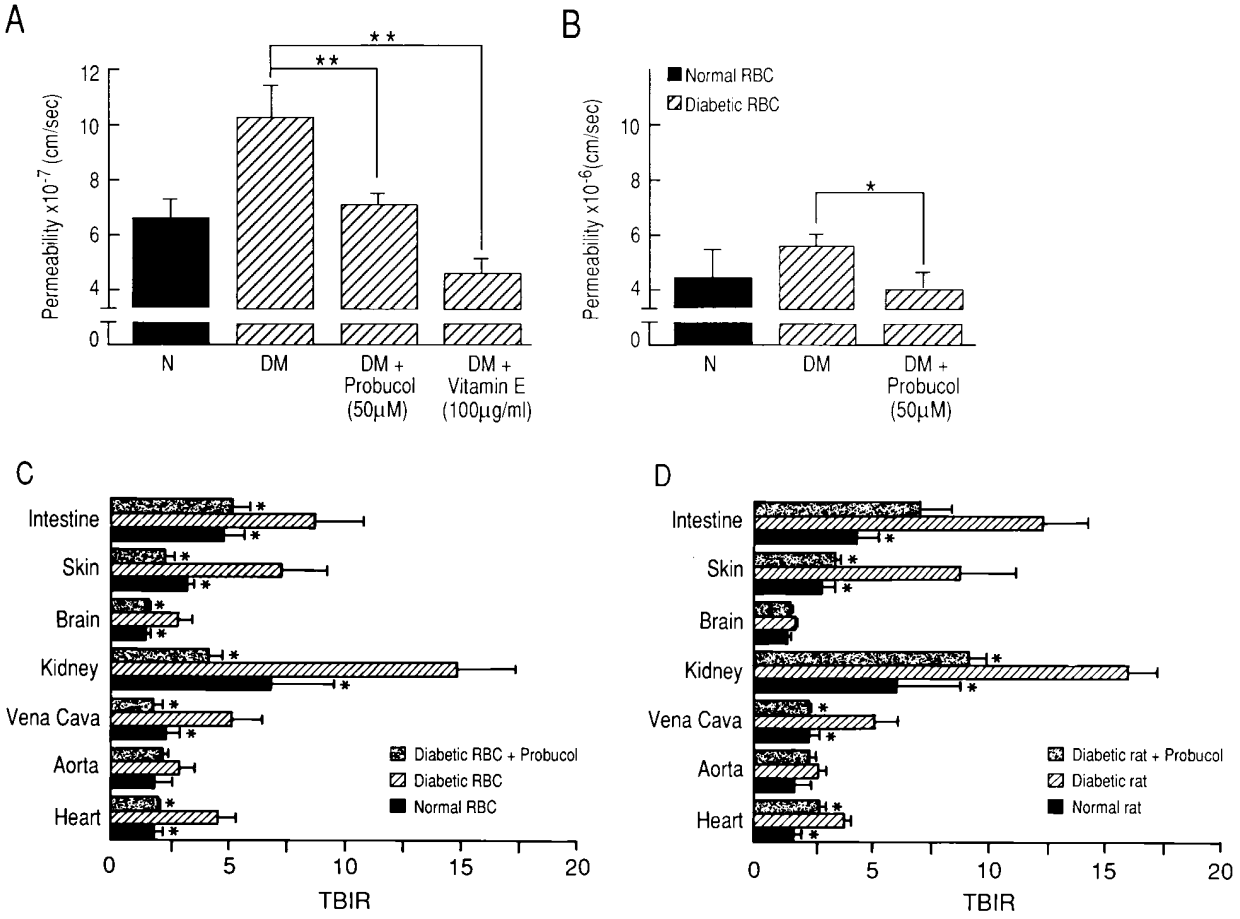

Figure 4. Effect of antioxidants on hyperpermeability induced by diabetic red cells on the barrier function of cultured EC monolayers (*A* and *B*) and on hyperpermeability induced by infusion of diabetic red cells (*C*) and in diabetes (*D*). (*A* and *B*) Effect of vitamin E and/or probucol on diffusional transit of ^{125}I-albumin (*A*) or ^{3}H-inulin (*B*) across EC monolayers. Postconfluent bovine aortic ECs were incubated for 24 h at 37°C with normal red cells (*N*) or diabetic red cells (*DM*) in the presence or absence of 16 h of preincubation with vitamin E (100 μg/ml) and/or probucol (50 μM) as indicated in the figure and permeability measured by diffusional transit of ^{125}I-albumin (*A*) or ^{3}H-inulin (*B*). Red cells from six patients were used in each case per experimental condition. Data were analyzed by two-way ANOVA followed by Dunnetts' test (*A*) and Student's unpaired *t* test (*B*). $*P < 0.05$ and $**P < 0.01$. (*C*) Normal rats were infused with diabetic red cells alone as above or in the presence of probucol (25.8 μg/kg body wt dissolved in ethanol, 0.1 ml; intravenous). Six rats were used in each condition, and statistical analysis of data was performed using one-way ANOVA followed by Dunnett's test. $*P < 0.05$. (*D*) Diabetic animals, prepared as described in the text, were infused with probucol for 5 d (25.8 μg/kg body wt dissolved in ethanol, 0.1 ml; intravenous) and permeability was then assessed by TBIR. Data were analyzed by one-way ANOVA followed by Dunnetts' test. $*P < 0.05$. Six rats were used per experimental condition. In *C* and *D*, control experiments with infusion of ethanol (0.1 ml) alone were without effect on hyperpermeability (data not shown).

RAGE IgG virtually restored dysfunctional barrier function in all the organs in normal animals infused with diabetic red cells, and sRAGE blocked hyperpermeability in diabetic animals. Anti-RAGE IgG could not be used because of selective toxicity in the diabetic animals only. We can only speculate on the mechanisms underlying this finding with respect to receptor regulation in diabetes, but these considerations certainly form the basis for present and future investigation in this area.

These data further suggest that central to the mechanism of AGE–RAGE interaction in the development of hyperpermeability is the involvement of oxidant-sensitive pathways. The surprisingly rapid reversal of hyperpermeability in diabetic animals after administration of sRAGE (within 1 h) suggests that labile mediators are likely involved in ongoing cellular dysfunction. One such mediator could be reactive oxygen species. Previous work (11) has established that binding of AGEs to RAGE induces endothelial oxidant stress (4, 11), based on generation of thiobarbituric acid–reactive substances, increased heme oxygenase 1 mRNA levels, and activation of the transcription factor NF-kB, each of these events being blocked by anti-RAGE IgG or antioxidants. Furthermore, oxidant stress in other contexts has been associated with diminished EC barrier function (20). Given the inhibitory effect of antioxidants on hyperpermeability in these studies, it is likely that oxidant stress, mediated by AGE–RAGE interaction, is central in the development of ongoing, chronic vascular dysfunction.

In this context, previous clinical studies have correlated plasma markers of oxidant stress and EC perturbation in diabetes with both vascular dysfunction and subsequent vascular complications (21). Alternatively (or in addition), the pathogenetic effect of AGEs could also be indirect, such as by induction of cytokines (e.g., tumor necrosis factor-α) (22) or growth factors (vascular permeability factor/vascular endothelial growth factor). Although mechanisms underlying increased perme-

ability in diabetes are likely to be complex, with contributions from hemodynamic, endothelial cell, and basement membrane perturbations (13, 23, 24) differing across the spectrum of organs (for example, kidney versus intestine) and changing with duration of disease, the reversal of vascular leakage by blockade of RAGE and antioxidants emphasizes a critical role for AGE–RAGE interaction. Indeed, our studies were performed after $\sim$ 12 wk of diabetes, and a component of the vascular leak observed is likely to be reversible at this stage versus that observed in animals with more long-standing diabetes.

Our results prompt further questions concerning intracellular signal transduction mechanisms activated by AGE–RAGE interaction. They emphasize the importance of evaluating efficacy of RAGE blockade in diverse diabetic models of vascular disease, including animals diabetic for longer periods of time and whose glucose levels are regulated by exogenous insulin to more closely simulate those in clinical diabetes, and point to the need for experiments to determine the duration of physiologic consequences of blocking RAGE. In this context, sRAGE probably acts by binding to soluble and tissue-bound AGEs, potentially blocking its interaction with RAGE as well as other potential AGE-binding proteins (5, 6, 25), although these data point to a significant role for RAGE in view of the protective effect of anti-RAGE IgG which directly binds to the receptor. These data constitute a first step in delineating the contribution of RAGE to a known diabetic complication, i.e., hyperpermeability, an important pathogenetic mechanism in the development of diabetic vasculopathy. Furthermore, we suggest that blockade of RAGE provides a novel target for therapeutic intervention in diabetic vascular complications.

Acknowledgments

We are grateful to Dr. Gabriel Godman (Dept. of Pathology, Columbia University) for his suggestions throughout the course of this work. The authors gratefully acknowledge the expert assistance with statistical analysis of the data by Dr. M. Debray and the expert assistance of Mary Osborne-Pellegrin.

This work was supported by a Research Fellowship to C. Zoukourian provided by Naturalia and Biologia, a fellowship from the ADR Biologie Vasculaire et Cellulaire, Association de Langue Française Etude du Diabete et Maladies Metaboliques, Public Health Service grants (AG00602, HL21006, and HL42507), Juvenile Diabetes Foundation, Council for Tobacco Research, New York Affiliate of the American Heart Association, and the Surgical Research Fund.

References

1. Ruderman, N., J. Williamson, and M. Brownlee. 1992. Glucose and diabetic vascular disease. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:2905–2914.

2. Baynes, J. 1991. Role of oxidative stress in development of complications in diabetes. *Diabetes.* 40:405–412.

3. Brownlee, M., A. Cerami, and H. Vlassara. 1988. AGEs in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.* 318:1315–1320.

4. Schmidt, A. M., O. Hori, J. Brett, S.-D. Yan, J. L. Wautier, and D. Stern. 1994. Cellular receptor for AGEs. *Arterioscler. Thromb.* 14:1521–1528.

5. Yang, Z., Z. Makita, Y. Horii, S. Brunelle, A. Cerami, P. Sehajpal, M. Suthanthiran, and H. Vlassara. 1991. Two novel rat liver membrane proteins that bind AGEs: relationship to macrophage receptor for glucose-modified proteins. *J. Exp. Med.* 174:515–524.

6. Khoury, J., C. Thomas, J. Loike, S. Hickman, L. Cao, and S. Silverstein. 1994. Macrophages adhere to glucose-modified basement membrane via their scavenger receptors. *J. Biol. Chem.* 269:10197–10200.

7. Brett, J., A. M. Schmidt, S. D. Yan, Y. S. Zou, E. Weidman, D. Pinsky, R. Nowygrod, M. Neeper, C. Przysiecki, A. Shaw, et al. 1993. Survey of the distribution of a newly characterized receptor for AGEs in tissues. *Am. J. Pathol.* 143:1699–1712.

8. Ritthaler, U., N. Roth, A. Bierhaus, R. Ziegler, A. M. Schmidt, R. Waldherr, P. Wahl, D. Stern, and P. Nawroth. 1995. Expression of RAGE in peripheral occlusive vascular disease. *Am. J. Pathol.* 146:688–694.

9. Viberti, G. 1983. Increased capillary permeability in diabetes mellitus and its relationship to microvascular angiopathy. *Am. J. Med.* 75:81–84.

10. Mattock, M., N. Morrish, G. Viberti, H. Keen, A. Fitzgerald, and G. Jackson. 1992. Prospective study of microalbuminuria as predictor of mortality in NIDDM. *Diabetes.* 41:736–741.

11. Yan, S. D., A. M. Schmidt, G. M. Anderson, J. Zhang, J. Brett, Y. S. Zou, D. Pinsky, and D. Stern. 1994. Enhanced cellular oxidant stress by the interaction of advanced glycation endproducts with their receptors/binding proteins. *J. Biol. Chem.* 269:9889–9897.

12. Esposito, C., H. Gerlach, J. Brett, D. Stern, and H. Vlassara. 1989. Endothelial receptor-mediated binding of glucose-modified albumin is associated with increased monolayer permeability and modulation of cell surface coagulant properties. *J. Exp. Med.* 170:1387–1407.

13. Albelda S., P. M. Sampson, F. R. Haselton, J. M. McNiff, S. N. Meller, S. K. Williams, and A. P. Fishman. 1988. Permeability characteristics of cultured endothelial cell monolayers. *J. Appl. Physiol.* 64:308–322.

14. David, G., and R. Reisfeld. 1974. Protein iodination with solid state lactoperoxidase. *Biochemistry.* 13:1014–1020.

15. Schmidt, A.-M., S.-D. Yan, J. Brett, R. Mora, R. Nowygrod, and D. Stern. 1993. Regulation of human mononuclear phagocyte migration by cell surface binding proteins for advanced glycation end products. *J. Clin. Invest.* 91: 2155–2168.

16. Williamson, J. R., K. Chang, R. G. Tilton, C. Prater, J. R. Jeffrey, C. Weigel, and W. R. Sherman. 1987. Increased vascular permeability in spontaneously diabetic BB/W rats and in rats with mild versus severe streptozotocin-induced diabetes. *Diabetes.* 36:813–821.

17. Wautier, J. L., M. P. Wautier, A. M. Schmidt, G. M. Anderson, O. Hori, C. Zoukourian, L. Capron, O. Chappey, J. Brett, P. J. Guillausseau, and D. Stern. 1994. AGEs on the surface of diabetic erythrocytes bind to the vessel wall via a specific receptor inducing oxidant stress in the vasculature: a link between surface-associated AGEs and diabetic complications. *Proc. Natl. Acad. Sci. USA.* 91:7742–7746.

18. Schmidt, A. M., M. Hasu, D. Popov, J. H. Zhang, J. Chen, S. D. Yan, J. Brett, R. Cao, K. Kuwabara, G. Costache, et al. 1994. The receptor for AGEs has a central role in vessel wall interactions and gene activation in response to circulating AGE-proteins. *Proc. Natl. Acad. Sci. USA.* 91:8807–8811.

19. Wautier, J. L., C. Paton, M. P. Wautier, D. Pintigny, E. Abadie, P. Passa, and J. Caen. 1981. Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications. *N. Engl. J. Med.* 305:237–242.

20. Shasby, D., and R. Roberts. 1987. Transendothelial transfer of macromolecules in vitro. *Fed. Proc.* 46:2506–2510.

21. Yaqoob, M., A. W. Patrick, P. McClelland, A. Stevenson, H. Mason, M. C. White, and G. M. Bell. 1993. Relationship between markers of endothelial dysfunction, oxidant injury and tubular damage in patients with insulin-dependent diabetes mellitus. *Clin. Sci.* 85:557–562.

22. Vlassara, H., M. Brownlee, K. Manogue, C. Dinarello, and A. Pasagian. 1988. Cachectin/TNF and IL-1 induced by glucose-modified proteins: role in normal tissue remodeling. *Science (Wash. DC).* 240:1546–1548.

23. Zatz, R., B. Dunn, T. Meyer, S. Anderson, H. Rennke, and B. Brenner. 1986. Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J. Clin. Invest.* 77:1925–1930.

24. Parving, H., G. Viberti, H. Keen, J. Christiansen, and N. Lassen. 1983. Hemodynamic factors in the genesis of diabetic microangiopathy. *Metab. Clin. Exp.* 32:943–949.

25. Schmidt, A. M., M. Vianna, M. Gerlach, J. Brett, J. Ryan, J. Kao, C. Esposito, H. Hegarty, W. Hurley, M. Clauss, et al. 1992. Isolation and characterization of binding proteins for advanced glycation endproducts from lung tissue which are present on the endothelial cell surface. *J. Biol. Chem.* 267:14987–14997.

26. Yamoaka, K., T. Nakagawa, and T. Uno. 1978. Application of Akaike's information criterion (AIC) in the evaluation of linear pharmacokinetic equations. *J. Pharmacokinet. Biopharm.* 6:165–175.