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Recombinant Advanced Glycation End Product Receptor Pharmacokinetics in Normal and Diabetic Rats

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SUMMARY

Vascular dysfunction in patients with diabetes mellitus is related to advanced glycation end product (AGE) formation. We previously showed that AGEs produce an increase in vascular permeability and generated an oxidant stress after binding to the receptor (RAGE) present on endothelium. RAGE, a 35-kDa protein that belongs to the immunoglobulin superfamily, has been cloned from a rat lung cDNA library, and recombinant rat soluble RAGE (rR-RAGE) has been produced in insect cells. The sequence of RAGE is highly conserved between human and rat. We studied the biological effect of rR-RAGE and pharmacokinetics of ^{125}I-rR-RAGE after intravenous or intraperitoneal administration in normal and streptozotocin-induced diabetic rats. rR-RAGE prevented albumin or inulin transfer through a bovine aortic endothelial cell monolayer, restored the hyperpermeability observed in diabetic rats or induced in normal rats by diabetic rat red blood cells, and corrected the reactive oxygen intermediate production after intravenous or intraperitoneal administration. After intravenous injection of ^{125}I-rR-RAGE, the distribution half-life was longer ($p \leq 0.01$) in diabetic (0.15 and 4.01 hr) than in normal (0.02 and 0.21 hr) rats, as was the case for the elimination half-lives (diabetic, 57.17 hr; normal, 26.02 hr; $p \leq 0.01$). Distribution volume was higher in diabetic than in normal rats (6.94 and 3.24 liter/kg, respectively; $p = 0.049$). Our study showed that rR-RAGE was biologically active *in vivo* and slowly cleared, which suggests it could be considered as a potential therapy.

Vascular dysfunction is one of the main complications in patients with diabetes mellitus. Several hypotheses have been proposed to explain vascular abnormalities linked to hyperglycemia, including sorbitol toxicity (1), diacylglycerol protein kinase activation (1), AGE formation (1, 2), and reactive oxygen species production (3). AGEs are the ultimate products of a nonenzymatic glycation of primary amino groups on proteins or lipids. These compounds are yellow-brown with characteristic fluorescence spectrum (1–3). The RAGE has been purified and cloned; it is a protein of 35 kDa that belongs to the immunoglobulin superfamily. It is composed of extracellular domains (one V-type domain followed by two C-type domains), a single transmembrane spanning domain, and a highly charged cytosolic tail (4). The RAGE is present on several cell types, including ECs, smooth muscle

cells, monocytes/macrophages, cardiac myocytes, neural tissue, and hepatocytes (5).

We recently showed that AGE engagement to a receptor present on ECs produces an oxidant stress and results in an increased vascular permeability (6, 7). We showed that sRAGE form could be used to prevent the consequences of the presence of AGE in the plasma and demonstrated that the newly described sRAGE prevents the effects of AGEs on EC functions and on the hyperpermeability observed in streptozotocin-induced diabetic rats or in normal rats that had been transfused with diabetic rat RBCs (7). sRAGE acts by binding to AGEs, which blocks their interaction with endogenous RAGE and restores vascular functions (7).

Because we previously established that AGE-RBCs induced an oxidant stress and were responsible for the increase in vascular permeability, in the current study we explored the properties of soluble rR-RAGE produced in insect cells on vascular permeability and reactive oxygen intermediate for-

ABBREVIATIONS: AGE, advanced glycation end product; EC, endothelial cell; RAGE, receptor for AGE; sRAGE, soluble form of RAGE; RBC, red blood cell; rR-RAGE, recombinant rat RAGE; TBARS, thiobarbituric acid-reactive substances; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Fab, fragment antigen binding; TBIR, tissue-blood isotope ratio; TCA, trichloroacetic acid; $t_{1/2}\lambda 1$, first distribution half-life; $t_{1/2}\lambda 2$, second distribution half-life; $t_{1/2}\lambda z$, elimination half-life; CL, total clearance; V_z, volume of distribution of the elimination phase; CL_R, renal clearance; *Sf9, Spodoptera frugiperda*.

mation. We observed that as was the case with purified RAGE, rR-RAGE prevented the increased albumin or inulin transfer through a bovine aortic EC monolayer and corrected the hyperpermeability found in diabetic rats or induced by infusion of diabetic rat RBCs into normal rats. Because rR-RAGE seems to be efficient, we studied the pharmacokinetic parameters to evaluate the possible therapeutic use of such a recombinant protein.

Materials and Methods

Cloning, Expression, and Purification of Soluble rR-RAGE

Full-length rat RAGE cDNA was cloned by screening a lung cDNA library (Clontech Laboratories, Palo Alto, CA) with a 187-bp DNA probe corresponding to the signal sequence of human RAGE (8). Filters were hybridized in a buffer of 50% formamide/5× SSC/10 mM sodium phosphate, pH 6.4, and 0.1% SDS, 1× Denhardt's solution, and 50 μg/ml sonicated sperm DNA for 16 hr at 42°. Filters were washed in 6× SSC/0.1% SDS four times each at room temperature, followed by washing in 1× SSC/0.1% SDS at 48° and exposure for 48 hr to X-ray film. Approximately 1×10^6 plaques were screened. The longest insert DNA from positive plaques was sequenced according to the dideoxy chain termination method (9). A DNA fragment coding for soluble RAGE was obtained using a polymerase chain reaction with primers 5′-CATGCCAGCGGGGACAG-CAGCT-3′ and 5′-TAGCGTACCCAGCCCAGACTC-3′. The polymerase chain reaction product was first subcloned into the pCR II vector (InVitrogen, San Diego, CA), and the EcoRI fragment of the resultant plasmid was cloned into the pBacPAK8-based vector (Clontech), which contained an in-frame stop codon.

Baculovirus expression of rR-RAGE was performed by cotransfecting the plasmid pBacPAK8/RAGE with a linearized BacPAK6 viral DNA (Clontech) into Sf9 cells according to the manufacturer's instructions. Recombinant plaques were identified and purified by their β-galactosidase-negative phenotype. rR-RAGE was purified from Sf9 media by chromatography on a SP Sepharose fast-flow column (Pharmacia, Uppsala, Sweden) equilibrated with sodium phosphate (20 mM, pH 7.5) and eluted with a salt gradient (0–0.5 M NaCl in the equilibration buffer). Fractions containing rR-RAGE were applied to Superdex 200PG (Pharmacia) in PBS to obtain an homogeneous sample of ~35 kDa on SDS-PAGE. This protein corresponded to that expected for the extracellular domain (2).

Radiolabeling of Proteins

rR-RAGE, albumin (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France), and murine Fab were labeled with ^{125}I-Na according to the iodogen method (10). One hundred micrograms of protein was incubated with 0.5 mCi of ^{125}I-Na in Eppendorf tubes coated with 10 μg of iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma-Aldrich Chimie) for 5 min at room temperature. Free iodine was removed by chromatography on a Sephadex G 25-M PD 10 column (Pharmacia) equilibrated with PBS. Precipitation of the iodinated protein by TCA (10%) at +4° gave >95% of bound iodine. Specific activities were in the range of 0.9 μCi/μg for rR-RAGE, 2.5 μCi/μg for albumin, and 1.15 μCi/μg for murine Fab. The purities of ^{125}I-rR-RAGE, ^{125}I-albumin, and ^{125}I-Fab preparations were also analyzed by SDS-PAGE.

Human Diabetic RBCs, Cultured ECs, and In Vitro Permeability Assays

RBCs were obtained from normal subjects and diabetic patients in accordance with the provisions of the Declaration of Helsinki and the rules of our institution. Diabetic patients and normal subjects were selected according to criteria previously described (7). In vitro permeability assays were performed according to our previous protocol (7). ECs were incubated with medium alone, normal RBCs, or dia-

betic RBCs (2.5×10^9 cells/ml) for 24 hr. To study the effect of rR-RAGE, ECs were incubated with diabetic RBCs with or without rR-RAGE (60 μg/ml). Permeability coefficient (P) was determined by using ^{125}I-albumin and ^{3}H-inulin in medium: $P = J \times (1/A) \times [1/(C_T - C_B)]$, 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 (7, 11).

In Vivo Permeability Studies

In vivo permeability studies were carried out as previously described (7). Normal rats received either normal RBCs, diabetic RBCs, or diabetic RBCs plus rR-RAGE (5.15 mg/kg), and diabetic rats received the same dose of R-RAGE by an intravenous bolus. In vivo permeability in normal and diabetic rats was determined using the TBIR method (7, 12).

TBARS Assay

The effect of rR-RAGE (2.25 mg/kg) on the oxidant stress was determined by measurement of TBARS, which form a fluorescent compound after a reaction with thiobarbituric acid (13) (Sobioda, Grenoble, France). Serum was stored at −20° until analysis; before assay, t-butyl-4-hydroxyanisole (Sigma-Aldrich Chimie) was added to the specimens in a final concentration of 10 μmol/liter to prevent artificial auto-oxidation.

Pharmacokinetics of ^{125}I-rR-RAGE or Murine ^{125}I-Fab

Plasma, urine, and tissue kinetics of ^{125}I-rR-RAGE were performed in normal and diabetic male Wistar rats (200–250 g) placed into metabolic cages after the injection. Animals had free access to food and water before the experiments and were anesthetized with ether before intravenous or intraperitoneal administration of ^{125}I-rR-RAGE (250 μg/kg, volume ≤1 ml). For intravenous administration, ^{125}I-rR-RAGE was injected in bolus via the femoral vein, and blood samples (50 μl) were collected in heparinized tubes via the tail vein at 2, 5, 10, 30, and 45 min and 1, 1.5, 2, 3, 6, 8, 24, 30, 48, 54, 72, and 96 hr. After intraperitoneal administration, blood samples were collected at the same times except that the first sample was taken at 10 min. The blood hematocrit measured at 6, 24, 54, and 96 hr after ^{125}I-rR-RAGE injection did not differ from physiological values.

Rats were killed at 96 hr, and radioactivity in organs (kidney, liver, spleen, intestine, vena cava, aorta, lung, and skin) was determined. Furthermore, tissue distribution of ^{125}I-rR-RAGE was studied in normal and diabetic rats at the time corresponding to 87.5% of the ^{125}I-rR-RAGE distribution (i.e., 38 min and 12 hr for normal and diabetic rats, respectively). The amount of ^{125}I-rR-RAGE determined in organs was corrected by the presence of radioactivity in the residual blood remaining in the tissue (14). Urine was collected at 1 and 2 hr and then at each blood collection. Blood samples were centrifuged at $3000 \times g$ for 10 min, and plasma was isolated. The TCA-precipitable fraction of plasma (20 μl) and urine (150 μl) was counted in a gamma counter (Minaxi Gamma 5000; Packard Instruments, Rungis, France). Creatinine clearance in normal and diabetic rats was calculated using creatinine concentration in urine and plasma at the midpoint of the urine collection interval.

To assess whether ^{125}I-rR-RAGE could induce the urinary excretion of AGEs in diabetic rats, urinary samples were incubated with anti-AGE antibodies (molar ratio of ^{125}I-rR-RAGE to anti-AGE antibodies = 1) for 1 hr at +37° and then overnight at +4°. The ^{125}I-rR-RAGE/AGE complexes were then separated from ^{125}I-rR-RAGE unbound with polyethylene glycol precipitation (7% of polyethylene glycol in borate buffer).

Pharmacokinetics of a molecule belonging to the immunoglobulin superfamily was also performed to assess whether pharmacokinetic parameters determined in diabetic rats could be influenced by AGEs or by the hyperpermeability observed in diabetic animals. Pharmacokinetics of a monoclonal murine ^{125}I-Fab (250 μg/kg) was performed in normal (five rats) and diabetic (three rats) rats after

intravenous bolus injection according to the experimental protocol described for ^{125}I-rR-RAGE.

Pharmacokinetic Analysis

Plasma concentration-time data for ^{125}I-rR-RAGE were analyzed using Siphar Software (SIMED, Créteil, France) and fitted to a three-compartment model. We used the Powell minimization algorithm, and the model was fitted to data using weighed least-squares with a $1/y^2$ weighing factor. To select the most appropriate model, an examination of the standard deviation of distribution and elimination rate constants ($\lambda 1$, $\lambda 2$, λz) and half-lives ($t_{1/2}\lambda 1$, $t_{1/2}\lambda 2$, $t_{1/2}\lambda z$) was performed. In addition, the likelihood test and Akaike criterion were tested. The distribution ($t_{1/2}\lambda 1$ and $t_{1/2}\lambda 2$) and terminal ($t_{1/2}\lambda z$) half-lives were calculated as $0.693/\lambda$. The area under the plasma ^{125}I-rR-RAGE concentration-time curve from zero to infinity was determined by linear trapezoidal estimation from 0 to the last measured time with extrapolation to infinity by adding the value of the last measured plasma concentration divided by the terminal rate constant. The CL, steady state volume of distribution, V_z, and mean residence time were calculated using standard equations (15).

The maximal concentration (C_{max}) and corresponding experimental time (T_{max}) after the intraperitoneal injection of ^{125}I-rR-RAGE were experimentally observed values. The absorption rate coefficient, distribution and elimination half-lives ($t_{1/2}\lambda 1$, $t_{1/2}\lambda z$), and absolute bioavailability (F) were determined using Siphar Software. Plasma concentration-time data were fitted to a two-compartment model with the Powell minimization algorithm and a $1/y^2$ weighing factor. The absolute bioavailability was calculated by the area ratio method using the mean values of pharmacokinetic parameters (16).

The ^{125}I-rR-RAGE CL_R was calculated between 0 and 96 hr after ^{125}I-rR-RAGE injection (15), and CL_R mechanisms were analyzed by plotting the urinary excretion rate of ^{125}I-rR-RAGE versus the corresponding plasma concentration at the midpoint of the urine collection interval. These concentrations were calculated from the plasma ^{125}I-rR-RAGE concentration-time curve. CL_R of ^{125}I-rR-RAGE was also estimated after linear regression analysis on the basis of the slope of the urinary excretion rate versus plasma concentration.

Plasma pharmacokinetics of ^{125}I-Fab was described by a two-compartment model, and pharmacokinetic parameters were calculated as previously calculated for ^{125}I-rR-RAGE.

Identification of ^{125}I-rR-RAGE

Immunoidentification of ^{125}I-rR-RAGE. Specific anti-RAGE antibodies (6) were used in an immunoprecipitation assay to determine the immunoreactive fraction of radiolabeled rR-RAGE before and after injection in rats. ^{125}I-rR-RAGE sample (25 μl) was incubated with the anti-RAGE antibodies (50 μl; dilution, 1/12) and PBS (225 μl) for 1 hr at 37° and 1 hr at 4°. Polyethylene glycol 8000 (7% in borate buffer) (Sigma-Aldrich Chimie) was added and incubated overnight. The precipitate isolated by centrifugation was counted in a gamma counter (Packard Instruments).

SDS-PAGE analysis. Plasma and urine samples containing ^{125}I-rR-RAGE were analyzed by SDS-PAGE on a 15% acrylamide gel under nonreducing conditions. Radiolabeled proteins and metabolites were autoradiographed using X-ray film (Amersham, Les Ulis, France) and intensifying screens for 8 weeks. The migration zone of labeled proteins or metabolites were compared with prestained standards (phosphorylase b, 101 kDa; bovine serum albumin, 83 kDa; ovalbumin, 50.6 kDa; carbonic anhydrase, 35.5 kDa; soybean trypsin inhibitor, 29.1 kDa) (BioRad, Paris, France).

Statistical Analysis

Results are presented as mean ± standard error. Two-way analysis of variance followed by parametric Dunnett's test in the event of significant differences was used to compare permeability of ECs in the presence of normal or diabetic RBCs and the results of *in vivo* permeability studies. Mean values of pharmacokinetic parameters

were compared using the nonparametric Mann-Whitney two-sample test.

Results

Cloning of Full-Length Rat RAGE cDNA

To isolate full-length rat RAGE cDNA, a rat lung cDNA library was screened with a probe corresponding to the signal sequence of human RAGE with moderately high stringency. Approximately 100 positive clones were obtained from 1 million screened plaques; this is similar to the number of positive clones observed in a human lung cDNA library (4). Several clones were further characterized. The DNA sequence and the deduced amino acid sequence of the longest insert are shown in Fig. 1. Rat RAGE has 81% sequence similarity to that of human RAGE at the nucleic acid level and 88% at the amino acid level. All the cysteine residues are conserved between rat and human RAGE, indicating their structural importance.


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AGCCACAGTA GGAAGTGGGG CAGACAGAAC CAGGAGCCTG GGAAGGAAGC   50
         M   P   T    G   T   V   A   R   A    W   V   L   V    L   A   L
ACCATGCCAA CGGGGACAGT AGCTAGAGCC TGGGTACTGG TTCTTGCTCT  100
 W   G   A    V   A   G   G    Q   N   I   T   A   R    I   G   E   P
GTGGGGAGCC GTAGCTGGTG GTCAGAACAT CACAGCCCGG ATCGGAGAGC  150
 L   M   L    S   C   K    G   A   P   K    K   P   T    Q   K   L
CACTTATGCT GAGCTGTAAG GGGGCCCCTA AGAAGCCAAC CCAGAAGCTA  200
 E   W   K   L    N   T   G    R   T   E    A   W   K   V    L   S   P
GAATGGAAAC TGAACACAGG AAGGACTGAA GCTTGGAAGG TCCTCTCTCC  250
 Q   G   D    P   W   D   S    V   A   R    I   L   P    N   G   S   L
CCAGGGAGAC CCCTGGGACA GTGTGGCTCG AATCCTCCCC AATGGTTCAC  300
 L   L   P    A   I   G    I   V   D   E    G   T   F    R   C   R
TCCTCCTTCC AGCTATCGGA ATTGTCGATG AGGGGACTTT CCGGTGTCGG  350
 A   T   N   R    L   G   K    E   V   K    S   N   Y   R    V   R   V
GCAACTAACA GGCTTGGGAA GGAGGTCAAG TCCAACTACC GAGTCCGAGT  400
 Y   Q   I    P   G   K   P    E   I   V    N   P   A    S   E   L   T
CTACCAGATT CCTGGGAAGC CGGAAATTGT GAATCCTGCC TCTGAACTCA  450
 A   N   V    P   N   K    V   G   T   C    V   S   E    G   S   Y
CAGCCAATGT CCCTAATAAG GTGGGGACGT GTGTGTCTGA GGGGAGCTAC  500
 P   A   G   T    L   S   W    H   L   D    G   K   P   L    I   P   D
CCTGCAGGGA CCCTTAGCTG GCACTTGGAT GGGAAACCTC TGATTCCTGA  550
 G   K   G    T   V   V   K    E   E   T    R   R   H    P   E   T   G
TGGCAAAGGG ACAGTTGTGA AGGAGGAGAC CAGGAGGCAC CCTGAGACGG  600
 L   F   T    L   R   S    E   L   T   V    T   P   A    Q   G   G
GACTCTTCAC GCTTCGGTCA GAGCTCACAG TGACCCCAGC CCAAGGAGGG  650
 T   T   P   T    Y   S   C    S   F   S    L   G   L   P    R   R   R
ACCACTCCTA CCTATTCCTG CAGCTTCAGT CTGGGCCTTC CTCGGCGCAG  700
 P   L   N    T   A   P   I    Q   P   V   R   V   E    P   L   P   P
ACCCCTGAAC ACAGCCCCCA TCCAGCCCCG AGTCAGGGAG CCCCTGCCTC  750
 E   G   I    Q   L   L    V   E   P   E    G   G   T    V   A   P
CAGAGGGCAT TCAGCTGTTG GTTGAGCCTG AAGGTGGAAC AGTCGCTCCT  800
 G   G   T   V    T   L   T    C   A   I    S   A   Q   P    P   P   Q
GGTGGGACCG TGACCCTGAC CTGTGCCATC TCTGCCCAGC CTCCCCCTCA  850
 I   H   W    I   K   D   G    T   P   L    P   L   A    P   S   P   V
AATCCACTGG ATAAAAGATG GCACACCCCT GCCCCTTGCC CCCAGCCCTG  900
 L   L   L    P   E   V    G   H   E   D    E   G   I    Y   S   C
TGCTGCTCCT CCCTGAGGTA GGGCATGAGG ATGAGGGCAT CTACAGCTGC  950
 V   A   T   H    P   S   H    G   P   Q    E   S   P   P    V   N   I
GTGGCCACCC ACCCTAGCCA TGGACCTCAG GAAAGCCCTC CTGTCAACAT 1000
 R   V   T    E   T   G   D    E   G   Q    A   A   G    S   V   D   G
CAGGGTCACA GAAACCGGTG ATGAAGGACA AGCTGCAGGC TCTGTGGATG 1050
 S   G   L    G   T   L    A   L   A   L    G   I   L    G   G   L
GGTCTGGGCT GGGAACGCTA GCCCTGGCCT TAGGGATCCT GGGGAGGCCTG 1100
 G   I   A   A    L   L   I    G   A   I    L   W   R   K    R   Q   P
GGAATAGCCG CTCTGCTCAT TGGGGCCATC CTGTGGCGAA AACGACAACC 1150
 R   L   E    E   R   K   A    P   E   S    Q   E   D    E   E   E   R
CAGACTCGAG GAGAGGAAGG CCCCAGAAAG CCAGGAGGAC GAGGAGGAAC 1200
 A   E   L    N   Q   S    E   E   A   E    M   P   E    N   G   A
GTGCAGAGCT GAATCAGTCA GAGGAAGCGG AAATGCCAGA GAATGGTGCA 1250
 G   G   P
GGGGGACCTT AAGAGCGCCC AGGCAAACCC TGTCTCCTTC AGCTTCCGAC 1300

CTCAGCTGTG CTGGCTCCAG ACGAGCTCCC CCACTCTACG ATCCCAATTC 1350

AACCTCGAGC CACTTTCTTC TCCAACCAGA GCCCACATGA TCCATGCTGA 1400

GTAAACACCT GACACATGTT AAAAAAAAAA AAAA
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Fig. 1. Nucleotide and deduced amino acid sequence of rat RAGE. The nucleotide sequence was obtained from both strands using the dideoxy chain termination method. *Underlined*, stop codon.

Expression and Characterization of rR-RAGE

To express a large amount of soluble rR-RAGE for the permeability studies, a DNA fragment coding for sRAGE was obtained using a polymerase chain reaction and cloned into a baculovirus expression vector. After the cotransfection of plasmid pBacPAK8/RAGE with BacPAK 6 viral DNA into *Sf 9* cells, recombinant plaques were identified, and media were screened for the expression of recombinant protein by Western blotting using monoclonal antibody raised against rR-RAGE.

rR-RAGE was purified from the conditioned media (Fig. 2A, *lane 02*) on an SP Sepharose fast-flow column and equilibrated with 20 mM sodium phosphate buffer, pH 7.5. Using a 0–0.5 M salt gradient, rR-RAGE was eluted from the column between fractions 17–28 (Fig. 2A, *lanes 03–14*). Contaminants were removed from the pooled fractions using gel filtration chromatography (Fig. 2B). The major band corresponds to purified rR-RAGE, with a molecular mass of 42 kDa. The faint band seen slightly below 30 kDa protein marker is a cleaved form of rR-RAGE because it also binds to monoclonal anti-RAGE antibody (result not shown). The fractions containing rR-RAGE were pooled and used to test the effect of sRAGE on permeability.

***In Vitro* Permeability.** The transfer of molecular tracers (^{125}I-albumin and ^{3}H-inulin in Fig. 3, A and B, respectively) through the EC barrier was similar in minimal essential
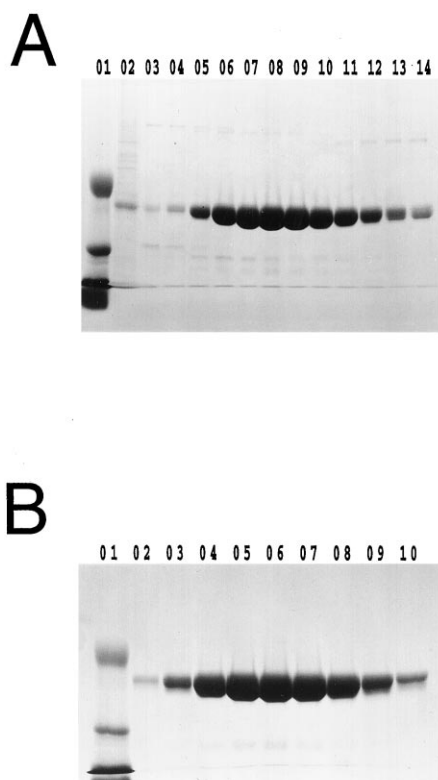

Fig. 2. SDS-PAGE of fractions from SP Sepharose fast-flow chromatography (A) and gel filtration chromatography (B) of *Sf9* cell-conditioned media containing rR-RAGE. All the samples are denatured, reduced with 50 mM dithiothreitol, and boiled before the application. A, *Lane 01*, molecular mass markers. *Lane 02*, conditioned media. *Lanes 03–14*, fractions 17 to 28. B, *Lane 01*, molecular mass markers. *Lanes 02–10*, fractions 25–33. The largest molecular mass marker is 45 kDa, and the band below is 30 kDa.
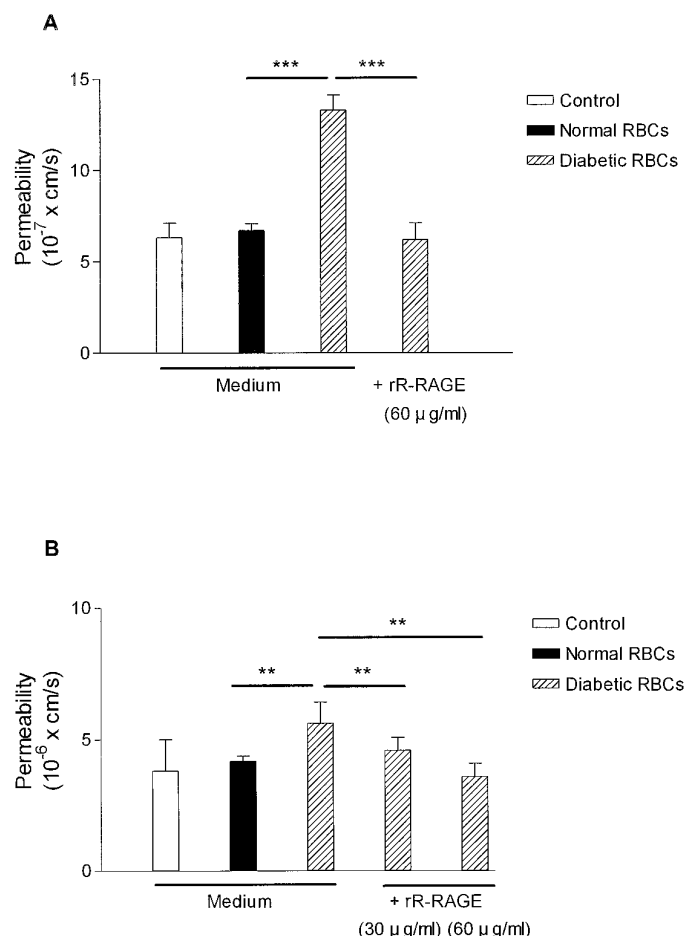

Fig. 3. Effect of diabetic RBCs on the barrier function of bovine cultured EC monolayers. ECs monolayers were incubated with minimal essential medium (seven rats), with minimal essential medium plus normal RBCs (10 rats), or diabetic RBCs (nine rats). Effect of rR-RAGE was studied in minimal essential medium plus diabetic RBCs and rR-RAGE ($n = 5$). A, Permeability of cultured EC monolayers to ^{125}I-albumin. B, Permeability of cultured EC monolayers to ^{3}H-inulin. The results are presented as mean values. *Bars*, mean ± standard error. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

medium plus albumin or in the presence of normal RBCs. The addition of diabetic RBCs to ECs significantly increased by 2-fold ($p \leq 0.001$) and 1.3-fold ($p \leq 0.01$) the permeability to ^{125}I-albumin or ^{3}H-inulin, respectively. Preincubation of RBCs with rR-RAGE prevented the effect of diabetic RBCs on permeability of the EC monolayer; this effect was dependent on the rR-RAGE concentration for transfer of ^{3}H-inulin.

***In Vivo* Permeability.** Diabetic rats had a vascular hyperpermeability to albumin compared with normal rats. The vascular leakage was more pronounced in skin, intestine, kidney, vena cava, and heart, being 2.8-, 2.6-, 2.4-, 2.2-, and 2.1-fold increased, respectively (Fig. 4A). A bolus injection of rR-RAGE (5.15 mg/kg) corrected the enhanced permeability observed in diabetic rats. The correction of hyperpermeability was observed 1 hr after rR-RAGE injection and was higher in skin, intestine, vena cava, heart, and aorta (Fig. 4A). Infusion of diabetic RBCs in normal rats increased the permeability to albumin compared with normal RBCs infused in normal rats (Fig. 4B). The administration of rR-RAGE with the injection of diabetic RBCs prevented the increase of albumin transfer and extravasation (Fig. 4B) in
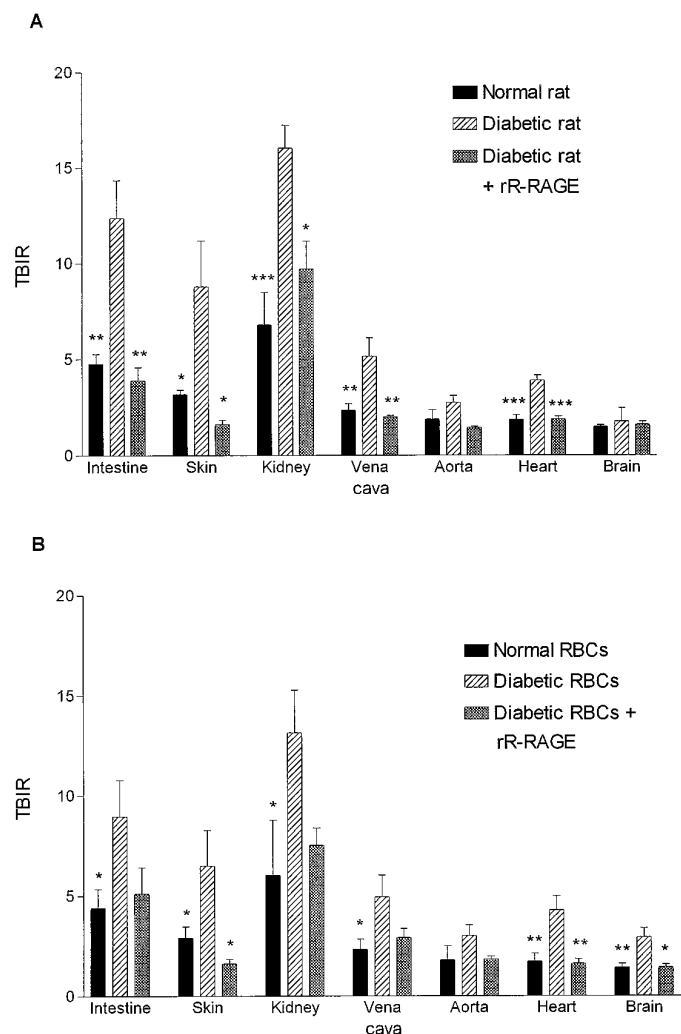
A

B

Fig. 4. A, Effect of rR-RAGE on vascular permeability. TBIR was determined in normal ($n = 6$), diabetic ($n = 7$), and diabetic rats pretreated with rR-RAGE (5.15 mg/kg). ^{125}I-albumin- and ^{51}Cr-labeled RBCs were injected in rats 40 min after RBCs or RBCs plus rR-RAGE, and TBIR was calculated 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 arterial blood sample harvested before excision of the heart. B, Effect of a transfusion of diabetic RBCs on vascular permeability. Normal (seven rats), diabetic RBCs (seven rats), or diabetic RBCs plus rR-RAGE (60 μg/ml) (seven rats) were injected in normal rats, and TBIR was determined 1 hr after the injection. The results are presented as mean values. *Bars*, mean $\pm$ standard error. ∗, $p < 0.05$. ∗∗, $p < 0.01$. ∗∗∗, $p < 0.001$.

skin, heart, brain, kidney, and vena cava. The results are in agreement with our previous study (7).

Effect of rR-RAGE on Oxidant Stress. Normal rats had significantly lower plasma TBARS levels than diabetic rats (1.76 ± 0.08 and 2.76 ± 0.12 μmol/liter, respectively; $p = 0.002$). Both intravenous and intraperitoneal administration of rR-RAGE significantly reduced plasma TBARS levels (2.08 ± 0.09 μmol/liter, $p = 0.011$, and 2.12 ± 0.04 μmol/liter, $p = 0.007$, respectively). This showed that rR-RAGE was efficacious in reducing oxidant stress after intravenous or intraperitoneal injection.

Plasma and Urine Pharmacokinetics. After intravenous bolus, ^{125}I-rR-RAGE plasma disposition in normal or diabetic rats was described by a three-exponential decay

(rats 25 and 26, respectively; Fig. 5). The mean pharmacokinetic parameters are presented in Table 1. Distribution and elimination half-lives were significantly different in normal and diabetic rats. A rapid initial distribution phase was observed in normal and diabetic rats (0.02 ± 0.01 and 0.15 ± 0.03 hr, respectively; $p = 0.008$), followed by a slower distribution phase in diabetic rats compared with that in normal rats (4.01 ± 0.87 and 0.21 ± 0.07 hr, respectively; $p = 0.008$). Finally, plasma concentrations decreased with different elimination half-lives of 26.02 ± 2.36 and 57.17 ± 11.62 hr for normal and diabetic rats, respectively ($p = 0.008$). The V_z of ^{125}I-rR-RAGE was significantly higher in diabetic rats than in normal rats (6.9 ± 1.8 and 3.24 ± 0.76 liter/kg, respectively; $p = 0.049$). Total body clearance of ^{125}I-rR-RAGE was higher but not significantly different in diabetic rats compared with normal rats ($p = 0.690$).

The total amount of ^{125}I-rR-RAGE in urine was not significantly different between the two groups of animals (5661 ± 2188 and 4893 ± 3141 ng for diabetic and normal rats, respectively; $p = 0.846$), and the precipitation of AGEs in the urine of diabetic rats that had received ^{125}I-rR-RAGE showed that only 0.3–1% of ^{125}I-rR-RAGE present in urine was bound to AGEs. CL_R was higher but not significantly different in diabetic rats compared with normal rats (2.52 ± 0.99 and 1.58 ± 0.75 ml/hr/kg, respectively; $p = 0.471$) and lower than creatinine clearance (263 ± 68 and 453 ± 139 ml/hr/kg, respectively; $p = 0.280$). This result was confirmed by the analysis of urinary excretion rate versus plasma concentration, which showed nonproportional relationships, indicating that glomerular filtration and tubular reabsorption characterized CL_R of ^{125}I-rR-RAGE in normal and diabetic rats. For normal rats, CL_R was 1.40 ± 0.50 ml/hr/kg for plasma concentration of ^{125}I-rR-RAGE below 35.32 ± 11.11 ng/ml and 57.53 ± 13.04 ml/hr/kg for plasma concentrations above 35.32 ± 11.11 ng/ml (five rats). For two diabetic rats, CL_R was 8.43 ± 4.75 ml/hr/kg for plasma concentrations of ^{125}I-rR-RAGE below 55 ± 0.63 ng/ml and 66.98 ± 19.73 ml/hr/kg for plasma concentrations above 55 ± 0.63 ng/ml. This tubular reabsorption did not exist in the three other diabetic rats and suggested that diabetes mellitus altered renal physiology to different extents and led to different urinary excretion in normal and diabetic animals.

Plasma pharmacokinetics of ^{125}I-rR-RAGE after intraperitoneal administration in normal or diabetic rats (rats 29 and 18, respectively; Fig. 5) was described by a rapid resorption phase with T_{max} of 1–2 hr followed by a two-exponential decay. Pharmacokinetic parameters (Table 2) were not significantly different in normal and diabetic rats, and the absolute bioavailability of ^{125}I-rR-RAGE was lower in diabetic rats (35%) than in normal rats (61%).

Plasma disposition of ^{125}I-Fab was characterized by a biexponential decline in normal and diabetic rats, with steady state volumes of distribution of 0.19 ± 0.01 and 0.12 ± 0.01 liter/kg, respectively ($p = 0.006$). All other pharmacokinetic parameters did not differ statistically.

Biodistribution of ^{125}I-rR-RAGE

In a limited group of animals (three normal rats and two diabetic rats), we studied the distribution of ^{125}I-rR-RAGE at the end of the distribution phase. ^{125}I-rR-RAGE showed a distribution profile between the different organs similar in normal and diabetic rats. Meanwhile, the amount of ^{125}I-rR-
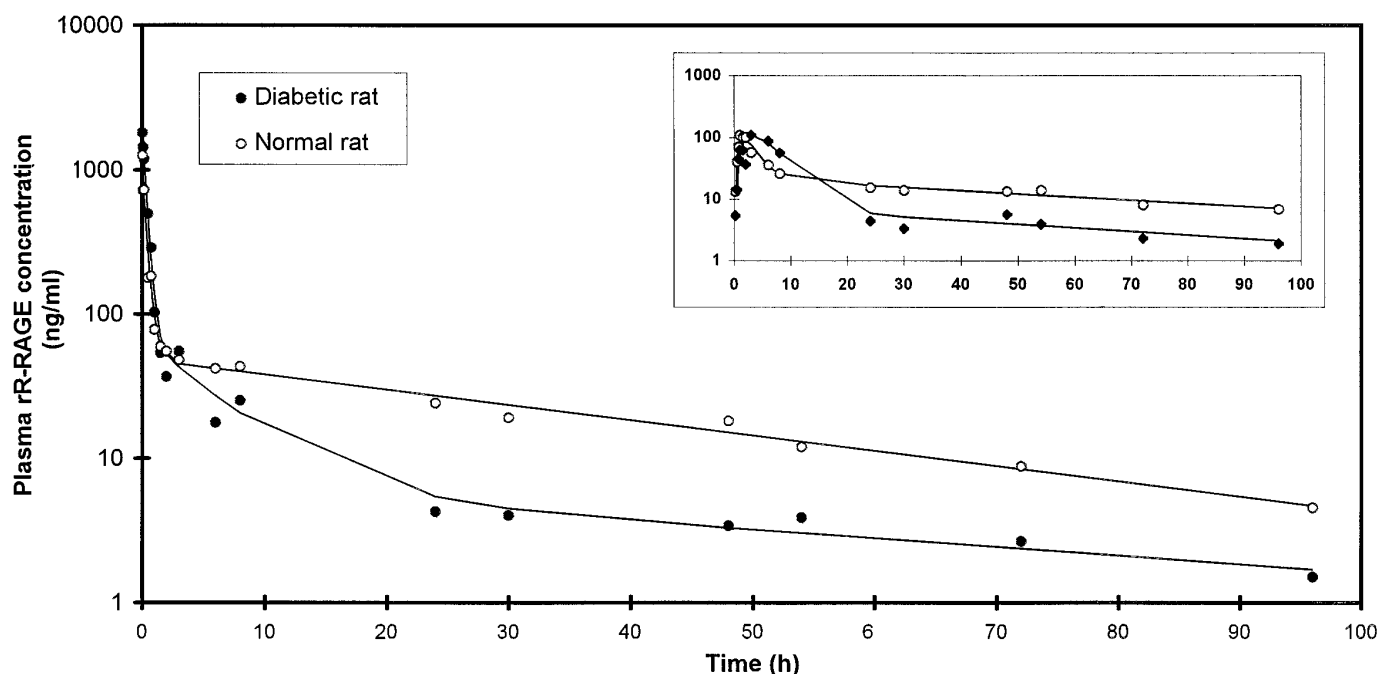
Fig. 5. Plasma concentration-time curves after intravenous injection of ^{125}I-rR-RAGE (250 μg/kg) in normal (rat 25) and diabetic (rat 26) rats and after intraperitoneal injection in normal (rat 29) and diabetic (rat 18) rats.

TABLE 1

Pharmacokinetic parameters of ^{125}I-rR-RAGE after intravenous administration

Values are mean $\pm$ standard error.

	$t_{1/2}\lambda 1$	$t_{1/2}\lambda 2$	$t_{1/2}\lambda z$	CL	V_z	V_{ss}	MRT	AUC
	hr	hr	hr	ml/hr/kg	liter/kg	liter/kg	hr	ng/hr/ml
Diabetic rat ($n = 5$)	0.15 ± 0.03	4.01 ± 0.87	57.17 ± 11.62	108.89 ± 50.09	6.94 ± 1.40	4.85 ± 1.24	34.11 ± 9.17	2856 ± 1120
Normal rat ($n = 5$)	0.02 ± 0.01	0.21 ± 0.07	26.02 ± 2.36	88.15 ± 22.92	3.24 ± 0.76	2.60 ± 0.61	32.41 ± 3.71	3152 ± 916
p	0.008	0.008	0.008	0.690	0.049	0.032	1.000	0.690

V_{ss}, steady state volume of distribution; MRT, mean residence time; AUC, area under the curve.

TABLE 2

Pharmacokinetic parameters of ^{125}I-rR-RAGE after intraperitoneal administration

Values are mean $\pm$ standard error.

	C_{max}	T_{max}	K_a	$t_{1/2}\lambda 1$	$t_{1/2}\lambda z$	AUC	F
	ng/ml	hr	hr	hr	hr	ng/hr/ml	%
Diabetic rat ($n = 4$)	158.40 ± 49.33	2.06 ± 0.56	1.25 ± 0.72	3.38 ± 1.16	51.98 ± 6.31	1579 ± 242	35
Normal rat ($n = 3$)	126.57 ± 33.99	1.00 ± 0.00	0.58 ± 0.28	1.17 ± 0.50	48.98 ± 6.38	2102 ± 68	61
p	0.645	0.400	0.629	0.184	0.857	0.114	

AUC, area under the curve; F, bioavailability.

RAGE distributed in organs was higher in diabetic rats than in normal rats, which in agreement with the higher distribution volume of ^{125}I-rR-RAGE in diabetic rats (Fig. 6A). The difference between normal and diabetic rats did not reach the statistical level of significance for each organ, probably due to the limited number of animals studied. In normal and diabetic rats, ^{125}I-rR-RAGE distribution was higher in spleen (3501 $\pm$ 73 versus 2372 $\pm$ 416 ng/g of organ, $p = 0.123$), liver (1770 $\pm$ 31 versus 1454 $\pm$ 47 ng/g of organ, $p = 0.016$), kidney (1636 $\pm$ 123 versus 1264 $\pm$ 73 ng/g of organ, $p = 0.067$), and aorta (1414 $\pm$ 74 versus 745 $\pm$ 121 ng/g of organ, $p = 0.027$) than in other organs. At the end of the experiment, we did not find any significant difference between normal and diabetic rats. At the end of the distribution phase, the maximum

radioactivity corresponding to ^{125}I-rR-RAGE was found in the spleen, and after 96 hr, most of the radioactivity was in the kidney and, to a lesser extent, the liver (Fig. 6B). After intravenous or intraperitoneal administration, distribution of ^{125}I-rR-RAGE in organs was similar at the end of the experiment except that after intraperitoneal administration ^{125}I-rR-RAGE was 50% less tissue distributed.

Identification of ^{125}I-rR-RAGE

Immunoidentification of ^{125}I-rR-RAGE. After rR-RAGE labeling, >95% of the radioactivity was recovered after TCA precipitation. To further assess whether the radioactivity corresponded to rR-RAGE, we performed immunoprecipitation studies. The specific anti-RAGE antibodies precipitated 75–
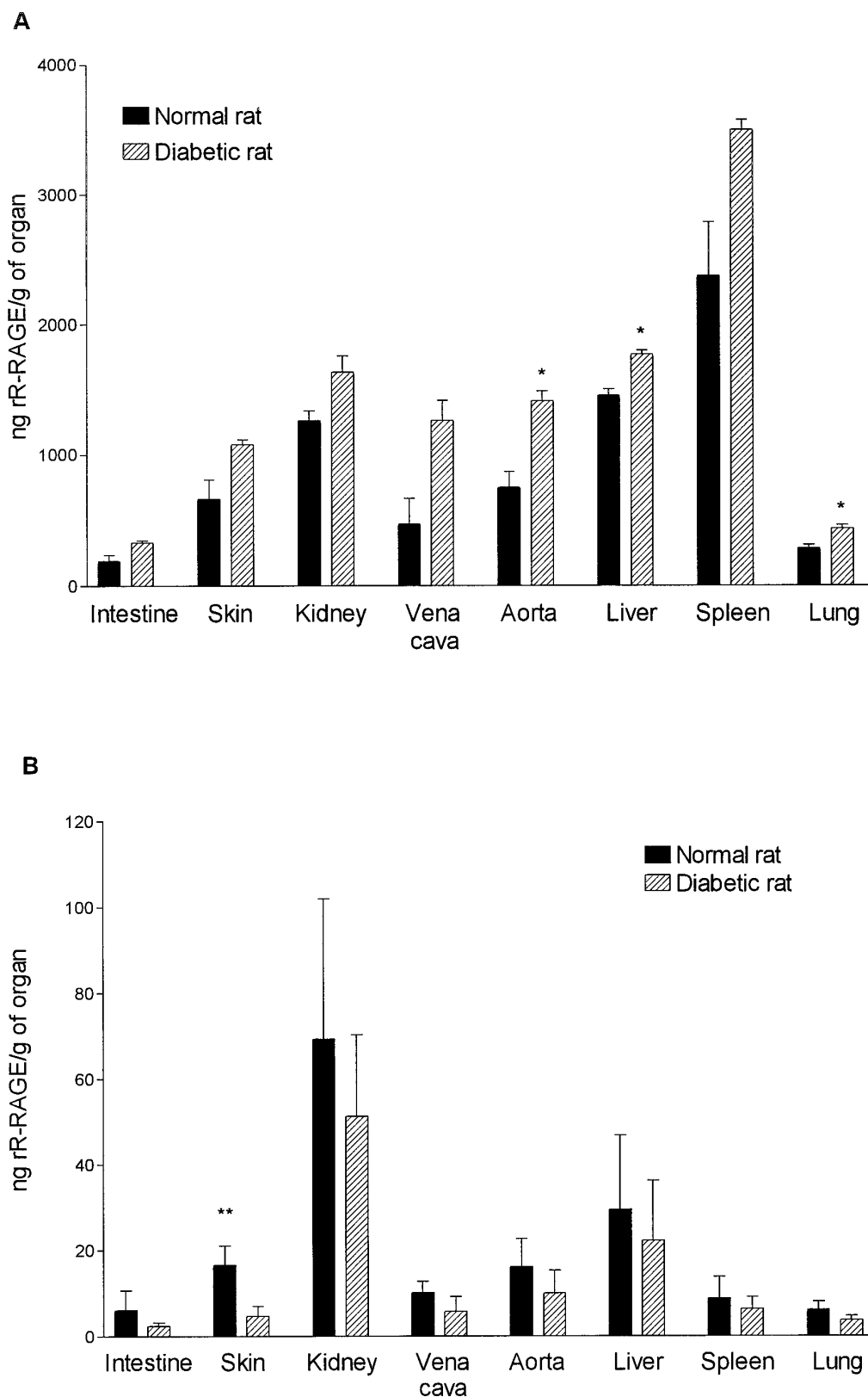
A

B

Fig. 6. Biodistribution in normal (three rats) and diabetic (two rats) rats was determined at the end of the distribution phase, 38 min after ^{125}I-rR-RAGE injection for normal rats, 12 hr after ^{125}I-rR-RAGE injection for diabetic rats (A), and 96 hr after ^{125}I-rR-RAGE injection for normal and diabetic rats (B). The results are presented as mean values. *Bars*, mean ± standard error.

80% of the radioactivity measured after TCA precipitation in the samples collected after injection in animals. Two hours after injection of ^{125}I-rR-RAGE, 93% of the radioactivity was immunoreactive with the anti-RAGE antibodies (1380 ± 50 versus 1480 ± 20 cpm). From the results, we can conclude that the radioactivity corresponded to rR-RAGE and not to another protein of a similar molecular mass.

SDS-PAGE analysis. Autoradiography of SDS-PAGE (Fig. 7) of plasma samples after ^{125}I-rR-RAGE administration showed one band of ~35 kDa in normal and diabetic rats
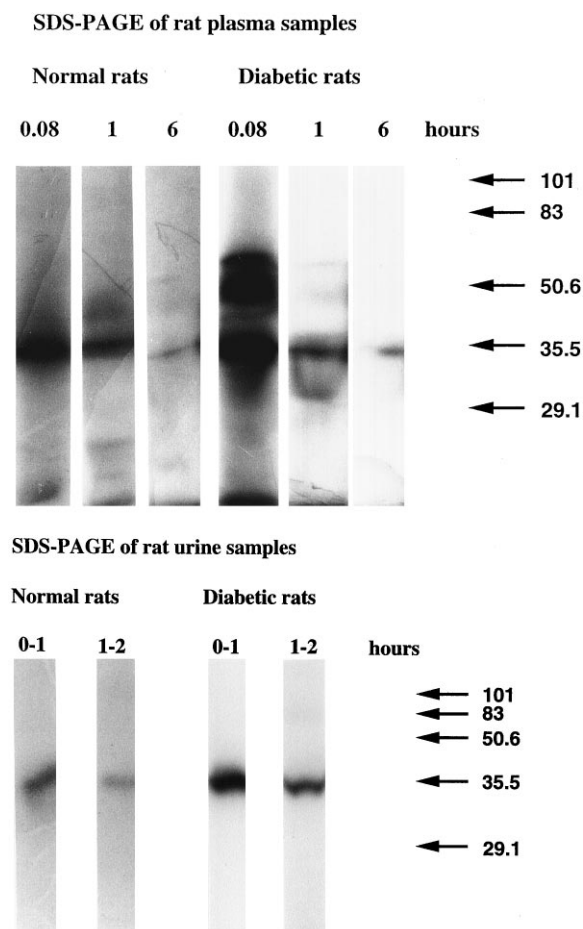
SDS-PAGE of rat plasma samples

Normal rats   **Diabetic rats**

0.08   1   6   0.08   1   6   hours

← 101
← 83

← 50.6

← 35.5

← 29.1

SDS-PAGE of rat urine samples

Normal rats   **Diabetic rats**

0-1   1-2   0-1   1-2   hours

← 101
← 83
← 50.6

← 35.5

← 29.1

Fig. 7. SDS-PAGE of rat plasma (*top*) and urine (*bottom*) samples after intravenous injection of ^{125}I-rR-RAGE. *Right*, standard protein molecular masses (kDa).

and two additional bands of 50 and 60 kDa in plasma of diabetic rats. The 35-kDa band corresponds to ^{125}I-rR-RAGE, and the two compounds of higher molecular masses could be the ^{125}I-rR-RAGE bound to AGEs present in vascular compartments of diabetic rats. In urine samples, only one band of 35 kDa was observed in normal and diabetic rats.

Discussion

Diabetic and normal rats that had received RBCs from syngenic diabetic rats developed a vascular hyperpermeability to albumin. In both animal models, the vascular hyperpermeability was corrected by the infusion of rR-RAGE, as previously shown with purified sRAGE (7). These experiments showed that the effect on the hyperpermeability was specific of RAGE and not due to contaminants in the preparation of RAGE because such contaminants were likely to be different in the preparations of RAGE from bovine lung extracts and of recombinant RAGE from insect cell culture media. The effect of rR-RAGE on vascular hyperpermeability further supports the importance of binding of AGEs to RAGE as a determinant factor of vascular dysfunction in diabetes. Furthermore, using bovine ECs in culture to study endothelial barrier function, we previously showed that RBCs from diabetic patients could provoke an increased albumin or inulin transfer. This effect was prevented by blocking the RAGE-AGE interaction by sRAGE or incubating the AGE RBCs with sRAGE (7). To investigate in

detail the effect of sRAGE on the albumin transport across endothelium in rat models, we cloned and expressed rR-RAGE. The sequence of RAGE was found to be highly conserved between species. Using purified rR-RAGE instead of sRAGE from the lung extracts, similar results were obtained, indicating that the observed effect of RAGE on permeability is not due to contaminants in the preparations of RAGE because such contaminants would likely be different in the preparations of RAGE from lung extracts and recombinant RAGE from insect cell culture media. Furthermore, rR-RAGE has kept the functional properties of the purified soluble form and blocked the alteration of EC function by AGEs present on diabetic RBCs.

After intravenous administration in normal and streptozotocin-induced diabetic rats, ^{125}I-rR-RAGE had longer distribution and elimination half-lives than ^{125}I-sRAGE previously studied (7). Two hypotheses could explain the differences between ^{125}I-rR-RAGE and ^{125}I-sRAGE pharmacokinetics. First, the exploration time of 54 hr in the previous study was not sufficient to find a three-compartment model as in the present study, and this drawback could explain the longer distribution and elimination half-lives of ^{125}I-rR-RAGE. Second, although there is little difference in amino acid contents (90% of homology) (4), we do not exclude that clearance of ^{125}I-sRAGE purified from bovine lung was higher than that of ^{125}I-rR-RAGE because of its bovine origin. Furthermore, as we previously observed with ^{125}I-sRAGE (7), distribution and elimination half-lives of ^{125}I-rR-RAGE were longer in diabetic rats than in normal rats, especially the second distribution half-life in diabetic rats (4.01 ± 0.87 hr). The presence of AGEs probably modifies the pharmacokinetics of ^{125}I-rR-RAGE in diabetic rats.

The volume of distribution of ^{125}I-rR-RAGE in normal rats was greater than the total body water (0.7 liter/kg), suggesting a rapid diffusion in tissues. The distribution volume of ^{125}I-rR-RAGE was 2-fold larger in diabetic rats than in normal rats and larger than the volume of the extracellular fluid in rats. Moreover, the duration of the process was considerably longer in diabetic rats (4.01 ± 0.87 hr) than in normal rats (0.21 ± 0.07 hr). This suggests that ^{125}I-rR-RAGE had an additional distribution compartment in diabetic rats. The higher volume of distribution of ^{125}I-rR-RAGE in diabetic rats than in normal rats was apparently not related to the vascular hyperpermeability due to diabetes because murine Fab, a 45-kDa protein that also belongs to the immunoglobulin superfamily, did had have a higher steady state volume of distribution in diabetic rats than in normal rats (0.12 ± 0.01 and 0.19 ± 0.01 liter/kg, respectively; $p = 0.006$). The more elevated volume of distribution of ^{125}I-rR-RAGE in diabetic rats can be explained by the interaction of ^{125}I-rR-RAGE with AGEs formed on diabetic RBCs (6) and on extracellular proteins. This could constitute an additional compartment of distribution for ^{125}I-rR-RAGE in diabetic rats. Furthermore, the concentration of ^{125}I-rR-RAGE measured at the end of the distribution phase in the different organs was increased in all organs of the diabetic rats compared with normal rats, and this difference was no longer observed when the tissue content was determined 96 hr after ^{125}I-rR-RAGE injection. These results were in agreement with the hypothesis that AGE formation in diabetic rats involves a higher organ distribution and leads to an enhanced volume of distribution in diabetic rats.

Study of the CL_R of ^{125}I-rR-RAGE showed that CL_R accounted for only 2% of the CL, suggesting that ^{125}I-rR-RAGE

was extensively reabsorbed at the kidney level and that peptidic hydrolysis, which characterizes the activity of tubular proximal cells for reabsorbed proteins (17), probably was an important source of ^{125}I-rR-RAGE catabolism. The same analysis of CL_R showed that the reabsorption process was less extensive in diabetic than in normal rats. The plasma threshold of the tubular reabsorption was more elevated in two diabetic rats and not observed in the three other diabetic rats. Nevertheless, the study of the urinary excretion rate versus plasma concentration showed that diabetic pathology influenced the urinary excretion of ^{125}I-rR-RAGE.

SDS-PAGE of diabetic rat plasma revealed ^{125}I-rR-RAGE complexes of ~60 kDa, a molecular mass that may correspond to ^{125}I-rR-RAGE/AGE complexes. The molecular mass of the possible ^{125}I-rR-RAGE/AGE complexes is above the glomerular filtration threshold, which is consistent with the fact that the precipitation of ^{125}I-rR-RAGE/AGE complexes with anti-AGE antibodies showed that only a small proportion (0.3–1%) of ^{125}I-rR-RAGE in urine was bound to AGEs. This could corresponds to ^{125}I-rR-RAGE/AGE complexes eliminated in urine, but it could be also underestimated, AGE epitopes recognized by AGE antibodies hidden in the ^{125}I-rR-RAGE/AGE complex formation. These results shown that ^{125}I-rR-RAGE/AGE complexes were not extensively eliminated in urine. We observed in diabetic animals that a higher proportion of ^{125}I-rR-RAGE was distributed in spleen and liver 96 hr after the ^{125}I-rR-RAGE injection, so this could indicate an elimination of ^{125}I-rR-RAGE/AGE complexes via these organs, but this remains to be demonstrated through special designed experiments using metabolically labeled AGEs.

In diabetic rats, pharmacokinetics of ^{125}I-rR-RAGE after intravenous or intraperitoneal administration were characterized by a very slow distribution phase (4.01 ± 1.16 and 3.38 ± 1.16 hr, respectively) and a similar elimination phase (57.17 ± 11.62 and 51.98 ± 6.31 hr, respectively), which confirms that AGEs influence ^{125}I-rR-RAGE pharmacokinetics in both routes of administration. The rate of resorption was quite rapid (T_{max} observed in the range 1–2 hr), but the extent was 2-fold less in diabetic rats (35%) than in normal rats (61%). This difference can be explained by the trapping of ^{125}I-rR-RAGE by AGE proteins in the peritoneal cavitae. In diabetic animals, the interaction between endogenous RAGE and AGEs leads to an oxidant stress assessed by the TBARS formation and blocked by administration of sRAGE (6). After intraperitoneal administration, rR-RAGE corrected plasma TBARS similarly to that obtained after intravenous injection, indicating that the absorbed fraction of rR-RAGE after intraperitoneal administration was biologically active.

rR-RAGE present in the vascular compartment could bind with AGEs present on proteins and lipoproteins and inhibit the binding to endogenous RAGE present on ECs. Consequently, it would block the transfer of AGE protein mediated by RAGE/lactoferrin-like receptor complexes through the endothelium (18).

In experimental or human pathologies, several attempts have been made to prevent ligand/receptor interactions, including the use of recombinant CD4 in human immunodeficiency virus infections (19) and of recombinant interleukin-1 receptor in rat autoimmune encephalomyelitis (20). Because RAGE seems to be a receptor not only for AGEs but also for amphoterin (21) and amyloid-β peptide (22), there is an interest in better understanding the pathophysiology of RAGE.

The use of recombinant RAGE as well as transgenic animals that overexpress or are deficient in RAGE will allow better delineation of what can be expected from the prevention of binding to endogenous RAGE.

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