Specific Macrophage Receptor Activity for Advanced Glycosylation End Products Inversely Correlates With Insulin Levels In Vivo

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A high-affinity macrophage receptor has been shown to mediate the removal of proteins modified by advanced nonenzymatic glycosylation end products (AGEs) in both animals and humans. To characterize the effect of diabetes on this receptor system, resident peritoneal macrophages from experimentally induced and genetically diabetic mice were studied. Binding and degradation of radioiodinated AGE–bovine serum albumin (AGE-BSA) were determined from saturation kinetics and compared with glucose and insulin levels of each subgroup. Scatchard plot analysis of nondiabetic mouse macrophages has indicated 1.5×10^5 receptors/cell, with a binding affinity of 1.7×10^7 M^{-1}. The *in vitro* exposure of macrophages to either elevated glucose or insulin concentrations failed to demonstrate a short-term regulatory effect on AGE-receptor function. However, macrophages from hypoinsulinemic alloxan-induced diabetic mice indicated a two- to threefold increase in AGE-receptor number per cell ($2.98 \pm 0.25 \times 10^5$/cell), and macrophages from C57BL/KsJ (*db*/*db*) mice showed an almost threefold greater receptor number ($2.86 \pm 0.2 \times 10^5$/cell), with binding affinity remaining essentially unchanged ($1.24 \pm 0.05 \times 10^7$ and $1.21 \pm 0.07 \times 10^7$ M^{-1}, respectively). In addition, a moderate increase (25–30%) of ^{125}I-labeled AGE-BSA degradation was observed in these two insulin-deficient diabetic macrophage groups compared with the normal control group. In contrast, macrophages from hyperinsulinemic and hyperglycemic C57BL/6J (*db*/*db*) mice demonstrated a distinct reduction in both AGE-receptor number ($0.67 \pm 0.03 \times 10^5$/cell) and binding affinity ($0.37 \pm 0.03 \times 10^7$ M^{-1}), with a decrease of AGE-BSA degradation of ~50% compared with the control group. This study provides evidence for a specific insulin-sensitive mechanism that modulates the function of the macrophage AGE receptors. High peripheral insulin levels may thus adversely affect the receptor-mediated clearance of nonenzymatically glycosylated proteins from sites of diabetic tissue damage. *Diabetes* 37:456–61, 1988

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Elevated plasma insulin levels have been associated with accelerated development of arteriosclerosis in both nondiabetic and diabetic patients (1,2). The narrowing of large vessels in such patients reflects the net accumulation of matrix and cellular components in the inner layers of the arterial wall (3). Although factors affecting the rate of vascular protein deposition and cellular proliferation have been studied extensively (3), the potential role of hyperinsulinemia and the processes involved in the removal of vascular deposits have not been well characterized.

Recently, we identified a new macrophage-mediated removal system that recognizes proteins modified by a common in vivo process (4), the long-term nonenzymatic reaction of glucose with proteins (5,6). In contrast to the short-term nonenzymatic reaction with proteins, which results in reversible Amadori adduct formation, the long-term glycosylation of proteins leads to the formation of complex irreversible adducts called advanced glycosylation end products (AGEs). These AGEs accumulate on long-lived proteins as a function of glycemic level and time. In diabetic blood vessels, increased formation of glucose-derived AGE appears to contribute to the process of vascular occlusion by chemically trapping low-density lipoprotein (LDL) and other plasma proteins that leak out of the circulation (7) and by progressive nonenzymatic cross-linking of collagen and basement membrane (7,8).

Removal of these glucose-modified proteins is accomplished by monocyte-derived macrophages through specific interaction with a high-affinity cell surface receptor (4). Scatchard plot analysis of binding data of AGE-albumin to mouse peritoneal macrophages has indicated that there are

TABLE 1
Macrophage binding, uptake, and degradation of AGE-BSA with different in vitro concentrations of glucose and insulin

	Binding (pmol/mg)	Uptake (μg/mg)	Degradation (μg/mg)
Glucose (mM)*			
10	1.35 ± 0.14	2.25 ± 0.34	7.08 ± 2.20
25	1.28 ± 0.22	1.70 ± 0.42	8.30 ± 1.61
50	1.32 ± 0.08	2.20 ± 0.32	7.42 ± 1.82
Insulin (U/ml)†			
10	1.38 ± 0.21	1.92 ± 0.42	7.38 ± 1.22
100	1.58 ± 0.26	1.48 ± 0.64	6.66 ± 0.93
500	1.46 ± 0.12	1.74 ± 0.33	8.47 ± 0.41
1000	1.20 ± 0.18	2.18 ± 0.55	6.83 ± 0.23

Resident peritoneal macrophages incubated for 2 h at 4°C (binding) or for 4 h at 37°C (uptake and degradation) in presence of ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA, 25 μg/ml, 378 nM) with or without excess AGE-BSA (see MATERIALS AND METHODS). Data are means ± SD. *P* values not significant in all comparisons between groups.
*No insulin added.
†Glucose concentration 10 mM.

~150 × 10^3 AGE receptors/cell, with an affinity constant of 1.7 × 10^7 M^{-1} (4). This AGE receptor is distinct from other scavenger receptors that recognize mannose-fucose, acetyl-LDL, and formaldehyde-treated albumin (9).

The AGE-receptor–mediated recognition system enables macrophages to bind to, and then specifically remove, only the proteins with accumulated advanced glycosylation products. This removal mechanism may be critically important in determining the rate at which arteriosclerosis develops, because factors that reduce its efficiency would also play a major role in determining the total amount of accumulated glucose-modified protein in blood vessel walls. Because of the association between hyperinsulinemia and arteriosclerosis in diabetic patients, we investigated the effect of hyperinsulinemia on the AGE receptor with normal mouse peritoneal macrophages under various in vitro concentrations of glucose or insulin and with macrophages from either alloxan-induced or genetically diabetic mice.

Our results suggest that high peripheral insulin levels in vivo compromise AGE-receptor–mediated removal and clearance of cross-linked AGE proteins from diabetic tissues, whereas low insulin levels enhance this macrophage-mediated process.

MATERIALS AND METHODS

Mice. We purchased 30 diabetic insulin-deficient C57BL/KsJ (*db/db*) mice, 30 diabetic hyperinsulinemic C57BL/6J (*db/db*) mice, 30 nondiabetic C57BL/6J (*db/+*) mice, and 30 normal C57BL/6J (+/+) mice from The Jackson Laboratory (Bar Harbor, ME), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All of the mice were male and 6–8 mo old. Genetically diabetic animals were hyperglycemic for at least 6 mo before use.

The normal age- and sex-matched C57/BL/6J (+/+) group was rendered diabetic by intraperitoneal injection of freshly prepared alloxan monohydrate (40 mg/kg) dissolved in 0.1 M sodium phosphate buffer (pH 3.0). Induction of diabetes was assessed by measuring serum glucose concentrations with a glucose analyzer (Bio-Dynamics, Indianapolis, IN) 48 h after injection and at weekly intervals thereafter. All alloxan-induced diabetic (ALX-D) mice had plasma glucose >300 mg/ml, which was maintained for at least 8 wk after induction of diabetes before being used in experiments. All mice were weighed weekly and screened for ketosis by a commercial dipstick of urine samples. None became ketotic, and none were treated with exogenous insulin. Throughout the studies, all mice appeared to be metabolically stable, and only four mice from the ALX-D group died before macrophage harvesting. Serum insulin levels were measured in all groups, after exsanguination by cardiac puncture, by radioimmunoassay (Clinical Pathology, Animal Medical Center, New York). All of the ALX-D and C57BL/KsJ (*db/db*) mice showed significant hyperglycemia and hypoinsulinemia compared with the control group ($P < .001$). The C57BL/6J (*db/db*) mice were markedly hyperinsulinemic and hyperglycemic ($P < .001$). These values are similar to those reported previously for this diabetic animal model (10–12).

Isolation of mouse peritoneal macrophages. Resident peritoneal macrophages were harvested in phosphate-buffered saline as described previously (12). Cells from 8–10 mice were pooled and centrifuged at 500 × *g* for 10 min, washed with Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY), and resuspended in medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. After total cell counts and differential, aliquots of 2 × 10^6 cells were seeded in Linbro plastic dishes (1.0 × 1.0 cm) and incubated at 37°C in 5% CO_2 for 2 h, after which nonadherent cells were removed by washing the monolayer with serum-free medium.

Preparation of protein with AGE. The AGE–bovine serum albumin (AGE-BSA) was prepared by incubating BSA in 50 mM glucose at 37°C for 6 wk, in the presence of protease inhibitors (1.5 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA) and antibiotics (100 U/ml penicillin and 40 μg/ml gentamicin) as described previously (4,9,13). The amount of AGE formed on BSA was assessed by measuring with radioimmunoassay (14) the amount of the chemically and structurally known AGE, 2-furoyl-4-(5)-(2-furanyl)-1*H*-imida-

TABLE 2
Fasting serum glucose and insulin concentrations in normal and diabetic mice

Group	*n*	Strain	Serum glucose (mg/ml)	Serum insulin (μU/ml)
1	26	C57BL/6J (+/+) (alloxan-induced diabetic)	440 ± 80	25.2 ± 4.1
2	30	C57BL/KsJ (*db/db*)	500 ± 71	8.4 ± 1.3
3	30	C57BL/6J (*db/db*)	480 ± 83	423.3 ± 56
4	30	C57BL/6J (*db/+*) (normal control)	74 ± 23	87 ± 21

Data are group means ± SD. $P < .001$ for glucose and insulin levels for group 4 vs. groups 1, 2, and 3 and insulin levels for group 2 vs. 3; *P* value for glucose level for group 2 vs. 3 is nonsignificant.

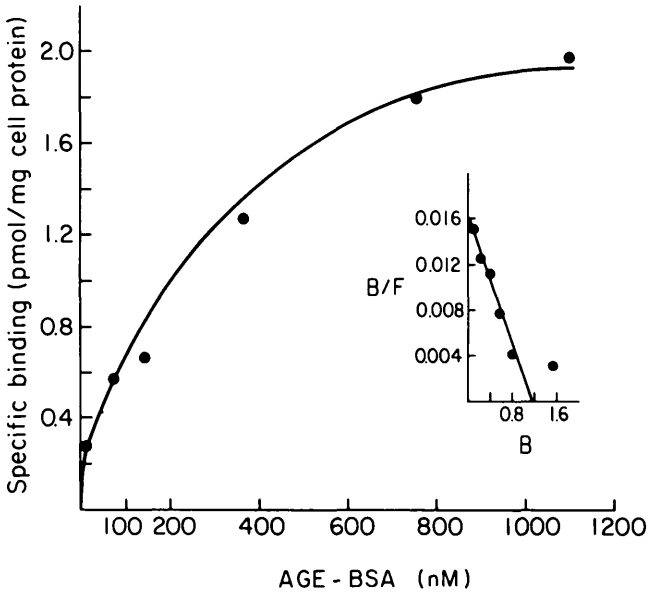

FIG. 1. Specific binding and Scatchard analysis (*inset*) of data with ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA), unlabeled AGE-BSA, and C57BL/6J (*db*/+) nondiabetic mouse peritoneal macrophages. Saturation binding profile obtained by incubating macrophages with various concentrations of ^{125}I-AGE-BSA at 4°C for 2 h. Nonspecific binding determined in presence of 100-fold excess of unlabeled AGE-BSA. Specific binding determined on duplicate samples as described in MATERIALS AND METHODS and expressed as mean of 3 independent determinations. Units for Scatchard analysis are: bound (B), pmol/mg cell protein; bound/free (B/F), pmol · mg^{-1} · nM^{-1}.

zole (FFI) (15). With this assay, the AGE-BSA preparation contained 360 pmol FFI/mg protein, whereas normal BSA contained 8–14 pmol FFI/mg protein before glycosylation. Radioiodination of AGE-BSA was carried out by the method of Fraker and Speck (16) to a specific activity of 854 counts per minute (cpm)/ng protein.

In vitro macrophage experiments. All cell cultures were incubated at 37°C for 72 h in 1.0 ml fresh medium containing 10% FCS and different concentrations of glucose (10–50 mM) or insulin (10–1000 μU/ml). Binding studies of ^{125}I-labeled AGE-BSA (378 nM, sp act 735 cpm/ng) to the peritoneal macrophage AGE receptors of each group of mice were carried out for 2 h at 4°C in the absence and presence of 100-fold excess unlabeled AGE-BSA, followed by Scatchard analysis (17) as previously described (4). Nonspecific binding was generally <20% of the total as determined previously (4,9). The intracellular accumulation and degradation of AGE-BSA were then determined as described previously (4,9). After the addition of different amounts of ^{125}I-AGE-BSA (378 nM, sp act 680 cpm/ng) in each well and a 4-h incubation at 37°C, the amount of ^{125}I-ligand accumulated by the cells and the trichloroacetic acid (TCA)-soluble (20%) material secreted in the medium were determined.

Statistics. Group data are expressed as means ± SD and were analyzed with one-way analysis of variance (17).

RESULTS

Because either hyperglycemia, hypoinsulinemia, or hyperinsulinemia could affect the AGE-receptor number or affinity, in vitro binding, uptake, and degradation experiments were performed with ^{125}I-AGE-BSA (50 μg, 750 nM) after preincubation of mouse peritoneal macrophages with various concentrations of glucose (10–50 mM) or insulin (10–1000 μU/ml) in the medium (Table 1). No significant differences in ^{125}I-AGE-BSA binding, uptake, or degradation were noted after 72 h of incubation.

To determine whether longer exposure of macrophages or macrophage precursors to similar glucose and insulin variations would induce AGE-receptor alterations, macrophages from ALX-D and genetically diabetic mice were examined. In Table 2, fasting blood glucose and insulin levels of the diabetic groups are compared with those of a normoglycemic normoinsulinemic control group.

Saturable specific binding of ^{125}I-AGE-BSA to resident peritoneal macrophages from the control group (Fig. 1) corresponds to an estimated 1.43 ± 0.10 × 10^5 receptors/macrophage and an affinity constant of 1.38 ± 0.18 × 10^7 M^{-1}, values similar to those we reported previously (4). As shown in Fig. 2, maximum binding of ^{125}I-AGE-BSA by macrophages from ALX-D, hyperglycemic, insulin-deficient diabetic mice was more than twofold higher than that observed with cells from normal control subjects (4.2 ± 0.39 vs. 1.9 ± 0.14 pmol/mg cell protein, $P < .001$). Scatchard analysis indicated a two- to threefold increase in AGE-receptor number (2.98 ± 0.25 × 10^5/cell, $P < .001$), whereas the affinity constant remained unchanged (1.24 ± 0.05 × 10^7 M^{-1}, $P < .29$).

Similarly, macrophages from the equally hyperglycemic, insulin-deficient, genetically diabetic C57BL/KsJ mouse

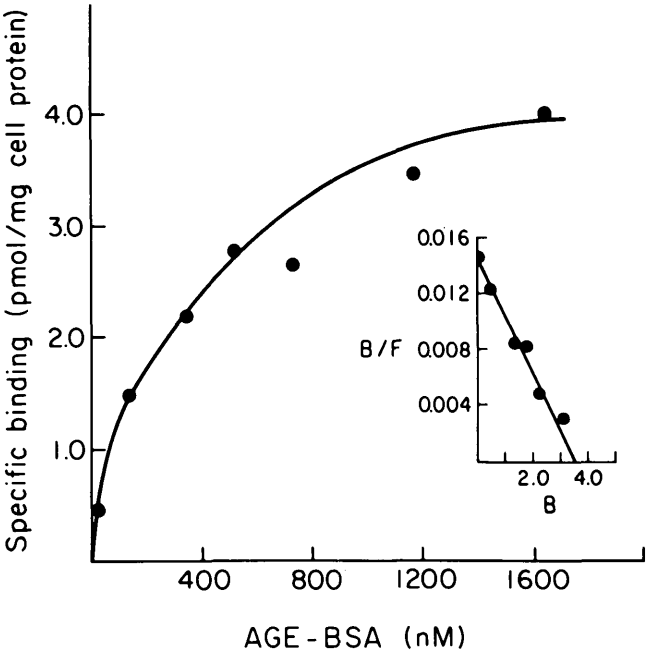

FIG. 2. Specific binding and Scatchard analysis (*inset*) of data with ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA), unlabeled AGE-BSA, and C57BL/6J (+/+) experimentally diabetic mouse peritoneal macrophages. Diabetes induced in mice via intraperitoneal injection of alloxan monohydrate (40 mg/kg). Saturation binding profile obtained by incubating macrophages with various concentrations of ^{125}I-AGE-BSA at 4°C for 2 h. Nonspecific binding determined in presence of 100-fold excess of unlabeled AGE-BSA. Specific binding determined on duplicate samples as described in MATERIALS AND METHODS and expressed as mean of 3 independent determinations. Units for Scatchard analysis are as in Fig. 1.

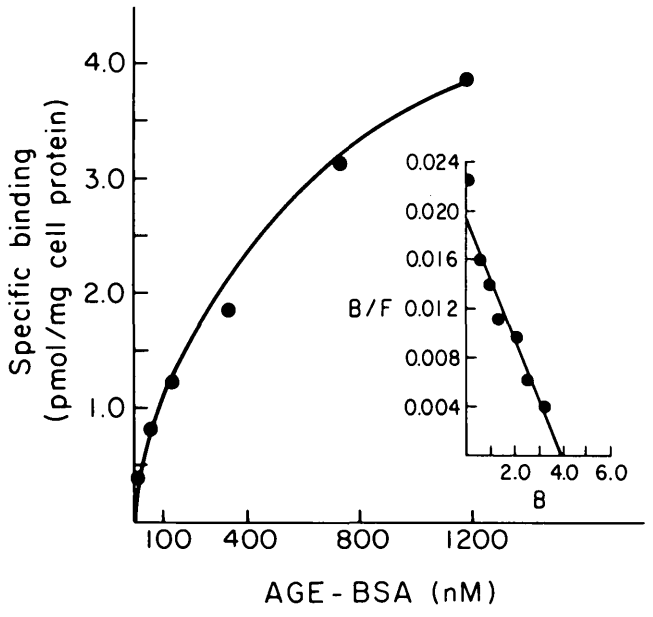

FIG. 3. Specific binding and Scatchard analysis (*inset*) of data with ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA), unlabeled AGE-BSA, and C57BL/KsJ (*db/db*) hypoinsulinemic mouse peritoneal macrophages. Saturation binding profile obtained by incubating macrophages with various concentrations of ^{125}I-AGE-BSA at 4°C for 2 h. Nonspecific binding determined in presence of 100-fold excess of unlabeled AGE-BSA. Specific binding determined on duplicate samples as described in MATERIALS AND METHODS and expressed as mean of 3 independent determinations. Units for Scatchard analysis are as in Fig. 1.

had an almost twofold higher ^{125}I-AGE-BSA–specific binding (3.8 ± 0.13 pmol/mg cell protein) than cells from age-matched nondiabetic control mice ($P < .001$; Fig. 3), with a more than twofold greater receptor number (2.86 ± 0.20 × 10^5/cell, $P < .001$) but a similar affinity (1.21 ± 0.07 × 10^7 M^{-1}, $P < .3$).

In contrast, macrophages from the diabetic C57BL/6J mouse, tested during the early hyperinsulinemic phase when glucose levels averaged 300 mg/dl and insulin levels were >350 μU/ml, demonstrated a distinct reduction in maximal binding capacity (1.3 ± 0.08 pmol/mg cell protein, $P < .01$; Fig. 4). This was associated with a significant decrease of both AGE-receptor number (0.67 ± 0.03 × 10^5/cell, $P < .001$) and binding affinity (0.37 ± 0.03 × 10^7 M^{-1}, $P < .001$).

Because the total AGE-BSA phagocytic capacity of macrophages is determined by the net effect of receptor and postreceptor mechanisms, the degradation of ^{125}I-AGE-BSA was determined by precipitation in TCA and measurement of the TCA-soluble radioactivity released in the medium after a 4-h incubation with macrophages at 37°C (Fig. 5). A moderate increase (25–30%) of ^{125}I-AGE-BSA degradation was observed in the two insulin-deficient diabetic macrophage groups compared with the normal control group. In contrast, degradation of AGE-BSA decreased by ~50% of control in the hyperinsulinemic diabetic group (Fig. 5).

DISCUSSION

Our study was undertaken to characterize the effect of hyperinsulinemia, hypoinsulinemia, and hyperglycemia on the binding and degradation of AGE by the specific high-affinity AGE receptor present on peritoneal macrophages. The results demonstrate that macrophage binding and degradation of AGE proteins vary inversely with circulating insulin levels but are independent of hyperglycemia and type of diabetes.

The in vitro exposure of macrophages to either elevated glucose or insulin concentrations failed to demonstrate a short-term regulatory effect on AGE-receptor function. Macrophages from ALX-D and genetically diabetic mice were then examined to determine whether longer exposure of macrophages was necessary for induction of AGE-receptor changes by hyperglycemia and/or altered insulin levels. The ALX-D mice were hyperglycemic and insulin deficient, as were genetically diabetic mice with the mutant *db/db* gene on the C57BL/KsJ background. In contrast, genetically diabetic mice with the mutant *db/db* genes on a different background (C57BL/6J) exhibited identical levels of hyperglycemia, with a characteristic marked elevation of insulin levels. The *db/+* mouse of either genetic background, heterozygous for both *db* and *m* genes (10–12), had normal glucose and insulin levels, and this mouse was the most appropriate control for these studies.

Binding and degradation of AGE proteins by macrophages from the two hyperglycemic insulin-deficient diabetic groups were enhanced. Upregulation of AGE-receptor function was primarily due to an increase in receptor number.

In contrast, AGE-receptor number, receptor-binding affinity, and intracellular degradation were all significantly reduced in the hyperinsulinemic diabetic group. Together, these in vitro and in vivo results suggest that longer exposure of macrophages to altered levels of insulin is necessary to induce significant changes in AGE-receptor properties and

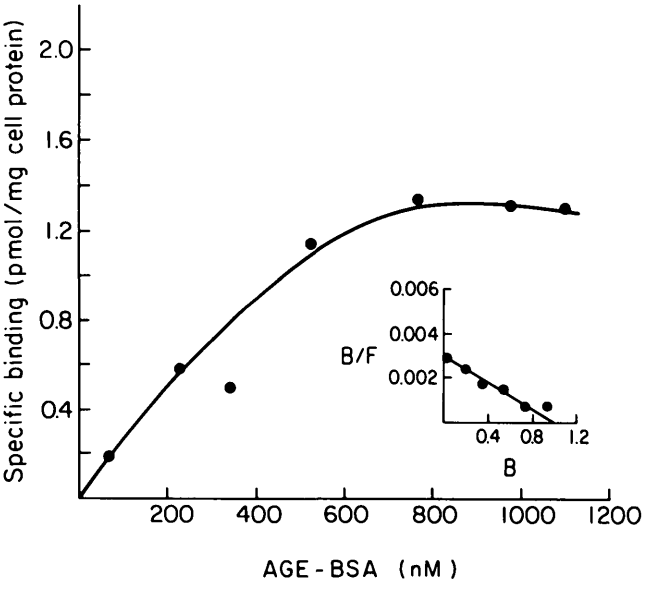

FIG. 4. Specific binding and Scatchard analysis (*inset*) of data with ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA), unlabeled AGE-BSA, and C57BL/6J (*db/db*) hyperinsulinemic mouse peritoneal macrophages. Saturation binding profile obtained by incubating macrophages with various concentrations of ^{125}I-AGE-BSA at 4°C for 2 h. Nonspecific binding determined in presence of 100-fold excess of unlabeled AGE-BSA. Specific binding determined on duplicate samples as described in MATERIALS AND METHODS and expressed as mean of 3 independent determinations. Units for Scatchard analysis are as in Fig. 1.

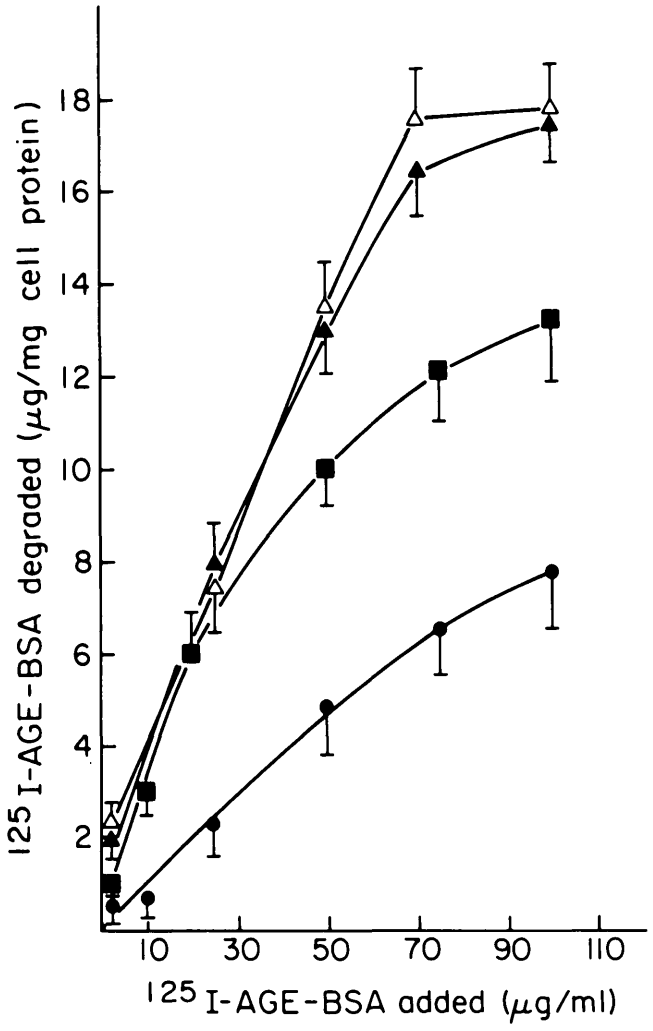

FIG. 5. Degradation of ^{125}I-labeled advanced glycosylation end products–bovine serum albumin (AGE-BSA) by macrophages from normal (■), alloxan-induced hypoinsulinemic diabetic (△), hypoinsulinemic diabetic C57BL/KsJ (*db*/*db*) (▲), and hyperinsulinemic diabetic C57BL/6J (*db*/*db*) (●) mice as determined by measurement of trichloroacetic acid–soluble radioactivity released in medium after 4-h incubation at 37°C. Values are means ± SD of 3 different experiments, each performed in triplicate.

function. However, it is also possible that exposure of an insulin-sensitive macrophage precursor is required, or that hyperinsulinemia generates a more proximal regulatory signal in vivo.

Multiple metabolic abnormalities are present in diabetes, and several of these may play a role in AGE-receptor regulation. That insulin itself is directly responsible for the observed AGE-receptor alterations is suggested by the reported insulin-induced decrease in the number of Fc receptors on guinea pig macrophages incubated in vitro (18). Similarly, peritoneal macrophages from insulin-treated diabetic rats also demonstrated downregulation of both Fc-receptor number and total postreceptor intracellular degradation of endocytosed proteins, although fractional catabolism of the smaller amount bound was increased (19). Hyperphagocytosis was not observed in macrophages from hypoinsulinemic animals, however. In contrast, reticular endothelial system phagocytes from hypoinsulinemic rats given streptozocin exhibited hyperphagocytosis, which was reversed by prolonged insulin replacement (20). In this model, acute replacement of insulin with a single large dose did not alter the hyperphagocytosis, even though it reversed both hypoinsulinemia and hyperglycemia. The differences observed in macrophage phagocytosis from hypoinsulinemic animals appear to reflect differences in the degree of insulin deficiency (18–20).

Our study provides evidence for a specific insulin-sensitive mechanism that modulates the properties and function of the macrophage AGE receptors. In diabetes, where peripheral insulin levels are elevated in many non-insulin-dependent diabetic patients with moderate hyperglycemia and in most insulin-dependent patients whose peripherally injected doses must compensate for hepatic requirements and extraction, downregulation of this removal mechanism for proteins with advanced glycosylation products may be critically important. Factors that reduce its efficiency would also play a major role in determining the total amount and rate of hyperglycemia-accelerated glucose-modified protein accumulation in diabetic blood vessel walls. In nondiabetic subjects, such a mechanism may partly explain the well-documented association between hypocaloric intake or fasting and retardation of aging in animal models (21).

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