Low Phagocytic Activity of Resident Peritoneal Macrophages in Diabetic Mice

Relevance to the Formation of Advanced Glycation End Products

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Formation of advanced glycation end products (AGEs) is accelerated in diabetic subjects along with hyperglycemia. Although several lines of evidence indicate that AGEs stimulate macrophages to secrete several cytokines and growth factors, little is known about the effect of AGEs on the primary function of macrophages, such as phagocytosis. On the other hand, impairment of the phagocytic function of monocytes/macrophages is suggested to contribute to the low resistance to infection in diabetic subjects. In the present study, we examined the effect of AGEs on the phagocytic function of macrophages. Using flow cytometric analysis of mouse resident peritoneal macrophages, we showed that AGEs suppress phagocytosis of fluorescent microspheres by cultured macrophages. In addition, experiments using streptozotocin-induced diabetic mice demonstrated a significant decrease in the phagocytic activity of resident peritoneal macrophages 12 weeks after induction of diabetes compared with age-matched control mice. The phagocytic activity of peritoneal macrophages correlated inversely with AGE content in the adjacent peritoneal tissue. Furthermore, reduced phagocytic activity of macrophages was associated with a reduction in intracellular ATP content. Because phagocytosis is an important component of the defense system, suppression of such activity by AGEs may explain, at least in part, the increased susceptibility of diabetic patients to infection. *Diabetes* 48:2074–2082, 1999

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AG, aminoguanidine-hemisulfate salt; AGE, advanced glycation end product; AGE-BSA, AGE-modified bovine serum albumin; AU, arbitrary unit; BSA, bovine serum albumin; 3-DG, 3-deoxyglucosone; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride; PBS, phosphate-buffered saline; Pyr-BSA, pyrraline-modified BSA.

Nonenzymatic glycation (Maillard reaction) has been implicated in the pathogenesis of diabetic complications (1,2). Recent immunohistochemical studies have shown that the formation of advanced glycation end products (AGEs) is enhanced in diabetic subjects (3–5). Pyrraline is one of the AGEs derived from the reaction of glucose with amino groups in proteins (6,7). Plasma pyrraline levels are elevated in diabetic rats and humans as determined by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody to pyrraline (8). The presence of pyrraline in vivo has also been confirmed by chromatographic techniques (9,10), while immunohistochemical studies show localization of pyrraline in arteriosclerotic lesions of diabetic patients (8), as well as in lesions of Alzheimer's disease (11). Furthermore, we (12) and other investigators (13,14) have shown that the blood levels of 3-deoxyglucosone (3-DG), a potent precursor of several AGEs including pyrraline, are also elevated in diabetic animals and humans, indicating that the advanced step of glycation is accelerated in diabetic subjects.

AGE formation per se is known to alter the structural and functional properties of tissue proteins. In addition, interaction between AGE-modified proteins and various types of cells is thought to play a pathological role in the abnormalities observed in diabetic subjects (15–18). In this regard, the relation of AGEs to monocytes/macrophages has also been investigated, especially in terms of atherosclerosis. For example, Vlassara et al. (15) demonstrated that after recognition of AGEs by macrophages, the cells secrete various cytokines, such as tumor necrosis factor and interleukin-1. However, the exact structure of AGEs responsible for these actions remains unknown.

On the other hand, our previous findings showed that pyrraline-modified albumin tended to accumulate in monocytes/macrophages because of a reduced susceptibility to lysosomal proteolytic enzymes (19). It is not clear at present whether AGE-laden macrophages alter their primary function. Macrophages are multifunctional cells involved in both the development of atherosclerosis and host defense mechanisms. Although the immune system is very complex, macrophages initiate humoral- and cell-mediated immune responses and also have effector functions, such as the

removal of immune complexes and infective agents (20). In this regard, diabetes is associated with a dysfunction of the immune system, which enhances the development of infection (21). Several lines of evidence have suggested that impairment of phagocytic function of monocytes/macrophages contributes, at least in part, to the impaired resistance to infection in diabetes (22–24).

In the present study, we examined whether exposure of macrophages to an AGE-rich environment including pyrraline changes their phagocytic function both in vitro and in vivo. The phagocytic activity was objectively evaluated by counting the number of phagocytosed fluorescence-labeled latex particles using flow cytometry (25,26). Subsequently, we investigated the mechanism(s) by which AGEs affected the phagocytic activity of the cells.

RESEARCH DESIGN AND METHODS

Materials. RPMI 1640 medium and supplements such as L-glutamine, sodium pyruvate, and penicillin/streptomycin were purchased from Gibco BRL (Gaithersburg, MD). Fetal calf serum (FCS) was obtained from Dainippon Pharmaceutical (Osaka, Japan). Fluorescent latex particles (2.5% latex solids, 2.0 μm in diameter) were obtained from Polyscience (Warrington, PA). Bovine serum albumin (A-0281; Sigma, St. Louis, MO), free of globulin and free fatty acids, was used in the present study. Aminoguanidine-hemisulfate salt (AG) was also purchased from Sigma. N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) and potassium carbonate were purchased from Nacalai Tesque (Kyoto, Japan). Caproyl pyrraline and 3-DG were kindly supplied by Dr. V.M. Monnier (Case Western Reserve University, Cleveland, OH) and Dr. F. Hayase (Meiji University, Kawasaki, Japan), respectively. All other chemicals were of analytical grade and purchased from Wako Pure Chemical (Osaka, Japan), unless otherwise noted.

Preparation of AGE bovine serum albumin and pyrraline-modified bovine serum albumin. Bovine serum albumin (BSA) (50 mg/ml) was incubated with 50 mmol/l 3-DG, an intermediate of the advanced stage of glycation, in phosphate-buffered saline (PBS) (pH 7.4) at 37°C for 7 weeks under sterile conditions. This mixture theoretically contains only advanced stage products. The samples were then dialyzed against PBS to remove excess 3-DG at 4°C using Spectropor dialysis tubing (Spectrum Medical, Los Angeles, CA) with a molecular mass cutoff of 6,000–8,000 daltons. Native BSA alone was incubated under the same conditions and used as a control.

Pyrraline-modified BSA (Pyr-BSA) was prepared using the carbodiimide coupling reaction as described previously (7,8). The modification by pyrraline was confirmed by its characteristic ultraviolet spectrum. Control BSA was prepared using the same procedure as above but without adding caproyl pyrraline.

Portions of each protein sample were radioiodinated with carrier-free ^{125}I by using Iodo-Beads (Pierce, Rockford, IL) (27).

Cell preparation. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of our university. Female Balb/c mice (8 weeks old) were obtained from CLEA Japan (Osaka, Japan). Mouse resident peritoneal macrophages were obtained by the modified method of Cohn and Benson (28). Briefly, a normal mouse was killed under ether anesthesia. Approximately 10 ml of cold serum-free RPMI 1640 medium was injected intraperitoneally through the exposed peritoneum. After a gentle massage, peritoneal fluid containing cells was harvested and centrifuged at 250*g* for 10 min at 4°C. The pellet was reconstituted with 3 ml of RPMI 1640 containing 10% FCS (medium A) and cultured at 37°C in a 35-mm dish that had been coated with 15% FCS-containing RPMI 1640. After 1 h, only attached macrophages were used for the following experiments.

Determination of phagocytic activity of mouse resident peritoneal macrophages preincubated with AGE- or pyrraline-modified BSA in vitro. Mouse resident peritoneal macrophages were preincubated with AGE-modified BSA (AGE-BSA) and native BSA at a concentration of 100 μg/ml in serum-free RPMI 1640 medium under an atmosphere of 5% CO_2 at 37°C for 1, 2, 3, and 4 h. We additionally incubated the cells with a mixture of BSA and AGE-BSA (1:1) for 4 h at the same concentration of protein as above to estimate the dose dependency of the effect of AGEs. The similar experiments were performed with a set of control and Pyr-BSA. After each incubation period, the cells were washed three times with RPMI 1640 containing 2% FCS and harvested using a scraper. In this step, the cell viability was >90% as confirmed by the trypan blue exclusion test. The phagocytic activity of the cells was analyzed by the method of Steinkamp and colleagues (25,26) with some modifications. Briefly, the concentration of cell suspension was adjusted to 2×10^6 cells/ml in medium A. Next, we diluted the fluorescent latex particles 100 times with the same medium, then added 1.5 ml of the suspension of these particles to 0.5 ml of the above-mentioned cell suspension. The mixture was allowed to react at 37°C for 30 min with gentle shaking in a water bath. The cells were then washed three times with cold PBS (pH 7.4) to terminate the reaction. The final pellet was resuspended with PBS and applied to flow analytical cytometrics (FACS 440 system; Becton Dickinson, Mountain View, CA) to analyze the phagocytosed fluorescent particles. This method can discriminate phagocytes containing none, one, two, three, or more particles, since the fluorescence intensity of the cells is dependent on the number of ingested fluorescent particles. Two types of indexes were calculated from the obtained histogram. First, we determined the phagocytic rate, representing the percentage of cells with ingested particles relative to the total number of cells. We also calculated the phagocytic index representing the mean number of particles ingested per cell, which was expressed as 100 when a particle was ingested. The latter parameter served to evaluate the overall extent of phagocytosis achieved by each macrophage.

Phagocytic activity of mouse resident peritoneal macrophages in diabetic mice. Female Balb/c mice (8 weeks old) were subdivided at random into four groups: *1*) control (n = 16), *2*) AG-treated control mice (n = 16), *3*) STZ-induced diabetic mice (n = 16), and *4*) AG-treated diabetic mice (n = 16). Diabetes was induced by a single intravenous injection of STZ (160 mg/kg body wt) freshly dissolved in 100 μl of sterile citrate buffer (pH 4.5). Only animals with plasma glucose level over 20 mmol/l after 1 week of injection were included in the study. Control mice were sham injected with the citrate buffer. Animals were provided with water ad libitum and fed a standard laboratory diet. AG (1.0 g/l) was added to the drinking water of mice of groups 2 and 4. After 4 and 12 weeks, 8 animals from each group were killed under ether anesthesia. Resident peritoneal macrophages from individual animals were prepared and incubated with fluorescent latex par-

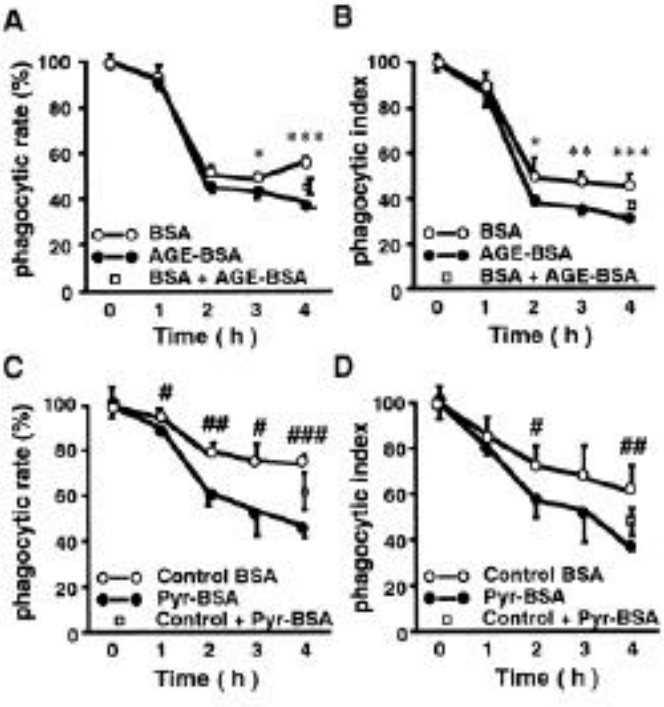

FIG. 1. Phagocytic activity of mouse resident peritoneal macrophages incubated with AGE-modified albumin or pyrraline-modified albumin in vitro. Resident peritoneal macrophages were obtained from 8-week-old Balb/c mice (*A* and *B*). Macrophages were preincubated with 100 μg/ml of native (n = 4) or AGE-BSA (n = 4) for 0, 1, 2, 3, and 4 h. After each incubation period, the phagocytic activity was estimated by evaluating the ability to intake fluorescent latex particles using flow cytometric analysis. Longer exposure to AGE-BSA significantly reduced the phagocytic rate (*A*) and index (*B*) compared with native BSA. Suppression effect of a 1:1 mixture of BSA and AGE-BSA (□) at 4 h was between those of BSA and AGE-BSA. *C* and *D*: Preincubation with a set of control (n = 4) and Pyr-BSA (n = 4) showed that Pyr-BSA significantly suppressed the phagocytic rate (*C*) and index (*D*) of macrophages. A 50% Pyr-BSA showed suppression effect between those of control and Pyr-BSA. Mean ± SD is plotted. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. native BSA, respectively. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. control BSA, respectively.

TABLE 1
Characteristics of mice used in the experiment

Experimental group	Body weight (g)		Plasma glucose (mmol/l)		Glycated Hb (%)	
	4 weeks	12 weeks	4 weeks	12 weeks	4 weeks	12 weeks
Control	23 ± 1	25 ± 1	4.7 ± 0.3	4.7 ± 0.4	3.3 ± 1.1	5.7 ± 1.1
AG-treated control	24 ± 1	26 ± 1	4.7 ± 0.3	4.7 ± 0.5	3.5 ± 1.2	5.5 ± 0.7
Diabetic	17 ± 3*	16 ± 2*	32.5 ± 2.2*	31.9 ± 2.6*	8.4 ± 1.2*	11.5 ± 2.1*
AG-treated diabetic	16 ± 4†	17 ± 4*	31.7 ± 2.9*	31.5 ± 2.5*	7.6 ± 1.2*	10.9 ± 2.7*

Data are means ± SD of eight animals in each group. Female Balb/c mice were subdivided at random into four groups: *1*) control, *2*) AG-treated control, *3*) diabetic, and *4*) AG-treated diabetic. Diabetes was induced by a single intravenous injection of streptozotocin (160 mg/kg body wt) freshly dissolved in 100 µl of sterile citrate buffer (ph 4.5). AG was orally administered in groups 2 and 4 by mixing the compound with drinking water at a concentration of 1 g/l. Body weight, plasma glucose level, and glycated Hb were measured 4 and 12 weeks after the induction of diabetes. *$P < 0.001$, compared with control mice; †$P < 0.005$, compared with control mice.

ticles as described above. The phagocytic activity of these cells was evaluated by analyzing the number of phagocytosed particles using flow cytometry. Blood samples were simultaneously withdrawn using a heparinized syringe and centrifuged at 2,000*g* for 10 min. Plasma samples were stored at –70°C until determination of plasma glucose level, while packed red blood cells were used to determine glycated hemoglobin level using a boronate affinity chromatography with Gly-Affin-Ghb (Seikagaku Kogyo, Tokyo).

Tissue preparation. Immediately after obtaining peritoneal macrophages, the peritoneum was dissected, removed, and stored at –70°C until use. The peritoneum was solubilized with type VII collagenase (Sigma) as described previously (29,30). The collagen content in collagenase soluble and insoluble fractions from each sample was estimated using hydroxyproline assay as described elsewhere (31), so that >95% of each tissue collagen was solubilized. The soluble fraction of each sample was stored at –70°C until determination of AGE content.

Measurement of AGE-derived fluorescence and pyrraline assay. The collagen concentration of each digested peritoneal tissue sample was adjusted to 1 mg/ml in Hepes buffer solution for measurement of fluorescence intensity and pyrraline level. Two distinct AGE-derived fluorescence intensities were measured in duplicate with excitation/emission at 370/440 nm (32) and 328/378 nm (33) using a fluorescence spectrophotometer (model F-4010, Hitachi, Tokyo). Fluorescence values were expressed in arbitrary units (AUs) (AU/mg of collagen). Pyrraline levels were determined by ELISA with antipyrraline monoclonal antibody (Pyr-B) according to the procedure for the collagenase digest of tissues as described previously (8,30).

Evaluation of direct effect of AG or L-NAME on phagocytic activity of mouse peritoneal macrophages. To examine if the efficacy of AG might be due to its direct effect as an inhibitor of inducible nitric oxide synthase (iNOS), we incubated the cells with AG at a concentration of 0, 25, 50, or 500 µmol/l in RPMI 1640 medium supplemented with 2% FCS for 4 h followed by analysis of their phagocytic activity as described above. In addition, the effect of L-NAME, an authentic inhibitor of iNOS, on the phagocytic activity of the cells was also evaluated under similar conditions. Furthermore, we examined if the above-mentioned iNOS inhibitors had ability to restore the phagocytic activities declined by the incubation with AGE- or pyrraline-modified albumin as follows: peritoneal macrophages were incubated with AGE-BSA or Pyr-BSA (100 µg/ml) in the presence and absence of 500 µmol/l of AG or L-NAME in vitro and were subsequently determined by their phagocytic activities as described above.

Evaluation of accumulated albumin in mouse peritoneal macrophages. To elucidate the relationship of the macrophage phagocytic function with the amount of internalized AGE- or Pyr-BSA, we incubated peritoneal macrophages from normal mice with ^{125}I-labeled protein samples (100 µg/ml) in the presence or absence of excess amounts of unlabeled ligand under the identical conditions to those for the evaluation of phagocytic activities as described above. The specific cell-associated radioactivity was measured to evaluate the accumulated albumin according to the methods described previously (19).

Measurement of intracellular ATP content of mouse peritoneal macrophages. Next, we investigated the mechanisms by which AGEs affected phagocytic function of macrophages. Because the phagocytic process requires energy, we evaluated the change of intracellular ATP content. For this purpose, peritoneal macrophages from normal mice were preincubated with AGE-BSA, Pyr-BSA, or their respective counterpart controls in the same way that the phagocytic activities were analyzed. The preincubated cells (2×10^4 cells) were extracted with 100 µl of 8% ice-cold perchloric acid. The extraction mixture was neutralized with an equal volume of 1 mol/l ice-cold potassium carbonate and cleared by centrifugation. The ATP content in the supernatant was determined by using an ATP assay kit (Toyo Ink, Tokyo) according to the protocol provided by the manufacturers, which is based on the ability of luciferase to convert luciferin to oxyluciferin in the presence of ATP (34). The emitted bioluminescence intensity was proportionate with the amount of ATP and was measured with an automated lumi-

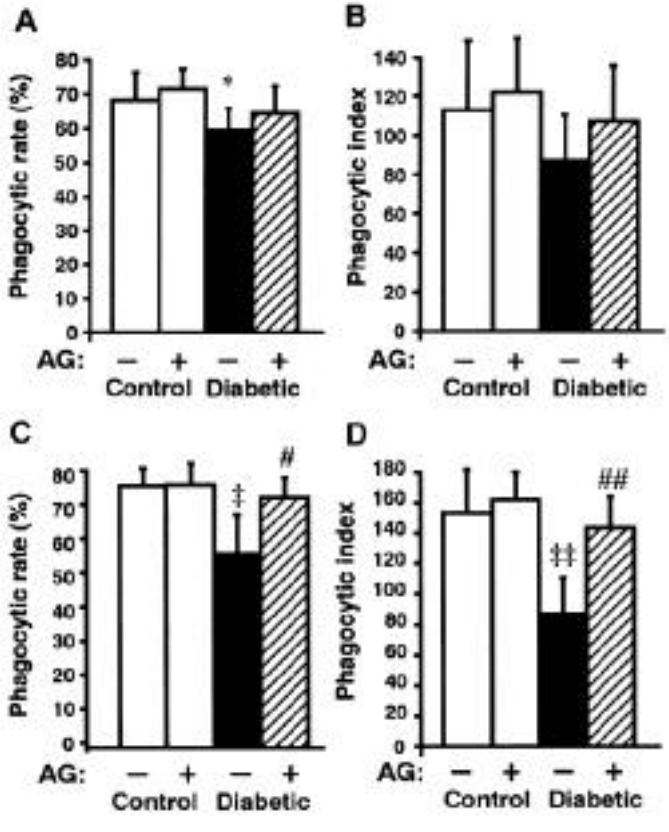

FIG. 2. Phagocytic activity of resident peritoneal macrophages of control and diabetic mice. Resident peritoneal macrophages were obtained from streptozotocin-induced diabetic mice 4 weeks (*A* and *B*) and 12 weeks (*C* and *D*) after induction of diabetes and from age-matched control mice. They were incubated with fluorescent latex particles in vitro. Analysis of the ingested particles was performed using flow cytometry. At week 4, both phagocytic rate (*A*) and index (*B*) were suppressed in diabetic mice, although changes in only the phagocytic rate were statistically significant. Effect of aminoguanidine administration was not statistically significant at this stage. *$P < 0.05$ vs. control mice. After 12 weeks of diabetic duration, both the phagocytic rate (*C*) and phagocytic index (*D*) were significantly suppressed in macrophages of diabetic mice. AG administration restored the reduced activity to almost control level. ‡$P < 0.005$ and ‡‡$P < 0.001$ vs. control mice, respectively. #$P < 0.005$ and ##$P < 0.001$ vs. untreated diabetic mice, respectively. Data are means ± SD.

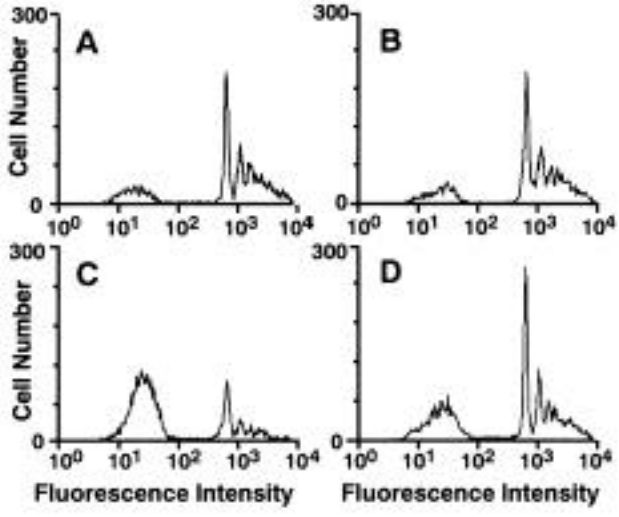

FIG. 3. Histograms of flow cytometric analysis of resident peritoneal macrophages at 12 weeks after induction of diabetes. Resident peritoneal macrophages were obtained from diabetic mice 12 weeks after induction of diabetes and from age-matched control mice. Macrophages from animals treated with aminoguanidine were also prepared. Macrophages were incubated with fluorescent latex particles, followed by flow cytometric analysis of the number of ingested particles. Representative histograms of animals from each group are shown. Because cell-associated fluorescence intensity is dependent on the number of the ingested fluorescent particles, the peaks correspond to cells that contained no ingested particles and 1, 2, 3, and more from left to right, respectively. The peaks in macrophages from diabetic mice shifted to the left, indicating that macrophages phagocytozed fewer particles. AG administration restored the shifted peaks to almost control pattern. *A*, control; *B*, AG-treated control; *C*, diabetic; and *D*, AG-treated diabetic mice. The phagocytic rate and index were calculated from these histograms.

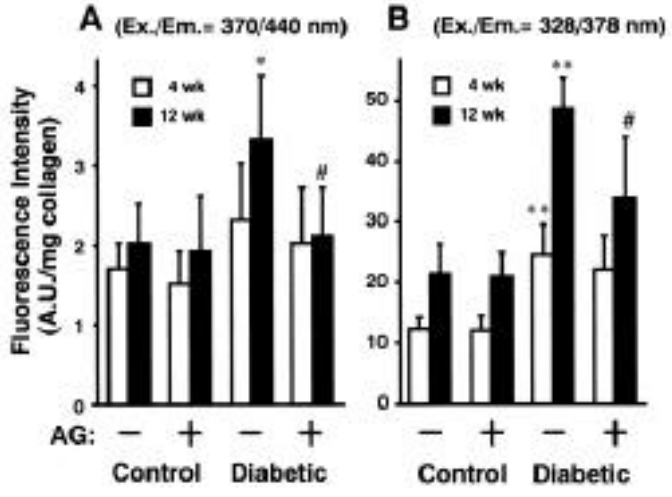

FIG. 4. AGE-derived fluorescence intensity in collagenase digest of mouse peritoneum. AGE-derived fluorescence intensity was measured at 4 weeks and 12 weeks. *A*: The intensity of one of AGE-derived fluorescence (excitation/emission = 370/440 nm) in peritoneal tissue increased significantly with time since the induction of diabetes, while control mice showed only marginal increase with time. The difference in the intensity between control and diabetic mice became significant at 12 weeks. The inhibitory effect of AG on the AGE formation was also observed at 12 weeks. *B*: The intensity of the other AGE-derived fluorescence (excitation/emission = 328/378 nm) in peritoneal tissue also increased with age. The extent of the increase was more significant in diabetic mice than in controls. Although the inhibitory effect of AG was not marked at 4 weeks, it became significant at 12 weeks. Data are means ± SD. *P < 0.005 vs. age-matched control mice, **P < 0.001 vs. age-matched control mice, #P < 0.005 vs. age-matched untreated diabetic mice.

nometer (Bio-Orbit 1253 Luminometer; Bio-Orbit, Turku, Finland). Data are expressed as nanomoles ATP per 1 million cells.

Statistical analysis. Data were expressed as means ± SD. Differences between groups were tested for statistical significance using Welch and Student's t tests. A P value <0.05 denoted statistical significance.

RESULTS

Phagocytic activity of mouse peritoneal macrophages exposed to AGE- or pyrraline-modified protein in vitro. Flow cytometric analysis showed that the phagocytic activity of mouse peritoneal macrophage after incubation with AGE-BSA was less than that of native BSA. The difference became marked with time, so that the phagocytic rate and index of peritoneal macrophages preincubated with AGE-BSA for 4 h were significantly lower (P < 0.001) than those preincubated with native BSA (n = 4 each, phagocytic rate: 48.7 ± 2.3 vs. 72.2 ± 1.1%; phagocytic index: 102.1 ± 4.5 vs. 157.8 ± 6.2; Fig. 1*A* and *B*). It was also shown that the suppressive effect of a mixture of BSA and AGE-BSA (1:1) was between those of 100% BSA and 100% AGE-BSA (rate: 56.9 ± 4.4%; index: 122.2 ± 6.8). This suppression effect of AGE-BSA was clearly reproduced by the incubation experiment with Pyr-BSA. At the incubation period of 4 h, the phagocytic rates of control BSA, Pyr-BSA, and the 1:1 mixture were 71.7 ± 2.5, 44.3 ± 4.9, and 59.2 ± 7.9%, respectively, while the phagocytic indexes were 173.0 ± 22.1, 96.9 ± 2.5, and 129.3 ± 16.5, respectively (Fig. 1*C* and *D*).

In vivo studies. Table 1 shows the profile of animals in the present study at 4 and 12 weeks after induction of diabetes. At 12 weeks, the mean blood glucose concentration was persistently higher in diabetic mice compared with the controls. The level of glycated Hb in diabetic mice at week 12 was significantly higher than at week 4, while control mice showed only a marginal increase. Treatment of mice with oral AG for 12 weeks did not alter blood glucose and glycated Hb levels, nor did it change body weight.

Phagocytic activity of resident peritoneal macrophages in mice. We also investigated the phagocytic activity of resident peritoneal macrophages. The phagocytic rate and index at the commencement of the observation period were 63.2 ± 4.5% and 87.6 ± 11.7, respectively (n = 8). At 4 weeks after induction of diabetes, the phagocytic rate in diabetic mice became significantly less than that of control mice (Fig. 2*A*), while no effect of AG was yet observed in diabetic mice (64.2 ± 7.7%). The phagocytic index in diabetic mice was also lower, albeit insignificantly, than in control mice (Fig. 2*B*). The effect of AG was also not yet significant at this stage. At 12 weeks, a marked difference in phagocytic activity between diabetic and control mice became evident. Representative histograms of flow cytometric analysis of macrophages from each group are shown in Fig. 3. The peak distribution of the histogram shifted to the left in diabetic mice, indicating that fewer fluorescent particles were ingested by macrophages (Fig. 3*C*). The calculated phagocytic rate and index in diabetic mice were significantly less than those in control mice (Fig. 2*C* and *D*). Treatment with AG restored the reduced levels of both parameters to control levels (phagocytic rate 73.7 ± 5.9%, P < 0.005 compared

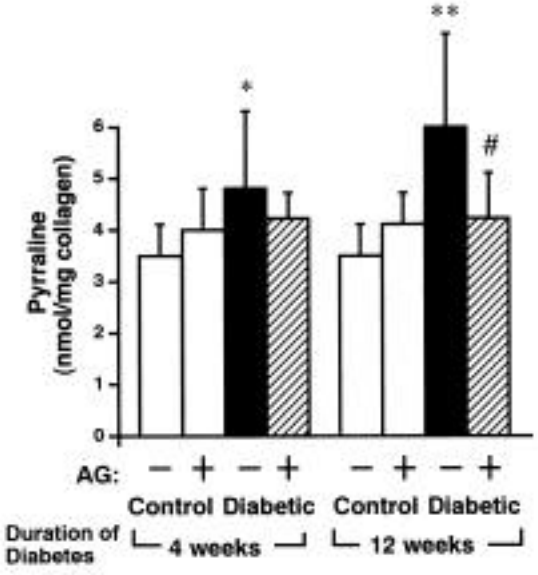

FIG. 5. Pyrraline levels in collagenase digest of mouse peritoneum. Pyrraline level in the peritoneal tissue was significantly higher in diabetic mice than in controls. The inhibitory effect of AG on pyrraline formation was observed at 12 weeks. Data are means ± SD. *P < 0.05 vs. control mice, **P < 0.005 vs. control mice, #P < 0.05 vs. untreated diabetic mice.

with untreated diabetic mice; phagocytic index 143.1 ± 20.9, P < 0.001 compared with untreated diabetic mice).

Measurement of AGE-derived fluorescence intensity of collagenase-digested peritoneal tissue samples. The extent of AGE formation in peritoneal tissue samples was determined by measuring two distinct AGE-derived fluorescence intensities at excitation/emission of 370/440 nm and 328/378 nm. After 4 weeks of diabetes, the fluorescence intensity (excitation 370 nm, emission 440 nm) of peritoneal tissue of diabetic mice was higher than that of the control group (2.3 ± 0.7 vs. 1.7 ± 0.3 AU/mg collagen), although the difference was not statistically significant. The inhibitory effect of AG on fluorescence formation in peritoneal tissues of diabetic mice was marginal (2.0 ± 0.7 AU/mg collagen; Fig. 4*A*). However, at 12 weeks, the fluorescence intensity of peritoneal tissues of diabetic mice was significantly higher than that in the control group (3.3 ± 0.8 vs. 2.0 ± 0.5 AU/mg collagen, P < 0.005). The increased fluorescence intensity was significantly (P < 0.005) quenched to the control level by treatment with AG (2.1 ± 0.6 AU/mg collagen). With regard to the other fluorescence intensity (excitation 328 nm, emission 378 nm), its level in diabetic mice was significantly higher (P < 0.001) than in controls, even at 4 weeks (24.5 ± 5.1 vs. 12.1 ± 2.2 AU/mg collagen), while no marked inhibitory effect of AG was evident at that stage (22.1 ± 5.6 AU/mg collagen; Fig. 4*B*). After 12 weeks, the difference in fluorescence intensity between peritoneal tissue samples of diabetic and control groups became larger (48.6 ± 5.1 vs. 21.5 ± 4.9 AU/mg collagen, P < 0.001). The inhibitory effect of AG became more pronounced at 12 weeks (34.0 ± 9.8 AU/mg collagen, P < 0.005 compared with untreated diabetic mice).

Peritoneal pyrraline levels. The level of pyrraline in collagenase digest of peritoneal tissues increased significantly in diabetic mice 4 weeks after induction of diabetes compared with control mice (Fig. 5). Treatment with AG prevented the increase in pyrraline level to some extent, though this effect

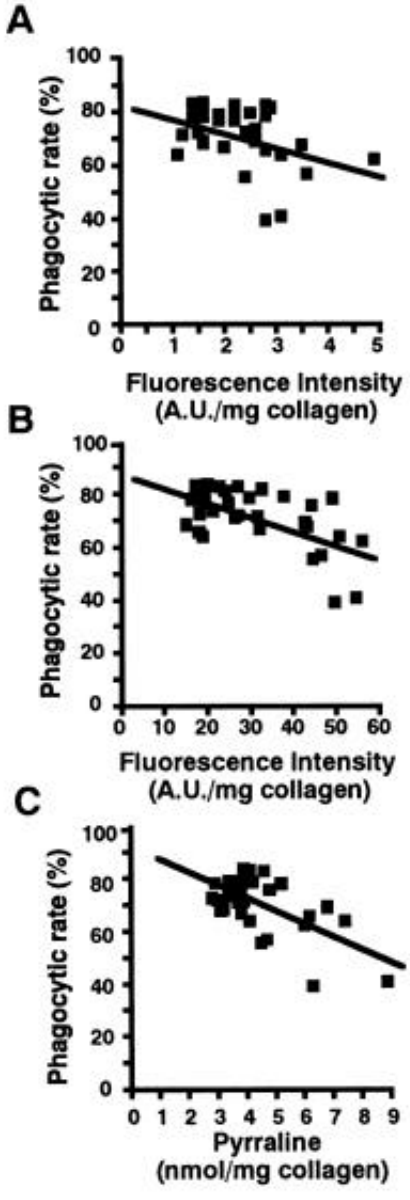

FIG. 6. Correlation between phagocytic rate of peritoneal resident macrophages and AGE content in peritoneal tissues. The phagocytic rates correlated inversely with fluorescence intensity (excitation/emission = 370/440 nm) (*A*), fluorescence intensity (excitation/emission = 328/378 nm) (*B*), and pyrraline levels (*C*) in peritoneal tissues in the same animal. r = –0.41 (P < 0.05), –0.60 (P < 0.001), and –0.61 (P < 0.001), respectively.

was not statistically significant. Pyrraline levels in the peritoneum of diabetic mice increased with time. After 12 weeks, the pyrraline levels were significantly higher than those in control mice (Fig. 5). Administration of AG in diabetic mice significantly quenched the increase in pyrraline level (Fig. 5).

Correlation between phagocytic activity of resident peritoneal macrophages and AGE content in the tissues. A reciprocal relationship was present between AGE content in peritoneal tissues and phagocytic activity of peritoneal macrophages. The correlation coefficients of the relationship between the phagocytic rate at 12 weeks and two distinct fluorescent intensities (excitation/emission = 370/440 and 328/378 nm) and pyrraline levels in the peritoneum were –0.41, –0.60, and –0.61, respectively (Fig. 6). The phagocytic

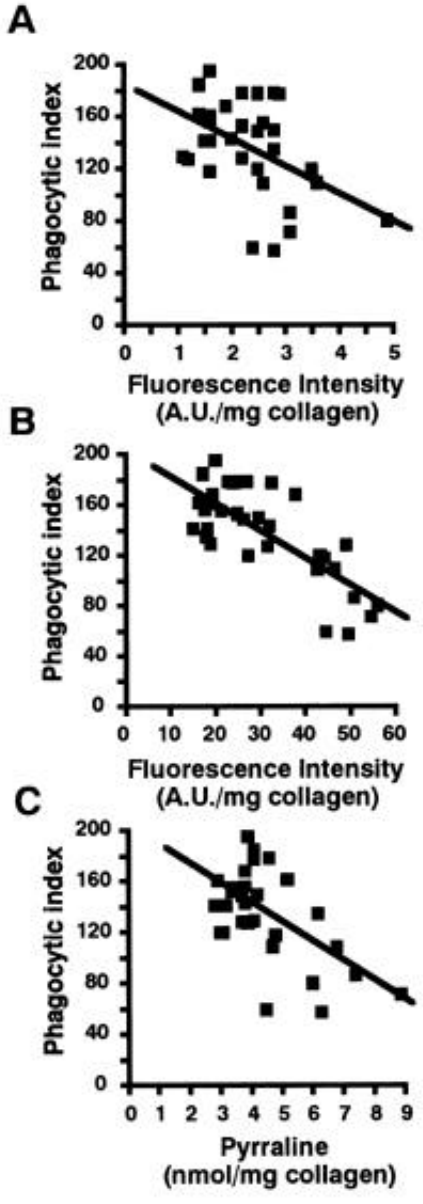

FIG. 7. Correlation between phagocytic index of peritoneal resident macrophages and AGE content in peritoneal tissues. The phagocytic indices correlated inversely with fluorescence intensity (excitation/emission = 370/440 nm) (*A*), fluorescence intensity (excitation/emission = 328/378 nm) (*B*), and pyrraline levels (*C*) in peritoneal tissues from the same animal. $r = -0.46$ ($P < 0.01$), –0.76 ($P < 0.001$), and –0.58 ($P < 0.001$), respectively.

index also inversely correlated with the fluorescence intensity (excitation/emission = 370/440 and 328/378 nm) and pyrraline levels in the tissue (r = –0.46, –0.76, and –0.58, respectively; Fig. 7).

Direct effect of AG or L-NAME on phagocytic activity of mouse peritoneal macrophages. We also examined if iNOS inhibitors directly reflected the phagocytic activity of peritoneal macrophages using flow cytometry (Table 2). The addition of AG alone did not significantly alter the phagocytic activity of the cells. At high concentrations of L-NAME, there was an adverse effect on the phagocytic activity rather than restoring such activity. Furthermore, our findings indicated that the declined phagocytic activity induced by AGEs and Pyr-BSA was not directly restored with either AG or L-NAME (Table 3).

Accumulation of AGE- or pyrraline-modified albumin in macrophage. Assays of internalization of each ^{125}I-labeled protein sample in the macrophages revealed that AGE-BSA was retained in the cells, while intact BSA collected was rapidly degraded. Consequently, a difference in the accumulated amounts of AGE-BSA and BSA became marked at 4 h (9.5 vs. 2.2 μg/mg cell protein; Fig. 8*A*). Pyr-BSA behaved in a similar way to AGE-BSA, resulting in the remarkable accumulation compared with control BSA at 4 h (1.4 vs. 0.5 μg/mg cell protein; Fig. 8*B*).

Alteration in intracellular ATP content of peritoneal macrophages by incubation with AGEs. Intracellular ATP content was gradually declined by the incubation with AGE-BSA in a time-dependent manner, while the suppressive extent of ATP levels in the cells incubated with native BSA was significantly less as shown in Fig. 9*A* (0.8 ± 0.2 vs. 1.8 ± 0.2 at 4 h, $P < 0.001$). This phenomenon was basically reproduced by Pyr-BSA, so that ATP levels in the cells after incubation with Pyr-BSA were significantly less than that of control BSA at 4 h (1.8 ± 0.5 vs. 2.6 ± 0.3 nmol/106 cells, $n = 4$ each, $P < 0.05$; Fig. 9*B*).

DISCUSSION

Impairment of the immune system in diabetic subjects is well defined, and its relevance to alteration of phagocytic properties of monocytes/macrophages has also been proposed (22–24). In the present study, we investigated the involvement of AGE formation in the reduction of phagocytic activity of diabetic macrophages by using flow cytometric analysis with fluorescence-labeled latex particles. This method allows the analysis of a larger sample of cells on a cell-by-cell basis compared with the conventional microscopic analysis (25,26). Furthermore, the method allows an objective quantitation of the phagocytic activity to give two types of parameters, such as phagocytic rate and index. The present results show that the phagocytic activity of cultured mouse resident peritoneal macrophages diminished after exposure to albumin modified by AGEs or pyrraline. The extent of pyrraline modification of the sample used was 3.0 mol pyrraline/mol albumin, estimated by the ultraviolet spectrum and a molar extinction coefficient of pyrraline at 297 nm (7). Calculating the pyrraline modification in vivo based on our previous findings (8), some diabetic patients showed ~0.6 mol pyrraline/mol albumin. Diabetic rats, showing higher blood glucose levels, indicated more extensive modification, such as 1.3 mol/mol. Because AGE structure is very heterogeneous, other AGEs concomitantly form in vivo. Considering that similar findings were obtained from both AGE-BSA and Pyr-BSA, it may be deduced that a covalent modification of the protein per se is a common mechanism mediating the phenomena observed in vitro. In this relation, Lapolla et al. (35) directly determined the extent of glycation in serum albumin from diabetic patients, using a matrix-assisted laser desorption ionization method. They proved that the total number of modified residues by AGEs in a mole of albumin was from 1.4 to 14.8. Thus, the extent of pyrraline modification in the current preparation seems to reflect AGE modification occurring in diabetic subjects in vivo. Therefore, we next designed the experiments to investigate the relationship between AGE accumulation in

TABLE 2
Direct effect of iNOS inhibitors on phagocytic activity of macrophages in vitro

Phagocytic activity	Buffer alone	Aminoguanidine (μmol/l)			L-NAME (μmol/l)		
		25	50	500	25	50	500
Rate (%)	66.8 ± 9.0	62.4 ± 4.6	60.0 ± 9.9	63.6 ± 12.9	57.9 ± 15.7	59.2 ± 16.1	53.0 ± 3.2*
Index	90.7 ± 12.5	90.9 ± 11.9	87.8 ± 13.6	94.3 ± 27.3	85.7 ± 23.8	86.6 ± 34.5	78.4 ± 4.0

Data are means ± SD. Residential peritoneal macrophages were incubated with AG or L-NAME at a concentration of 25, 50, or 500 μmol/l in vitro followed by flow cytometric analysis of their phagocytic activities indicated as phagocytic rate and index. There was no direct significant improvement of activity by these agents. L-NAME exerted a suppressive effect rather than restoring effect on the activity. *$P < 0.05$ vs. the phagocytic rate in control cells preincubated with medium alone.

tissues and phagocytic activity of macrophages using an animal model of diabetes. The AGE formation in the peritoneal tissue increased proportionately with the duration of diabetes, while age-matched control mice showed a negligible change in the amount of AGEs. On the contrary, the phagocytic activity of peritoneal macrophages of diabetic mice was lower than that of age-matched controls. Such suppression became more marked at 12 weeks after induction of diabetes than at 4 weeks, indicating that peritoneal macrophages in the more AGE-rich environment had a lower phagocytic activity.

The precise mechanism responsible for the defective phagocytic activity of macrophages of diabetic mice remains to be elucidated. The high level of glucose may by itself affect macrophage function. Glass et al. (22) had suggested that hyperglycemia might downregulate lectin receptors present on mouse peritoneal macrophages, leading to a defective recognition of the bacterial cell wall. However, results of their subsequent studies in type 1 diabetic patients indicated that hyperglycemia itself had no effect on the expression of lectin-like receptors on monocytes (23). They speculated that continuous interaction between glycated serum proteins with the receptor might disturb its normal cellular distribution and turnover. Because the latex particles used in our experiment are thought to be phagocytosed nonspecifically because of the surface hydrophobicity or through unknown receptors, neither involvement of lectin receptor nor opsonization is essential for the regulation of the phagocytosis. In addition, our current in vitro experiments confirmed that high glucose concentration did not influence the phagocytic activity of macrophages (data not shown).

In the present study, administration of AG, a glycation inhibitor (36), to diabetic mice restored the phagocytic activity of macrophages to almost control levels along with the suppression of AGE formation in the tissue. This effect was not associated with changes in glycohemoglobin level, confirming that blood glucose level was not an important factor in this process. These results suggest the involvement of advanced stages of the Maillard reaction after addition of Amadori product in the phagocytic activity of macrophages. Iavicoli et al. (24) reported that the colloid clearance was significantly reduced in diabetes associated with severe microangiopathy, compared with patients who had no sign of microangiopathy or with normal subjects. They suggested that increased circulating immune complexes in patients with severe microangiopathy may result from an impaired function of fixed phagocytes. Because AGEs are thought to be involved in the progression of microangiopathy (1,2), Iavicoli et al.'s findings may support the potential relationship between AGEs and reduced phagocytic activity.

In addition to the inhibitory effect of AG on the AGE formation, this agent is also known to inhibit iNOS (37). However, our results demonstrated that the contribution of the latter activity to amelioration of the phagocytic activity was negligible, since the phagocytic activity did not change by reaction with AG at a concentration equivalent to ~10-fold of 50% inhibitory concentration (IC_{50}) for iNOS-inhibiting activity. This result was further supported by the fact that L-NAME, an authentic iNOS inhibitor, did not ameliorate the phagocytic activity of macrophages.

TABLE 3
Effect of iNOS inhibitors on restoring the AGE-induced reduction of phagocytic activity of macrophages in vitro

Incubation	Phagocytic activity	
	Rate (%)	Index
Native BSA (100 μg/ml)	70.9 ± 2.9	159.6 ± 12.0
AGE-BSA (100 μg/ml) alone	64.7 ± 3.0	121.8 ± 18.5
Plus aminoguanidine (500 μmol/l)	66.1 ± 6.6	104.0 ± 37.1
Plus L-NAME (500 μmol/l)	65.2 ± 3.7	112.6 ± 5.9
Control BSA (100 μg/ml)	72.6 ± 4.6	190.7 ± 18.6
Pyr-BSA (100 μg/ml) alone	64.6 ± 3.8	143.5 ± 33.0
Plus aminoguanidine (500 μmol/l)	65.4 ± 1.8	111.6 ± 8.6
Plus L-NAME (500 μmol/l)	65.9 ± 4.9	123.5 ± 21.1

Data are means ± SD. There was no profitable effect of AG or L-NAME on restoring the reduced phagocytic activity of peritoneal macrophages induced by the incubation with AGE-BSA or Pyr-BSA.

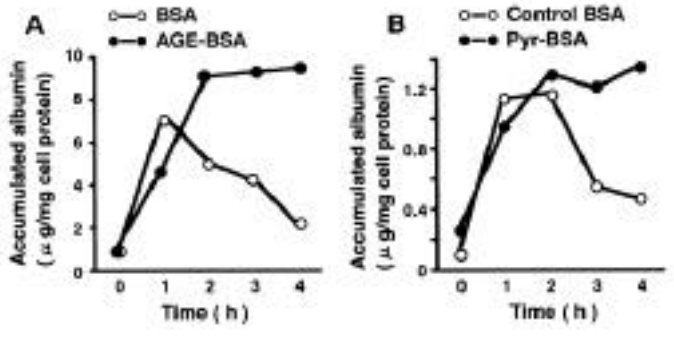

FIG. 8. Accumulation of AGE- or pyrraline-modified albumin in macrophages. Internalized AGE-BSA (*A*) or Pyr-BSA (*B*) was retained in the cells even after 4 h, while their respective counterpart controls were efficiently degraded.

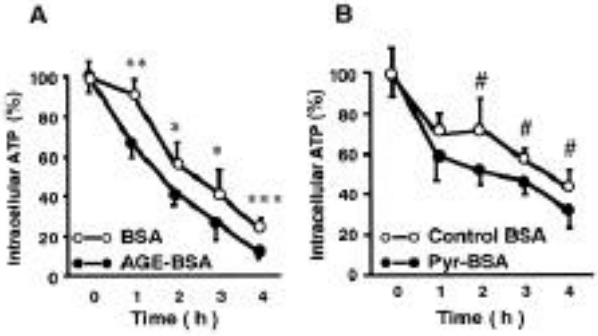

FIG. 9. Intracellular ATP content of peritoneal resident macrophages preincubated with AGE-modified and pyrraline-modified albumin. Peritoneal macrophages obtained from normal mice were preincubated with native BSA and AGE-BSA (*A*), as well as control and Pyr-BSA (*B*) for 0, 1, 2, 3, and 4 h, followed by determination of intracellular ATP content (n = 4, each). The results are presented as percent of that of untreated cells. ATP contents in macrophages preincubated with AGE-BSA and Pyr-BSA were significantly suppressed, compared with their respective counterpart controls. Mean ± SD is plotted. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. BSA, and #P < 0.05 vs. control BSA.

Our present findings show an inverse relationship between the phagocytic activity of resident peritoneal macrophages and AGE content, including pyrraline in the adjacent peritoneal tissue. Recent studies reveal that uptake of AGE-modified proteins by macrophages occurred through scavenger receptors as well as unknown receptors (38,39). Our previous (19) and current findings indicate that internalized pyrraline-modified albumin tended to accumulate in macrophages while the intact albumin was efficiently degraded (19). Furthermore, we found in the present study that AGE-BSA also shows a tendency to accumulate in macrophage cells in vitro. Thus, accumulation of AGEs in cells might lead to negative feedback on ingestion of substances. Alternatively, cells might be exhausted with degrading AGE-modified proteins that are resistant to proteolysis. We have also proved that pyrraline modification of albumin increased its resistance to degradation by lysosomal enzymes such as cathepsin D (19). In this regard, Pillai and Zull (40) indicated that lysosomal proteolysis by cathepsin D is ATP-dependent. On the other hand, it is known that phagocytosis is an active process that requires metabolic energy (41,42). Therefore, excess energy consumption at the proteolytic step may potentially limit the energy-dependent process of phagocytosis. This hypothesis may be supported by the present finding that intracellular ATP content in macrophages diminished after incubation with AGE proteins.

In conclusion, our results show that the accumulation of AGEs potentially influence macrophage phagocytic function, concomitant with the decrease in intracellular ATP content. Considering that phagocytosis is an important component of the defense system, suppression of such activity by AGEs may explain, at least in part, the increased susceptibility of diabetic patients to infection. Because macrophages exhibit a variety of functions, other forms of dysfunction might occur in a manner similar to this process. The present findings may provide a novel approach to elucidate the mechanisms of macrophage-related disorders in diabetes.

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