Advanced Glycation End Products Are Direct Modulators of β-Cell Function

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OBJECTIVE—Excess accumulation of advanced glycation end products (AGEs) contributes to aging and chronic diseases. We aimed to obtain evidence that exposure to AGEs plays a role in the development of type 1 diabetes (T1D).

RESEARCH DESIGN AND METHODS—The effect of AGEs was examined on insulin secretion by MIN6N8 cells and mouse islets and in vivo in three separate rodent models: AGE-injected or high AGE–fed Sprague-Dawley rats and nonobese diabetic (NODLt) mice. Rodents were also treated with the AGE-lowering agent alagebrium.

RESULTS—β-Cells exposed to AGEs displayed acute glucose-stimulated insulin secretory defects, mitochondrial abnormalities including excess superoxide generation, a decline in ATP content, loss of MnSOD activity, reduced calcium flux, and increased glucose uptake, all of which were improved with alagebrium treatment or with MnSOD adenoviral overexpression. Isolated mouse islets exposed to AGEs had decreased glucose-stimulated insulin secretion, increased mitochondrial superoxide production, and depletion of ATP content, which were improved with alagebrium or with MnTBAP, an SOD mimetic. In rats, transient or chronic exposure to AGEs caused progressive insulin secretory defects, superoxide generation, and β-cell death, ameliorated with alagebrium. NODLt mice had increased circulating AGEs in association with an increase in islet mitochondrial superoxide generation, which was prevented by alagebrium, which also reduced the incidence of autoimmune diabetes. Finally, at-risk children who progressed to T1D had higher AGE concentrations than matched nonprogressors.

CONCLUSIONS—These findings demonstrate that AGEs directly cause insulin secretory defects, most likely by impairing mitochondrial function, which may contribute to the development of T1D. ***Diabetes*** **60:2523–2532, 2011**

Type 1 diabetes (T1D) is characterized by insulin deficiency secondary to autoimmune-mediated destruction of insulin-producing β-cells in the pancreatic islets. Less than 50% of the familial clustering in T1D can be attributed to known genotypes (1), and the rising disease incidence in Westernized societies (2) is assumed to reflect changing environmental conditions. Indeed, the increased incidence of T1D over the past several decades can be entirely accounted for by case subjects with lower-risk HLA genes (3,4).

Advanced glycation end products (AGEs) may be environmental factors that contribute to the development of T1D. When reducing sugars such as glucose or carbonyls react nonenzymatically with amino groups, they result in glycated modifications such as HbA_{1c} (5), which after further biochemical rearrangements form AGEs. AGE formation occurs as part of the normal aging process, and AGE accumulation is accelerated by hyperglycemia. This explains why these sugar-dependent modifications have been extensively studied as pathogenic agents in diabetes complications (6–8). Nevertheless, they are also formed in the absence of hyperglycemia by homeostatic imbalances such as those with redox imbalances (9), aging (10), with kidney disease (11), or with other autoimmune diseases (12).

Apart from endogenously formed AGEs, exogenous AGEs are derived from foods (e.g., meat, milk, coffee, cheese), especially those prepared or processed under high temperature, stored for long periods, or containing food additives. Studies in animal models have demonstrated that restriction of dietary AGE intake significantly improves insulin sensitivity and extends lifespan in mouse models (13,14). In addition, low AGE–fed NOD mice have shown lower transmission of autoimmune diabetes across three generations (15). Furthermore, maternally transmitted AGEs prematurely elevate circulating AGE concentrations in infants to adult levels, thereby activating inflammatory pathways (16). A recent acute study has suggested that AGE injections can initiate β-cell dysfunction in vivo (17).

In the current study, we investigated the direct effects of AGEs on islet secretory function in cultured MIN6N8 cells, isolated rodent islets, and healthy rodents. Exposure via injection or oral intake in healthy rats and nonobese diabetic (NODLt) mice was used to determine whether AGEs impair β-cell function, which in the context of genetic susceptibility could promote development of T1D. We also investigated the effects of AGE-induced mitochondrial dysfunction on insulin secretion because excess

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superoxide generation in these organelles is known to lead to β-cell damage. In addition, circulating AGE concentrations were measured in at-risk children subsequently followed for progression to T1D.

RESEARCH DESIGN AND METHODS

Human subjects. Children and adolescents with pancreatic islet autoantibodies who were first-degree relatives of someone with T1D were monitored prospectively in the Melbourne Pre-Diabetes Family Study (18). Eighteen individuals (median age 13.4 years) developed diabetes over a median of 4.4 years follow-up. These were matched as closely as possible for sex, age, HLA-risk gene status, β-cell function, and number of islet autoantibody specificities with an equal number who had not developed diabetes over a median of 6.5 years' follow-up (19). The concentration of AGEs in serum samples obtained at the start of follow-up in each group was measured retrospectively. The study was approved by the Melbourne Health Human Research Ethics Committee.

Laboratory rodent studies. Groups of male Sprague-Dawley rats (n = 10/group) were given daily intraperitoneal injections of either AGE-modified rat serum albumin (AGE-RSA), RSA at 20 mg/kg/day, or saline (sham) for 4 months while on standard rat chow fed ad libitum. An additional subgroup of rodents injected with AGE-RSA (n = 10/group) received treatment with the AGE-lowering therapy alagebrium (4,5-dimethyl-3-(2-oxo2-phenylethyl)-thiazolium chloride; Synvista Pharmaceuticals, Ramsey, NJ) (10 mg/kg/day by oral gavage for the study duration [AGE-RSA+ALT]).

In the second rat study, groups of healthy male Sprague-Dawley rats (n = 20/group) were randomized to receive a diet low in AGE content (unbaked AIN-93G, containing the AGE N^{ε}(carboxymethyl)lysine (CML), 20.09 nmol/mol lysine/100 mg; Specialty Feeds, Perth, Australia); high in AGE content, (baked AIN-93G for 1 h at 165°C, 101.9 nmol/mol lysine/100 mg CML) or high in dextrose (glucose, 636 g dextrose per kg, Specialty Feeds) for 24 weeks (dietary analysis available in Supplementary Table 1). These rats were further randomized to receive sham gavage or 10 mg/kg/day alagebrium chloride by oral gavage for the study duration (n = 10/group).

In the third animal study, female NODLt mice fed standard mouse chow were studied for prediabetes at age 75 days (n = 20/group) with or without alagebrium chloride (1 mg/kg/day i.p.), administered from days 50–75 of age. For incidence studies, additional groups of littermate NODLt mice (n = 20/group) were studied until the diagnosis of diabetes was confirmed by glycosuria on three consecutive days, followed by a fasting blood glucose >15 mmol/L or until day 200 of age in the absence of diabetes.

All rats and mice were given ad libitum access to food and water and maintained on 12-h dark-light cycles. Food intake was monitored by metabolic caging for 24 h. Blood glucose by autoanalyzer (Biochemistry Department, Alfred Hospital, Prahran, Australia) and glycated hemoglobin were measured weekly. Serum CML concentrations were determined as previously described (20). All animal experiments were approved by the animal ethics committee of the Alfred Medical Research and Education Precinct.

Cell culture. All data shown are representative of two independent experiments, with n = 3–6 replicates per group per experiment. MIN6N8 cells, which are SV40-transformed insulinoma cells derived from NOD mice (21), were grown in Dulbecco's modified Eagle's medium (DMEM) and 15% fetal bovine serum (FBS). One percent FBS was used for 24 h before and during all experiments. Cells were incubated in the presence and absence of varying concentrations of AGE-BSA (10, 50, 100, or 500 μg/mL) or BSA control (10, 50, 100, or 500 μg/mL) for 28 days in normal glucose (5 mmol/L).

Manganese superoxide dismutase (MnSOD) haemagglutinating virus of Japan (HVJ)-liposome and empty vector adenoviruses were constructed by the Gene Transfer Vector Core (University of Iowa, Iowa City, IA). Cells were infected at 1 μl/million cells in 15 ml DMEM in 75 cm^2 flasks (1.2×10^{12} plaque-forming units [PFUs]/ml). HVJ liposomes were administered for 4 h before treatment with 100 μg/mL AGE-BSA or 100 μg/mL BSA for 7 days. Infection efficiency was determined by real-time RT-PCR, visualization of fluorescence using microscopy, and using the MnSOD activity assay as described below. Three independent experiments were performed.

Glucose uptake. Glucose uptake by MIN6N8 cells was determined using 2-deoxy-glucose (2DG) as previously described (22). Nonspecific uptake, measured in the presence of cytochalasin-B, was subtracted from total uptake.

Intravenous and intraperitoneal glucose tolerance tests. Intravenous glucose tolerance tests (IVGTTs) were performed on rats as previously described (23). Intraperitoneal glucose tolerance tests (IPGTTs) were performed as previously described (23) except that 1 g/kg glucose bolus was injected intraperitoneally and blood samples were taken at 0, 15, 30, 60, and 120 min.

Preparation of exogenous AGE albumin. Because the AGE CML is a modification of the parent amino acid lysine, albumin was used as the protein source of lysine because it the most abundant protein within the circulation and is also widely consumed in Western diets (24). Fatty acid–free cold ethanol precipitated fraction V of rat or bovine serum albumin (50 mg/ml; Sigma Chemical, St. Louis, MO) was incubated in the presence and absence of 0.5 mol/L D-glucose as previously described (25). The preparations were then dialyzed, and endotoxin was removed. The AGE CML was quantified by isotope dilution, selected ion-monitoring gas chromatography–mass spectrometry (SIM-GC/MS) (26), where the major AGE modification present on lysine residues within AGE-RSA and AGE-BSA was CML (38.2 ± 3.6 and 67.0 ± 1.2 mmol/mol lysine, respectively). There were also low levels of AGE moieties detected in native BSA (CML: 0.5 mmol/mol lysine) and RSA (0.3 mmol/mol lysine), corresponding to those produced during natural aging.

Calcium flux across MIN6N8 cells. MIN6N8 cells were cultivated on coverslips in various treatment conditions to confluence. On day 7, confluent cells were loaded with 2 μmol/L fura-2 (Molecular Probes)/DMSO for 30 min at 37°C in the dark in the presence of 2.8 mmol/L glucose. Cells were bathed with prewarmed Krebs-Ringer Bicarbonate buffer (KRBB) containing 2.8 mmol/L glucose and placed in a microchamber on the stage of a fluorescence microscope (Ion Optix, Olympus, Japan). Cells were excited at 1 Hz with 340 and 380 nm, and the emission signals at 510 nm were detected. Glucose (20 mmol/L) was injected at t = 30 s while the calcium ionophore ionomycin (Ca^{2+} channel opener) was added at the end of fluorescence measurements. The 340 (Ca^{2+}-bound chelator), 380 (Ca^{2+}-free), and 340/380 nm ratio signals were recorded continuously over a period of 300 s with IonOptix intensified charge coupled device video camera. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is expressed as the 340/380 nm ratio. The calcium channel blocker verapamil was used as a control (10 μmol/L).

Real-time RT-PCR. Gene expression for each of the sequences listed below was analyzed by real-time quantitative RT-PCR performed with the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Foster City, CA) as previously described (27). For rat proinsulin (NM_019130), the forward primer was 5′-TGGTTCTCACTTGGTG-GAAGCT-3′, the reverse primer 5′-GGACATGGGTGTGTAGAAGAATCC-3′, and the probe 6-FAM CCCACACACCAGGTAG-MGB. For mouse proinsulin (NM_008387), the probe was the same as that as for rat; however, the forward primer was 5′-TCAAGCAGCACCTTTGTGGTT-3′ and the reverse primer 5′-GGGACATGGGTGTGTAGAAGAAG-3′. For rat receptor for AGEs (RAGE) (L_33413), the probe was 6-FAM TGTGCCATCTCTGC-MGB, the forward primer was 5′-TCCTGGTGGGACCGTGAC-3′, and the reverse primer was 5′-GGGTGTGCCATCTTTTATCCA-3′.

Immunohistochemistry. A modification of the avidin biotin complex immunoglobulin enzyme bridge technique was used for immunohistochemistry as previously described (7). The primary antibodies used in this study were mouse anti-insulin/proinsulin (1:2,000; Biogenesis, Poole, U.K.), rabbit anti-CML (1:500) (28), goat-anti RAGE (1:500; Chemicon, Temecula, CA), and mouse anti-ED-1 (monocyte/macrophages, 1:50; Serotec, Kiddington, Oxford, U.K.). Quantitation of islet immunostaining was completed by computer-aided densitometry (Optimas 6.2 Video Pro-32; Bedford Park, Australia), where a minimum of 20 islets (×100) was counted per section. Results were expressed as proportional area of positive staining within islets (29).

Islet extraction. Pancreatic islets from C57BL/6J mice, NODLt mice or Sprague Dawley rats were isolated as previously described (30). Islets obtained from C57BL/6J were exposed to 100μg/mL AGE-BSA or 100μg/mL BSA in the presence and absence of the AGE inhibitor, alagebrium (1μmol/L) or the MnSOD mimetic MnTBAP (20μmol/L, Alexis Biochemicals, Switzerland).

Insulin secretion. MIN6N8 cells were seeded in 12-well plates and treated for 7 days. Isolated islets were plated at 10 islets per well. Insulin secretion assays were performed as previously described (23). Insulin content is the sum of insulin secreted during the first and second stimulation periods and intracellular insulin extracted from cells at the end of the glucose challenge. Glucose-stimulated insulin secretion (GSIS) during the first 10 min was expressed as percentage of total insulin content. The amount of insulin in the incubation buffer and cellular extracts/islets was measured using an insulin ELISA kit (Linco Research).

ATP content. Measurement of mitochondrial ATP content was performed in triplicate as previously described (31) using the bioluminescent ATP determination assay kit (Invitrogen, Carlsbad, CA). Mitochondria were isolated from 25-mm^2 flasks of cells using a mitochondrial isolation kit (Pierce, Rockford, IL) immediately prior to analysis. For measurements in islets, 20 islets were handpicked into 96-well plates.

Mitochondrial superoxide production. Superoxide production was determined in isolated mitochondria extracted from 75-mm^2 flasks of MIN6N8 cells using a Mitochondrial Isolation kit (Pierce) or in isolated islets (n = 30 islets/well) as previously described (20). For the AGE-BSA dose response in MIN6N8 cells, cells were harvested with trypsin and washed and resuspended with Hank's balanced salt solution (HBSS) and 5 μmol/L MitoSOX Red mitochondrial superoxide indicator (Molecular Probes, Eugene, OR) in HBSS was

added. Cells were incubated for 30 min at 37°C in the dark, washed with PBS, and analyzed on a FACS Calibur (BD Biosciences) flow cytometer using excitation at 400 nm with detection at 590 nm in the FL2 channel. A minimum of 15,000 events per sample was acquired.

MnSOD activity assay. Mitochondrial MnSOD activity was measured using a SOD assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's specifications.

Terminal dUTP nick-end labeling. Cell death was identified by 3′ in situ end labeling of fragmented DNA with biotinylated deoxyuridine-triphosphate as previously described (29).

Statistical analysis. Results are expressed as means ± SD unless otherwise specified. All analyses of rodent data with more than two groups were performed by ANOVA followed by post hoc analysis with Tukey's test. Student t or Mann-Whitney U tests were used to compare two groups where appropriate. A log-rank (Mantel-Cox) test was used to analyze diabetes incidence in NOD mice. A value of $P < 0.05$ was considered statistically significant. Differences in human age, islet autoantibodies, and metabolic parameters were analyzed with the Mann-Whitney U test. Frequencies of HLA types were assessed by the χ^2 test for trends. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

RESULTS

AGEs recruit pathways linked to β-cell damage. Incubation of MIN6N8 cells with 10, 50, or 100 μg/mL AGE-BSA for 28 days led to a significant dose-dependent reduction in GSIS compared with BSA-treated controls (Fig. 1*A*). Exposure to 10, 50, or 100 μg/mL AGE-BSA increased mitochondrial superoxide generation (Fig. 1*B*) in MIN6N8 cells compared with corresponding BSA controls. AGE-BSA treatment resulted in a loss of mitochondrial ATP content in MIN6N8 cells (Fig. 1*C*) compared with BSA controls at the same concentration. AGE-BSA exposure also decreased MnSOD activity (Fig. 1*D*) at concentrations of 50 or 100 μg/mL compared with cells treated with BSA alone. These experiments revealed a nonlinear dose response to AGE-BSA in MIN6N8 cells.

We then used 100 μg/mL AGE-BSA, a physiologically relevant concentration, for further in vitro studies in MIN6N8 cells. Both GSIS and mitochondrial ATP content were reduced with AGE-BSA treatment, and each was restored by treatment with the AGE inhibitor alagebrium (Fig. 2*A* and *B*). Cellular uptake of glucose was increased following exposure to AGE-BSA and returned to levels seen in the BSA control group following coincubation with alagebrium or with the glucose transport inhibitor cytochalasin B (Fig. 2*C*). A reduction in calcium flux (Fig. 2*D*) in AGE-BSA–treated cells was also demonstrated compared with BSA and alagebrium-treated MIN6N8 cells.

MnSOD activity in MIN6N8 cells was inhibited by coincubation with AGEs, but this was restored by concomitant alagebrium administration (Fig. 3*A*) or with infection by an MnSOD overexpressing adenoviral vector (Fig. 3*B*). Adenoviral overexpression of MnSOD also prevented the AGE-induced decline in GSIS (Fig. 3*C*) and decreased mitochondrial superoxide generation in response to AGEs in MIN6N8 cells (Fig. 3*D*).

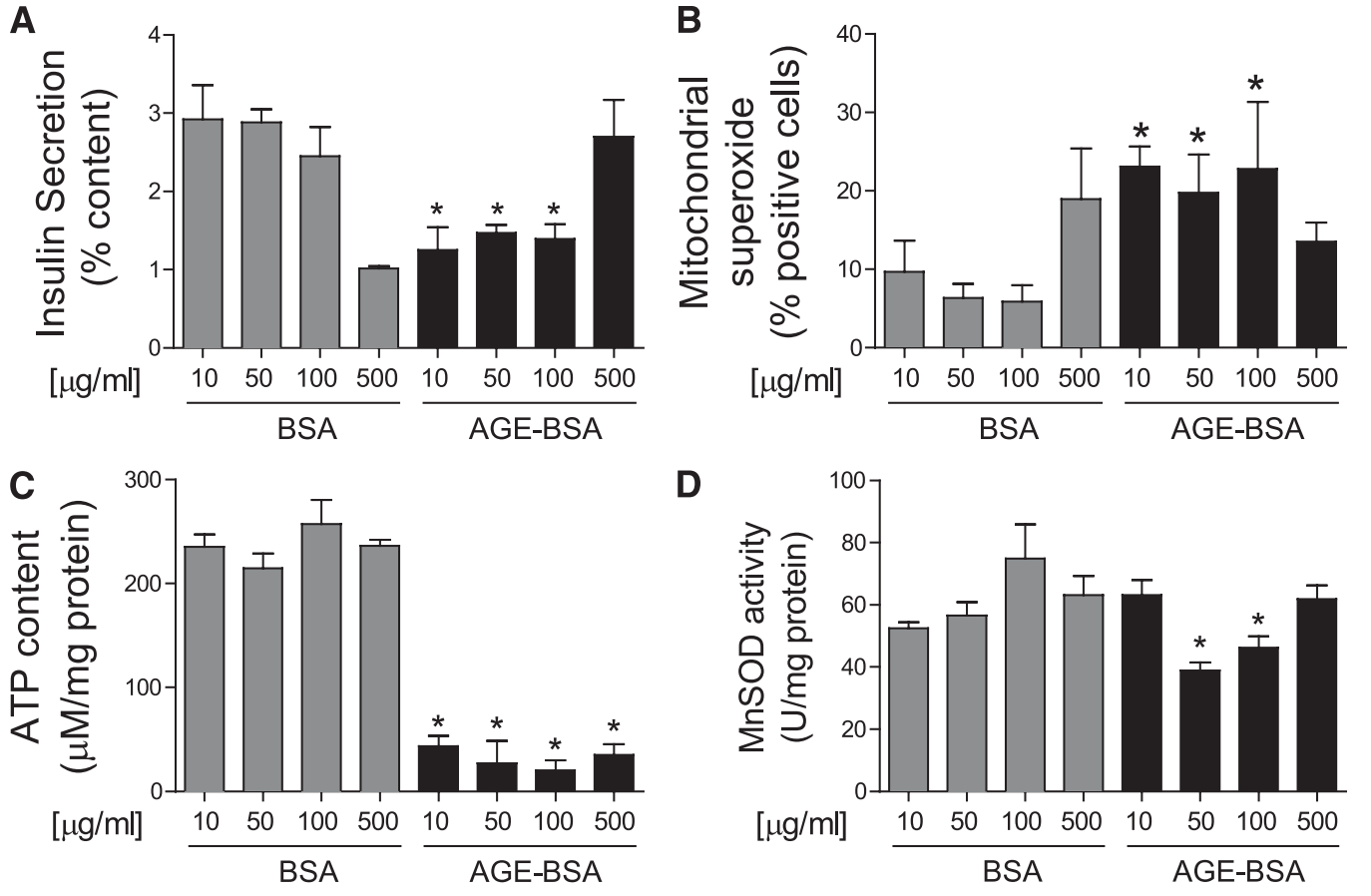

FIG. 1. AGEs recruit known pathways of β-cell damage to induce insulin secretory defects in MIN6N8 cells. MIN6N8 cells were grown in normal glucose (5 mmol/L) for 28 days with varying concentrations of AGE-BSA (10, 50, 100, or 500 μg/mL) or BSA (10, 50, 100, or 500 μg/mL) control. *A*: 20 mmol/L GSIS. *B*: Mitochondrial superoxide production. *C*: Mitochondrial ATP content. *D*: Mitochondrial MnSOD activity. All data shown are representative of two independent experiments (n = 4–6 replicates/group per experiment). *P < 0.05 vs. corresponding BSA control.

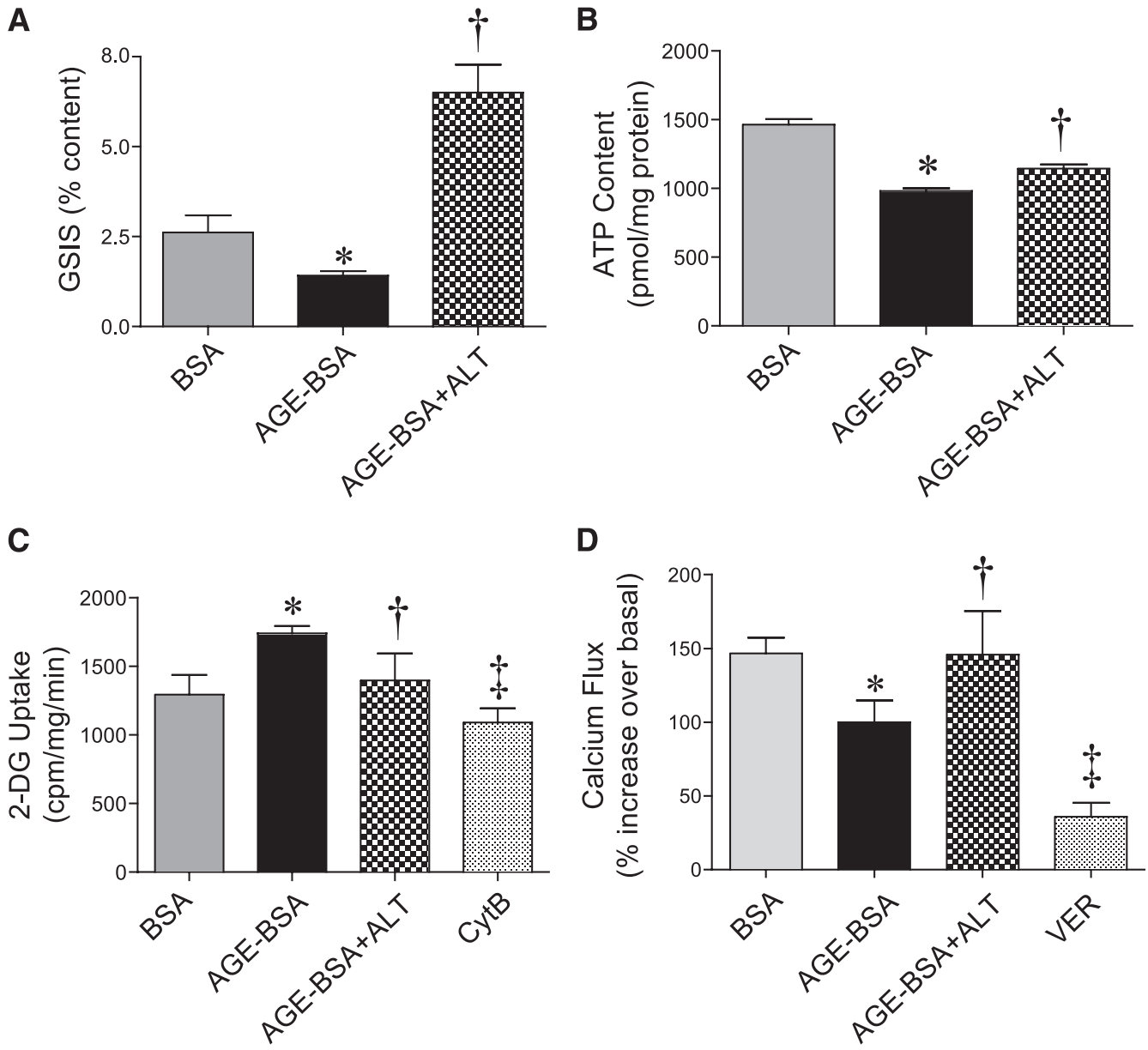

FIG. 2. AGE-induced pathological pathways are normalized with the AGE-lowering therapy alagebrium in MIN6N8 cells. MIN6N8 cells were grown in normal glucose (5 mmol/L) for 28 days with 100 μg/mL AGE-BSA or 100 μg/mL BSA with or without 1 μmol/L alagebrium. *A*: 20 mmol/L GSIS. *B*: mitochondrial ATP content. *C*: Glucose uptake using 2-DG. *D*: Calcium flux using Fura-2. *$P < 0.001$ vs. corresponding BSA control, †$P < 0.05$ vs. AGE-BSA, ‡$P < 0.001$ vs. AGE-BSA. CytB, cytochalasin B; VER, verapamil.

To complement studies performed in MIN6N8 cells, we isolated pancreatic islets from healthy mice and exposed them to 100 μg/mL AGE-BSA for 24 h. AGE exposure impaired GSIS, which was improved with either alagebrium or the MnSOD mimetic MnTBAP (Fig. 3*E*). AGE exposure also increased mitochondrial superoxide generation in isolated mouse islets (Fig. 3*F*) and lowered islet ATP content (Fig. 3*G*), both of these changes being attenuated by either alagebrium or MnTBAP coadministration.

Recurring transient or chronic elevations in circulating AGEs cause insulin insufficiency in healthy rats. In vivo studies were next performed to assess the effects of AGEs on pancreatic islet function in situ. Two experimental rat models were used. The first required intraperitoneal injections of AGE-RSA, or RSA as a control, to be administered daily to healthy male Sprague-Dawley rats for 16 weeks. AGE injections resulted in a transient daily increase in circulating AGEs (Fig. 4*A*). Daily AGE-RSA injections impaired insulin secretion during an IVGTT compared with RSA control-injected rats (Fig. 4*B*). This AGE-RSA–dependent insulin secretory defect was prevented by alagebrium (Fig. 4*B*). No differences were seen, however, in plasma glucose concentrations during the IVGTT in injected rat groups (Fig. 4*C*). AGE-RSA injections also decreased islet preproinsulin gene expression (0.46 ± 0.26-fold induction, $n = 4$/group), which was not observed in RSA-injected rats (1.25 ± 0.53; $P < 0.01$ vs. AGE-RSA) or in AGE-RSA–injected rats that received alagebrium therapy (1.35 ± 0.37; $P < 0.01$ vs. AGE-RSA). Although defects in insulin secretion and gene expression were identified, there were no changes in fasting plasma glucose, body weight, or glycated hemoglobin in the

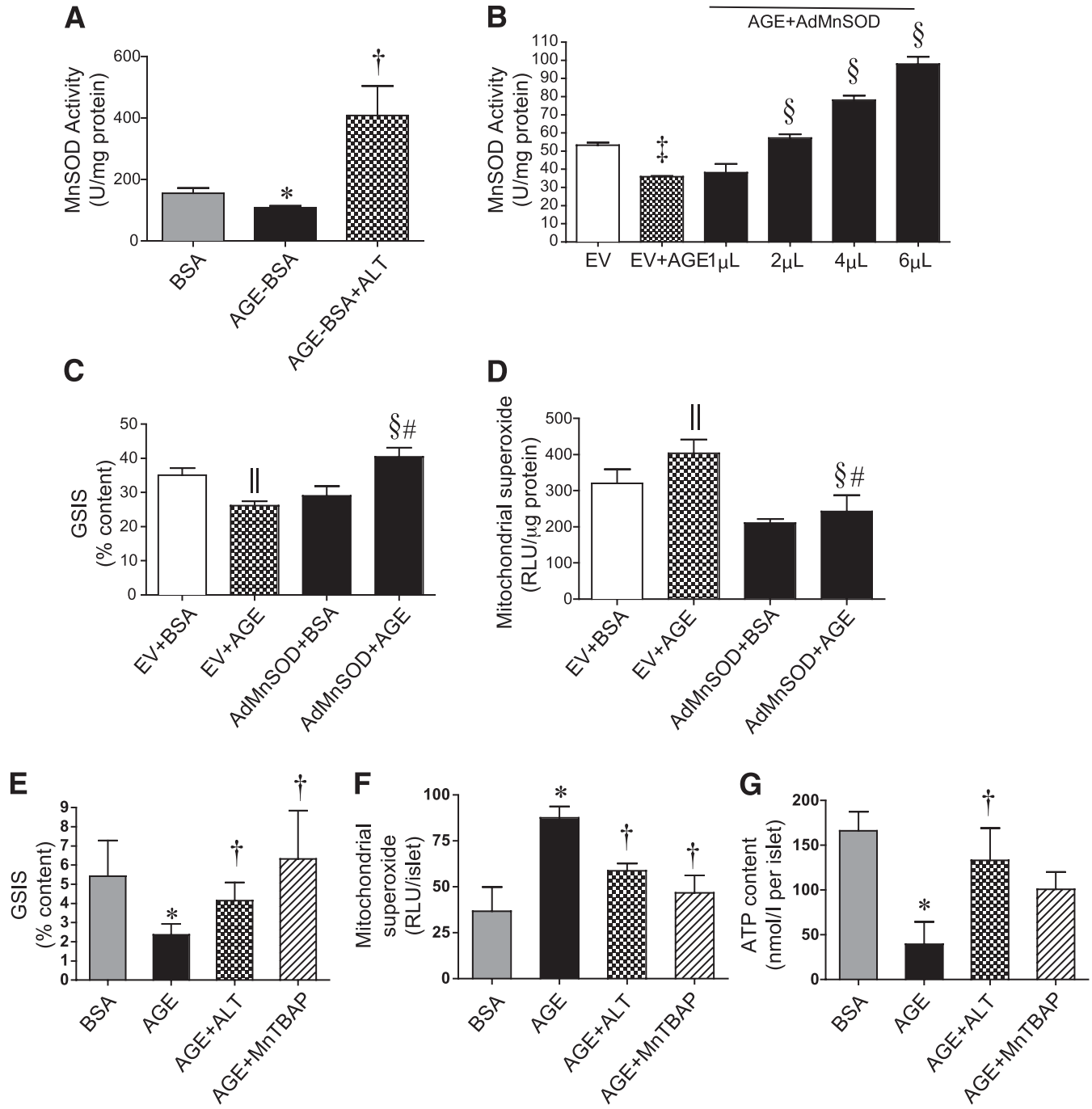

FIG. 3. Restoration of MnSOD activity prevents AGE-induced insulin secretory defects. *A*: MIN6N8 cells were exposed to AGE-BSA or BSA at 100 μg/mL for 28 days with or without 1 μmol/L alagebrium, and MnSOD activity was determined. *B–D*: MIN6N8 cells infected with MnSOD adenovirus (AdMnSOD). *B*: MnSOD activity in adenovirally infected cells at increasing concentrations of virus. All titrations of adenovirus were performed in triplicate. EV, empty vector. *C*: GSIS at day 7 in MIN6N8 cells infected with adenovirus on day 1 of 7 (1.2×10^{12} PFU/ml MnSOD adenovirus to overexpress MnSOD or empty adenoviral vector). *D*: Mitochondrial superoxide production in MIN6N8 cells infected with adenovirus on day 1 of 7 (1.2×10^{12} PFU/ml MnSOD adenovirus to overexpress MnSOD or empty adenoviral vector). All data shown are representative of three independent experiments (n = 4–6 replicates/group per experiment). *E–G*: Pancreatic islets were isolated from C57BL/6J mice at 8 weeks of age. Rested islets were incubated in the presence and absence of AGE-BSA (100 μg/mL) in DMEM/10% FCS containing 5 mmol/L glucose for 24 h. The MnSOD mimetic MnTBAP was also added to some groups (20 μmol/L). *E*: GSIS in isolated islets. *F*: Mitochondrial superoxide production. *G*: ATP content of islet mitochondria. *$P < 0.05$ vs. BSA; †$P < 0.05$ vs. AGE-BSA; ‡$P < 0.05$ vs. empty vector; §$P < 0.05$ vs. EV+AGE-BSA; ‖$P < 0.05$ vs. EV+BSA.

AGE-RSA compared with the RSA-injected rat group after 16 weeks (Table 1).

The second experimental rat model compared low and high dietary intake of AGEs for 24 weeks in healthy Sprague-Dawley rats in the presence and absence of alagebrium therapy. High dextrose–fed rats were also studied because there is ongoing debate about whether endogenous sources of AGEs, such as those generated from circulating glucose (in this case derived from dextrose), are more pathogenic than those manufactured exogenously through food processing (32). Consumption of both high-AGE and high-dextrose diets for 24 weeks increased

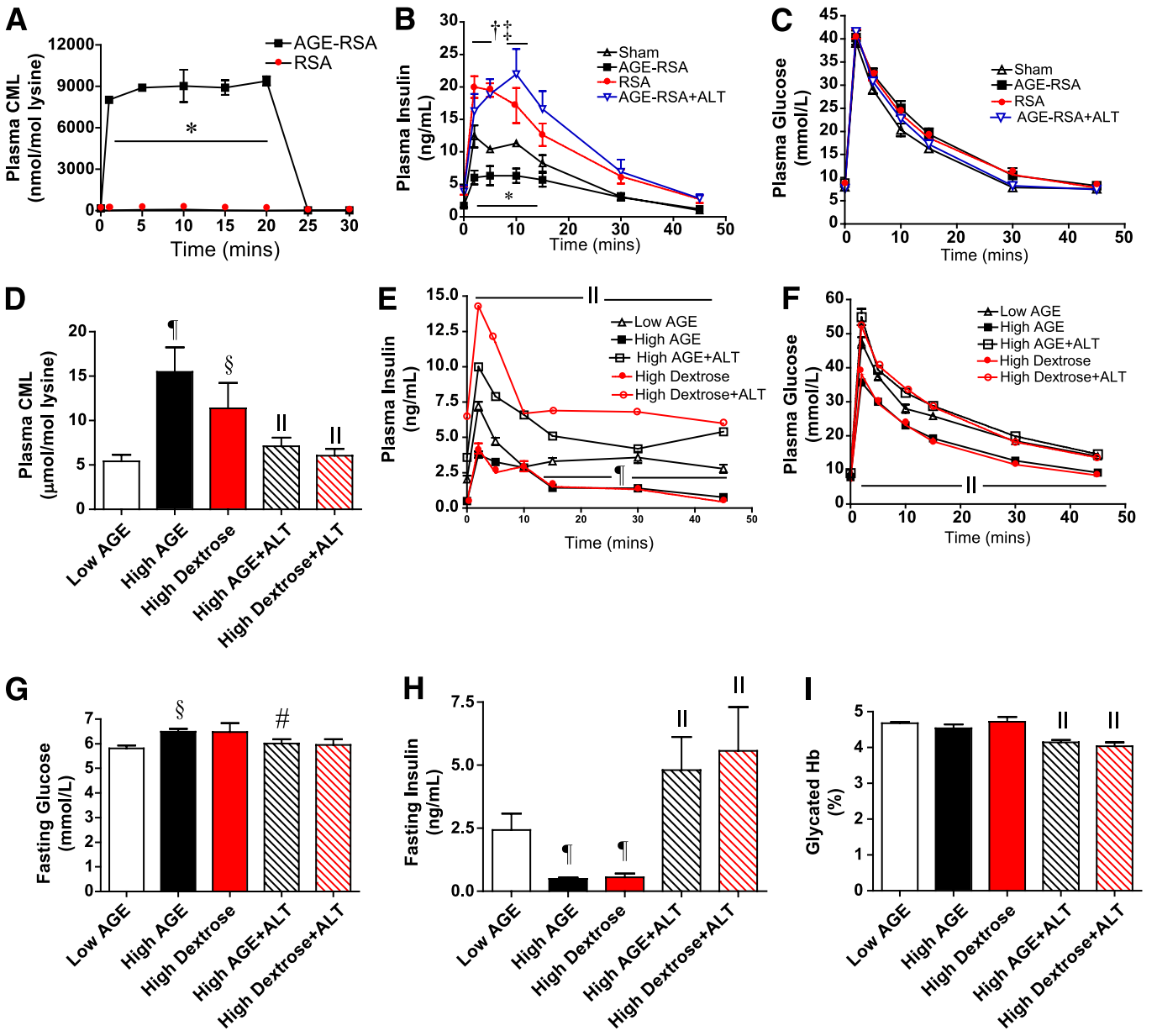

FIG. 4. Insulin secretory defects in healthy rodents result from either repeated daily or constant elevations in AGEs. Healthy Sprague-Dawley rats were exposed to a chronic AGE load via daily intraperitoneal injection (16 weeks) or ingestion of a high-AGE diet (24 weeks). *A–C*: AGE-RSA or RSA-injected rats at week 16 (n = 10/group), with some rats randomized to receive the AGE-lowering therapy alagebrium (10 mg/kg/day oral gavage; n = 10/group). *A*: Plasma AGE levels measured as the modification CML, following intraperitoneal injection. *B*: Plasma insulin concentrations during IVGTT. *C*: Plasma glucose concentrations during IVGTT. *D–I*: HighAGE or glucose (dextrose)–fed rats at week 24 (n = 10/group), with some rats randomized to receive alagebrium (n = 10/group). *D*: Plasma AGE (CML) levels. *E*: Plasma insulin concentrations during IVGTT. *F*: Plasma glucose concentrations during IVGTT. *G*: Fasting plasma glucose concentrations. *H*: Fasting plasma insulin concentrations. *I*: Glycated hemoglobin. *P < 0.01 vs. RSA or sham; †P < 0.01 AGE-RSA vs. RSA; ‡P < 0.01 AGE-RSA vs. AGE-RSA+ALT; ¶P < 0.05 vs. low AGE; §P < 0.05 vs. low AGE, Mann-Whitney test; ‖P < 0.05 vs. high AGE or high dextrose; #P < 0.05 vs. high AGE, Mann-Whitney test.

circulating AGE concentrations, which were ameliorated by alagebrium therapy (Fig. 4*D*). Consumption of either a high-AGE or high-dextrose diet resulted in impaired insulin secretion (Fig. 4*E*) and higher plasma glucose concentrations during an IVGTT (Fig. 4*F*), and these changes were prevented by alagebrium therapy. Preproinsulin gene expression in rat islets was also reduced by chronic high-AGE or high-dextrose feeding for 24 weeks (high AGE 0.57 ± 0.08-fold induction vs. low AGE 1.27 ± 0.26, P < 0.01; high dextrose 0.09 ± 0.02, P < 0.001 vs. low AGE). Increased fasting plasma glucose (Fig. 4*G*) and decreased fasting insulin (Fig. 4*H*) concentrations were demonstrated with high dietary AGE intake, and these changes in glucose and insulin were attenuated with concomitant administration of alagebrium. There was no significant difference in fasting plasma glucose seen with consumption of a high-dextrose diet (Fig. 4*G*), although these rats had a significant reduction in fasting plasma insulin concentrations, which were reversed with alagebrium therapy. Glycated hemoglobin concentrations were not elevated in high AGE– or high dextrose–fed rats but were significantly lowered by alagebrium therapy (Fig. 4*I*), consistent with the effects of this agent on glucose concentrations.

TABLE 1
Physiological and metabolic parameters in normal rats following AGE-RSA injections

	Plasma glucose (mmol/L)	Glycated hemoglobin (%)	Body weight (g)	Plasma CML (μmol/mol lysine)	Energy intake (kJ/day)
AGE-RSA	7.8 ± 0.5	4.7 ± 0.6*	548 ± 37*	359.2 ± 57.7	358.7 ± 40.1
RSA	7.4 ± 1.0	4.2 ± 0.8*	529 ± 58	311.7 ± 38.42	351.1 ± 80.1
AGE-RSA+ALT	7.6 ± 0.5	4.5 ± 0.5*	567 ± 50	217 ± 18.2*†	351.1 ± 56.3
Sham	7.4 ± 0.5	3.0 ± 0.3	629 ± 67	304.6 ± 25.6	316.0 ± 58.1

Data are means ± SD. Plasma glucose and glycated hemoglobin are included as measures of glycemic control at 4 months; n = 10 rats/group. Final body weight, plasma CML (AGE) concentrations, and energy intake following metabolic caging are also shown. *$P < 0.05$ vs. sham. †$P < 0.05$ vs. AGE-RSA.

Chronic in vivo exposure to AGEs increased pancreatic islet infiltration by monocytes and macrophages (Fig. 5*A–D*) compared with RSA-injected or AGE-RSA–injected rats treated with alagebrium. The β-cell content of AGEs (CML) also increased as seen by flow cytometry in AGE-RSA–injected rats compared with both RSA and AGE-RSA +ALT rats (Supplementary Fig. 1). In addition, AGE-RSA–injected rats also had excess generation of mitochondrial superoxide in islets compared with sham- or BSA-injected rats (Fig. 5*E*). Exposure to excess AGEs also caused β-cell apoptosis as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) in healthy rats (Fig. 5*F*), which was not seen in BSA-injected rats and was prevented with alagebrium.

Incidence of autoimmune diabetes in NOD mice is decreased with AGE-lowering therapies. Because AGEs were found to adversely affect pancreatic function in healthy rats, the effect of AGEs on diabetes development was next investigated in NODLt mice, which spontaneously develop autoimmune diabetes from approximately 100 days of age. Prediabetes by day 75 of age, plasma glucose concentration (Fig. 6*A*), and glycated hemoglobin (Fig. 6*B*) were both increased, compared with day 50 in NODLt mice, and were decreased by alagebrium therapy.

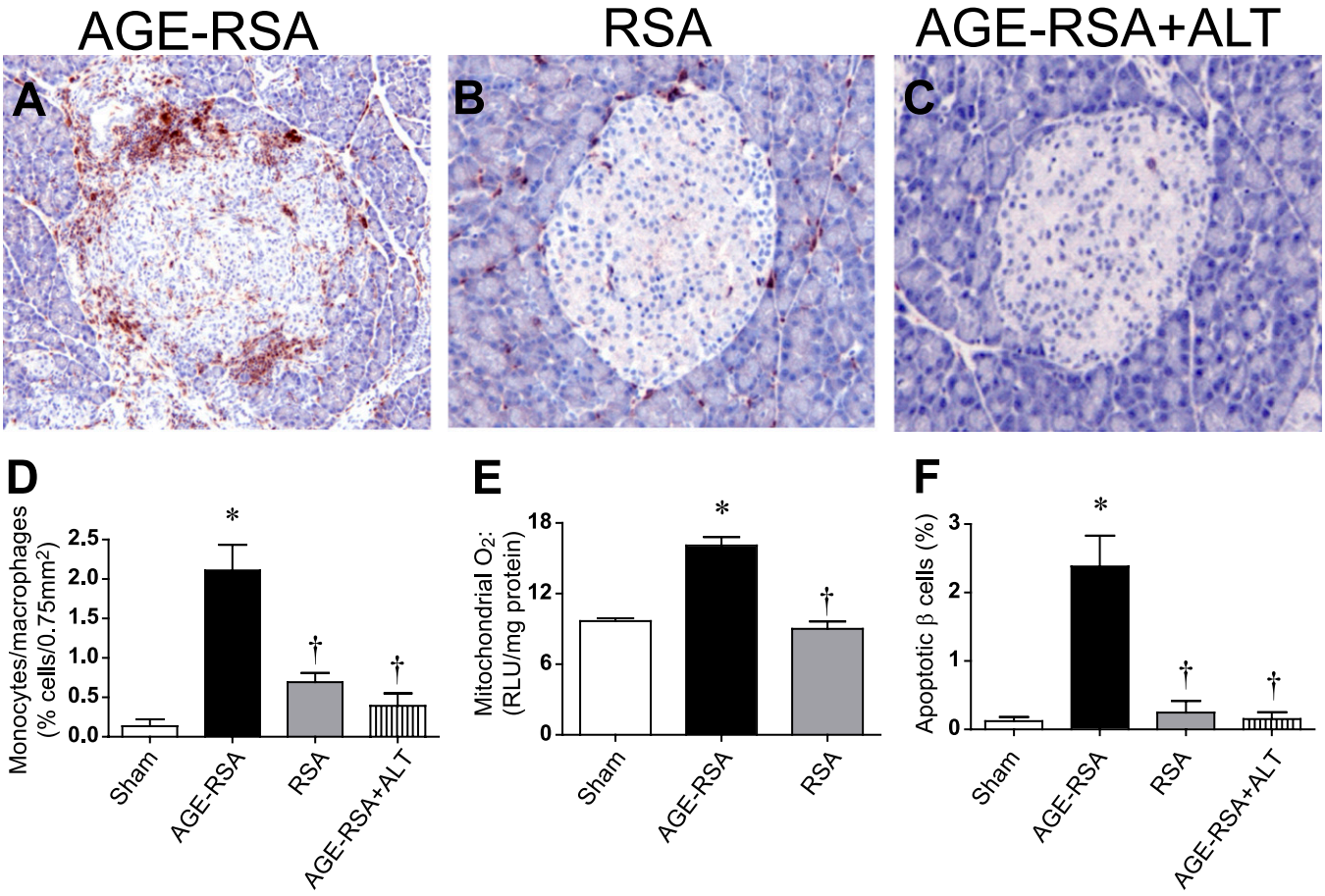

FIG. 5. Chronic in vivo AGE exposure causes islet damage in healthy rats. Healthy Sprague-Dawley rats were exposed to a chronic AGE load via daily intraperitoneal injection (16 weeks). AGE-RSA or RSA-injected rats at week 16 (n = 10/group). *A–D*: Islet monocyte/macrophage infiltration (ED-1 antigen) by immunohistochemistry at week 16 in AGE-RSA–injected rats (*A*), RSA-injected rats (*B*), and AGE-RSA–injected rats treated with alagebrium (*C*). *D*: Morphometric quantification of ED-1. *E*: Islet NADH-dependent mitochondrial superoxide generation in AGE-injected rats at week 16. *F*: β-Cell apoptosis determined by TUNEL. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. AGE-RSA. (A high-quality digital representation of this figure is available in the online issue.)

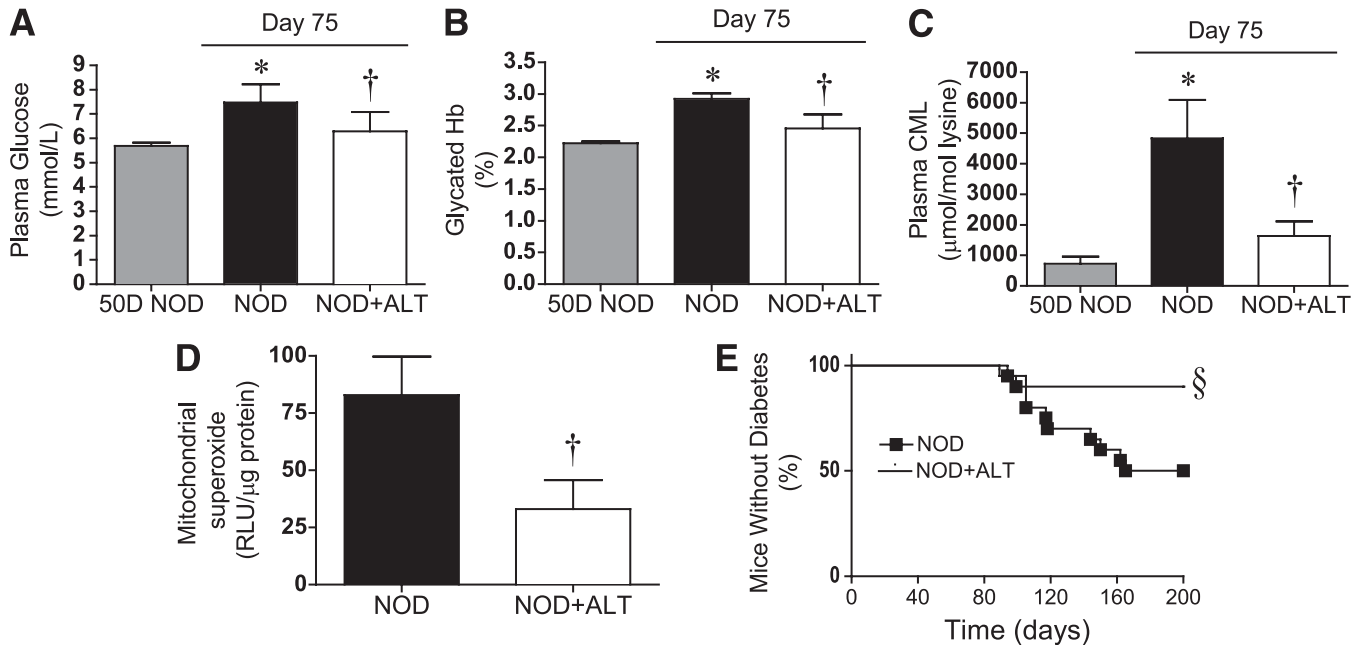

FIG. 6. The incidence of autoimmune diabetes in NOD mice is decreased with AGE-lowering therapy. Prediabetic NODLt mice were followed for 75 days (n = 10 mice/group). A group of mice (n = 10/group) was also treated from day 50 using the AGE-lowering therapy alagebrium chloride (1 mg/kg/day). *A*: Fasting plasma glucose. *B*: Glycated hemoglobin (Hb). *C*: Plasma AGE (CML) concentrations. *D*: Islet NADH-enhanced mitochondrial superoxide generation. *E*: Diabetes incidence in NODLt mice followed to day 200 (n = 20/group). *P < 0.05 vs. NODLt at day 50; †P < 0.01 vs. NODLt at day 75; §P = 0.009, log-rank (Mantel-Cox) test.

Prediabetic NODLt mice also had an increase of plasma AGE (CML) concentrations by day 75 of age, which was not seen in NODLt mice given alagebrium (Fig. 6*C*). These increases in circulating plasma glucose, glycated hemoglobin, and plasma AGEs in prediabetic NODLt were accompanied by increased islet mitochondrial superoxide production, which was prevented with alagebrium (Fig. 6*D*).

NODLt mice were also studied until the diagnosis of diabetes was confirmed or until day 200 of age. One group of NODLt mice was treated with alagebrium from day 50 of age. Approximately 50% of NODLt mice developed diabetes by day 200. Alagebrium reduced the incidence of diabetes in NODLt mice to approximately 5% (Fig. 6*E*).

At-risk children who progress most rapidly to T1D have increased serum AGE concentrations. We measured circulating AGE concentrations in a cohort of at-risk children and adolescents with islet autoantibodies in the Melbourne Pre-Diabetes Family Study (19). Eighteen who progressed to clinical diabetes over a median of 4 years were matched as closely as possible for age, sex, islet autoantibodies, HLA DR type, and β-cell function (first-phase insulin response to intravenous glucose) with another 18 who did not progress to diabetes over this time (see Table 3 in ref. 19). Those who progressed to diabetes during follow-up (progressors) had higher concentrations of serum AGEs (CML) compared with the nonprogressors (Fig. 7).

DISCUSSION

The current study provides evidence to support the hypothesis that exposure to AGEs impairs insulin secretion by stimulating the production of superoxide within mitochondria. In agreement with previous studies (33,34), we have shown that AGEs disrupt insulin secretion by MIN6N8 cells. However, more importantly, we have shown that impairment of insulin secretion by AGEs occurs via interruption of a number of critical sequential steps, including changes in cellular glucose uptake, alteration of cellular calcium flux, and loss of mitochondrial ATP content, which have not been previously identified. Consistent with an AGE-specific effect on insulin secretin in MIN6N8 cells, the administration of the AGE-lowering agent alagebrium prevented all of these changes. In addition, we demonstrated that isolated mouse islets exposed to AGEs also had impaired insulin secretion in response to glucose in the context of lower islet ATP content, consistent with a previous study (17). These islet abnormalities were restored by concomitant exposure to alagebrium.

With chronic administration of AGEs to healthy Sprague-Dawley rats, we observed a loss of insulin secretory function, including a decline in first-phase insulin secretion

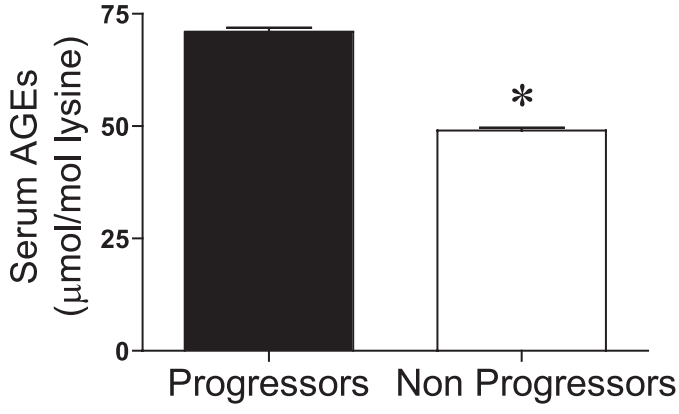

FIG. 7. Children with known genetic susceptibility who progress to type 1 diabetes have higher circulating AGE (CML) concentrations. Serum AGE (CML) concentrations were measured in HLA II–matched subjects recruited as part of the Melbourne Pre-Diabetes Family Study. N = 18/group. *P < 0.01 vs. nonprogressors.

in response to glucose, which is characteristic in humans who develop diabetes including T1D (35,36). Furthermore, rats that received a high-AGE diet had lower fasting plasma insulin concentrations, less preproinsulin gene expression in islets, and higher plasma glucose concentrations in the context of weight loss, which are often seen in children who develop T1D (35,36). Of interest, although healthy rats administered dextrose had increased circulating AGEs similar to those seen with the high-AGE diet, as well as a loss of insulin secretory function, this did not result in an increase in fasting plasma glucose concentrations. AGE injections for 16 weeks were also not associated with increased plasma glucose concentrations. This suggests that there may be effects of excess AGEs from dietary sources at other sites such as the gut, which ultimately impact on glucose homeostasis, given that the β-cell abnormalities were the most advanced in high AGE–fed rats. One could also speculate that insulin secretory defects may precede any AGE-induced effects on insulin sensitivity (14,37), given that there was no evidence of insulin resistance in rats administered AGEs, although this was beyond the scope of the current study.

Consistent with a role for AGEs in the development of autoimmune diabetes, NODLt mice had increased plasma glucose and AGE concentrations by day 75 of age, preceding the onset of diabetes. Furthermore, the incidence of autoimmune diabetes in NODLt mice was also reduced in the current study with the AGE-lowering agent alagebrium. In addition, healthy rats chronically administered AGEs showed increased β-cell cell death and islet invasion by monocytes and macrophages. Supporting a role for AGEs in the development of β-cell damage likely resulting in their destruction are previous studies suggesting that AGEs may have immunomodulatory roles, most likely via RAGE (38–40). Indeed, we have previously shown that changes in RAGE may contribute to the development of T1D in susceptible children (41). In the current study, plasma AGE concentrations were increased in susceptible children who progressed to T1D at a more accelerated rate, which is interesting given that these children were matched, including for the number of islet autoantibody specificities and β-cell function. All together, however, it is as yet unclear whether AGEs impair β-cell function directly or indirectly by enhancing adaptive (T-cell) immunity against β-cells.

Having determined that AGEs could impair insulin secretion, we next studied the effects of AGEs on oxidative stress, a pathway previously postulated to contribute to β-cell secretory dysfunction in both experimental models (42) and human disease (43). Indeed, patients with genetic abnormalities causing excess mitochondrial superoxide generation, such as Friedreich ataxia, develop insulin-requiring diabetes (44). Therefore, it is not totally surprising in the current study that excess mitochondrial superoxide generation, in concert with a deficiency in MnSOD activity, was identified following exposure to AGEs in cells and islets and within experimental in vivo models in the context of insulin secretory abnormalities. Indeed, changes in reactive oxygen species generation and MnSOD activity have been attributed to AGE exposure in previous studies in INS cells (17,45) and astrocytes (46). In the current study, defects in insulin secretion and a loss of mitochondrial ATP content in MIN6N8 cells and isolated mouse islets were prevented with adenoviral overexpression of MnSOD or by administration of the MnSOD mimetic MnTBAP, which also prevented AGE-mediated superoxide generation in mitochondria. This protective role for MnSOD has previously been observed both in β-cells (47,48) and in other contexts (49). It remains to be determined, however, whether reactive oxygen species–mediated damage to β-cells from insults such as those due to AGEs is directly responsible for β-cell death.

In conclusion, our studies demonstrate that exposure to excess AGEs activates pathways of β-cell damage which, via mitochondrial superoxide generation, can impair insulin secretion. We also show that interventions that decrease accumulation of AGEs decrease the incidence of autoimmune diabetes in mice. This leads us to postulate that protection against autoimmune diabetes may be afforded by prevention of mitochondrial dysfunction. Indeed, future studies are required to define how exposure to excess AGEs specifically contributes to T1D development and autoimmunity and whether this is via direct or indirect pathways. Taken together, our studies identify AGEs as a modifiable risk factor and therapeutic target for the prevention of T1D.

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M.T.C. performed the AGE injection and dietary studies and the analyses and assisted in preparation and drafting of the manuscript. F.Y.T.Y. completed the isolated islets and NODLt mouse studies and dose responses in MIN6N8 cells. D.C.K.T. completed the tissue culture studies including those using adenoviruses. S.A. was involved in glucose tolerance testing in all rodents and its interpretation and analyses. A.G. and V.T.-B. completed all of the RT-PCR and immunohistochemistry and assisted with AGE-injected rat studies. D.E.W. provided assistance with in vitro studies and drafting of the manuscript. J.-i.M. provided the MIN6N8 cells and assisted in their set-up and in interpretation of data from the studies of them. T.W.K. gave expert opinion on the NODLt data and studies and completed the flow-cytometry analyses. R.M.S. contributed to the discussion and reviewed and edited the manuscript. D.M.K. assisted with calcium flux experiments. B.G.D. and B.A.K. performed the glucose uptake experiments. S.F. was responsible for the recruitment, sample collection, and study of type 1 diabetic individuals as part of the Melbourne Pre-Diabetes Family Study. P.-H.G. assisted with manuscript preparation and data interpretation. L.C.H. was responsible for the recruitment, sample collection, and study of type 1 diabetic individuals as part of the Melbourne Pre-Diabetes Family Study and assisted with statistics, manuscript drafting, and interpretation of the studies. M.K. assisted with statistics, manuscript drafting, and interpretation of the studies. J.M.F. conceived the studies, interpreted and finalized the results, and assisted in the preparation of the manuscript.

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