Mitochondria are highly mobile and dynamic organelles that continuously fuse and divide. The fission and fusion processes are essential for preserving the stability of mitochondrial content and the integrity of mtDNA. Mitofusin 1 (Mfn1), direct implicated in the development of chronic complications of diabetes such as nephropathy, arteriosclerosis, retinopathy, and cataracts. AGEs can be formed via either the nonenzymatic “Maillard reaction” or increased polyol pathway flux. In particular, the reactive carbonyl group of methylglyoxal reacts with the amino group of lysine to produce protein conjugates with 3-deoxyglucosone-imidazolone, pyrraline, pentosidine, and \( N^\epsilon \)-carboxyethyl lysine-conjugated bovine serum albumin (CML-BSA) is a major component of advanced glycation end products (AGEs). We hypothesised that AGEs reduce insulin secretion from pancreatic \( \beta \)-cells by damaging mitochondrial functions and inducing mitophagy. Mitochondrial morphology and the occurrence of autophagy were examined in pancreatic islets of diabetic \( db/db \) mice and in the cultured CML-BSA-treated insulinoma cell line RIN-m5F. In addition, the effects of \( \alpha \)-lipoic acid (ALA) on mitochondria in AGE-damaged tissues were evaluated. The diabetic \( db/db \) mouse exhibited an increase in the number of autophagosomes in damaged mitochondria and receptor for AGEs (RAGE). Treatment of \( db/db \) mice with ALA for 12 wk increased the number of mitochondria with well-organized cristae and fewer autophagosomes. Treatment of RIN-m5F cells with CML-BSA increased the level of RAGE protein and autophagosome formation, caused mitochondrial dysfunction, and decreased insulin secretion. CML-BSA also reduced mitochondrial membrane potential and ATP production, increased ROS and lipid peroxide production, and caused mitochondrial DNA deletions. Elevated fission protein dynamin-related protein 1 (Drp1) level and mitochondrial fragmentation demonstrated the unbalance of mitochondrial fission and fission in CML-BSA-treated cells. Additionally, increased levels of Parkin and PTEN-induced putative kinase 1 protein suggest that fragmented mitochondria were associated with increased mitophagic activity, and ALA attenuated the CML-BSA-induced mitophagosome formation. Our study demonstrated that CML-BSA induced mitochondrial dysfunction and mitophagy in pancreatic \( \beta \)-cells. The findings from this study suggest that increased concentration of AGEs may damage \( \beta \)-cells and reduce insulin secretion.

Advancing glycated end products; mitochondrial dynamics; mitophagy; diabetes

\( N^\epsilon \)-carboxyethyl lysine-induced mitochondrial fission and mitophagy cause decreased insulin secretion from \( \beta \)-cells

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1Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan; 2School of Medical Laboratory Sciences and Biotechnology, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan; 3Department of Pathology, Saint Paul’s Hospital, Tao-Yuan, Taiwan; 4Institute of Biotechnology, National Tsing Hua University, Hsinchu, Taiwan; and 5Institute of Pharmaceutical Sciences and Technology, Central Taiwan University of Science and Technology, Taichung, Taiwan

Submitted 31 March 2015; accepted in final form 16 September 2015

Lo MC, Chen MH, Lee WS, Lu CL, Chang CR, Kao SH, Lee HM. \( N^\epsilon \)-(carboxyethyl) lysine-induced mitochondrial fission and mitophagy cause decreased insulin secretion from \( \beta \)-cells. Am J Physiol Endocrinol Metab 309: E829–E839, 2015. First published September 22, 2015; doi:10.1152/ajpendo.00151.2015.—\( N^\epsilon \)-(carboxyethyl) lysine-conjugated bovine serum albumin (CML-BSA) is a major component of advanced glycation end products (AGEs). We hypothesised that AGEs reduce insulin secretion from pancreatic \( \beta \)-cells by damaging mitochondrial functions and inducing mitophagy. Mitochondrial morphology and the occurrence of autophagy were examined in pancreatic islets of diabetic \( db/db \) mice and in the cultured CML-BSA-treated insulinoma cell line RIN-m5F. In addition, the effects of \( \alpha \)-lipoic acid (ALA) on mitochondria in AGE-damaged tissues were evaluated. The diabetic \( db/db \) mouse exhibited an increase in the number of autophagosomes in damaged mitochondria and receptor for AGEs (RAGE). Treatment of \( db/db \) mice with ALA for 12 wk increased the number of mitochondria with well-organized cristae and fewer autophagosomes. Treatment of RIN-m5F cells with CML-BSA increased the level of RAGE protein and autophagosome formation, caused mitochondrial dysfunction, and decreased insulin secretion. CML-BSA also reduced mitochondrial membrane potential and ATP production, increased ROS and lipid peroxide production, and caused mitochondrial DNA deletions. Elevated fission protein dynamin-related protein 1 (Drp1) level and mitochondrial fragmentation demonstrated the unbalance of mitochondrial fission and fission in CML-BSA-treated cells. Additionally, increased levels of Parkin and PTEN-induced putative kinase 1 protein suggest that fragmented mitochondria were associated with increased mitophagic activity, and ALA attenuated the CML-BSA-induced mitophagosome formation. Our study demonstrated that CML-BSA induced mitochondrial dysfunction and mitophagy in pancreatic \( \beta \)-cells. The findings from this study suggest that increased concentration of AGEs may damage \( \beta \)-cells and reduce insulin secretion.

Advanced glycated end products; mitochondrial dynamics; mitophagy; diabetes
mitofusin 2 (Mfn2), and optic atrophy 1 (OPA1) regulate the fusion of mitochondria. Recently, decreased mitochondrial fusion activity was found to be associated with type 2 diabetes, and reduced Mfn2 expression in skeletal muscle was directly proportional to insulin sensitivity (2). OPA1 activity is regulated by proteolytic processing, yielding five isoforms of OPA1 grouped as long (OPA1-L) and short (OPA1-S) forms (1). Proteolytic processing of OPA1-L to OPA1-S activates mitochondrial fission machinery (4). Mitochondria undergo rapid fragmentation via fission protein dynamin-related protein 1 (Drp1) with a concomitant increase in ROS formation after exposure to high glucose levels (42). This fusion-fission process may be deregulated in diabetes. Inhibition of fission machinery in cells expressing a dominant-negative form of the fission proteins Drp1 or fission 1 via RNAi decreased mitochondrial autophagy and caused the accumulation of oxidized mitochondrial proteins, reduced respiration, and impaired insulin secretion. Furthermore, fragmentation is an important step that facilitates the sequestration of damaged mitochondria by the autophagy pathway (35). Autophagy is a tightly controlled process that maintains homeostasis via the degradation of proteins and organelles in bulk (19). However, the autophagic machinery can be recruited to kill cells under certain conditions (6). Mitophagy describes the degradation of mitochondria through autophagy (21). One of the mechanisms that induce mitophagy in mammalian cells is the accumulation of PTEN-induced putative kinase 1 (PINK1) upon mitochondrial depolarization; PINK1 recruits Parkin, an E3 ubiquitin ligase, to the mitochondrial outer membrane (26). In general, PINK1-dependent recruitment of Parkin indicates the induction of mitophagy (32). Our hypothesis is that AGEs induced damage to mitochondria and their degradation by mitophagy, leading to β-cell dysfunction under hyperglycemia of diabetes.

α-Lipoic acid (ALA) is a disulfide compound found naturally in mitochondria as a coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. ALA also attenuated high-fat diet-induced hyperglycemia and insulin resistance significantly in mice (40). Dietary supplementation of ALA in aged rats was also reported to improve mitochondrial function and reduced oxidative insults in rat models (12). In the present study, we found mitochondrial defects and increased autophagosome formations in pancreatic islets of diabetic db/db mice. Oral administration of ALA improved insulin resistance and mitigated the morphological anomalies of mitochondria. We used an in vitro model of RIN-m5F cells dosed with Nε-carboxymethyl) lysine-conjugated bovine serum albumin (CML-BSA) to test our hypothesis. CML-BSA treatment decreased insulin secretion from RIN-m5F cells in vitro. Moreover, CML-BSA treatment increased receptor for AGE (RAGE) expression, impaired mitochondrial function, increased autophagy, and enhanced the generation of ROS and lipid peroxides. Pretreatment with ALA prevented these CML-BSA-associated effects. Mitophagy-specific markers Parkin and PINK1 were elevated in CML-BSA-treated cells. The in vitro data were parallel to the in vivo data. The results from the present study suggest that CML-BSA-induced mitochondrial dysfunction and excessive mitophagy may play a role in the pathogenesis of β-cell dysfunction.

**MATERIALS AND METHODS**

**Animal models.** Ten-week-old db/db male mice on a C57BL/6 background were gifts from the Development Center for Biotechnology of Taiwan. Wild-type C57BL/6J mice were obtained from the Taiwan National Laboratory Animal Center. The animal studies were approved by the Institutional Animal Care and Use Committees of Central Taiwan University of Science and Technology. The mice were housed in a temperature-controlled room and put on a 12-h light-dark cycle and were allowed ad libitum access to water and normal chow diet. Male db/db male mice on a C57BL/6 background and C57BL/6 male mice (body weight: 41.9 ± 2.5 g or 24.4 ± 1.6 g, respectively) were orally administered with ALA (200 mg/kg once/day; Sigma Chemical, St. Louis, MO), and water (vehicle) was orally administered to db/db mice for 12 wk. The mice were euthanized with pentobarbital sodium, and then their pancreas were removed and weighed. Blood glucose levels (Modular P800; Roche, Basel, Switzerland) and HbA1c concentrations (HLC-723G7; Tosoh, Tokyo, Japan) were analyzed. Plasma insulin (ng/ml) level was measured using a commercially available enzyme-linked immunosorbent assay kit (Millipore, St. Charles, MO).

**Transmission electron microscopy.** Pancreatic tissue sections (80 nm thick) were processed as described by Knells et al. for transmission electron microscopy (TEM) (20). Ultrastructure studies were carried out using a Hitachi H-600 electron microscope (Nissui Sango). Pictures of cellular mitochondria and autophagosomes were analyzed. Autophagosomes were recognized based on their ultrastructural appearance (41). The area and numbers of autophagosomes were quantified in randomly selected regions of pancreatic tissues taken from each mouse and imaged at ×8,000 magnification.

**Cell culture.** The insulin-secreting β-cell line RIN-m5F was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (3). For experiments, aliquots of 3–5 × 10⁵ cells were treated on the following day with CML-BSA or BSA for 24–72 h. If necessary, 300 μM ALA or 5 mM 3-methyladenine (3-MMA) (Sigma Chemical, St. Louis, MO) was added 1 h prior to treatment. 3-MA or chloroquine is a pharmacological inhibitor of autophagy.

**Preparation of CML-BSA.** CML-BSA was prepared as described previously (33). Briefly, 2 mg/ml of BSA was reacted with 0.75 M glyoxylic acid and 0.3 M NaCNBH3 in 0.5 M sodium phosphate buffer, pH 7.4, at 37°C for 24 h. The reactants were dialyzed overnight against 1,000 volumes of phosphate-buffered saline, pH 7.4, twice at 4°C. For CML-BSA preparation, the degree of modification was 34.5 mol of CML-BSA/mol BSA (25). For the control preparation, BSA was incubated without the reactants in 0.5 M sodium phosphate buffer, pH 7.4, at 37°C for 24 h. In this study, we used 6 μM CML-BSA treatment of RIN-m5F, which was equivalent to 42 U/ml CML-BSA.

**Western blotting.** Equal amounts of proteins from cell lysates were separated by electrophoresis on 7.5% sodium dodecyl sulphate polyacrylamide gels and then electrotransferred onto a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). Primary antibodies for RAGE, beclin 1, CML, Parkin, Mfn1, Mfn2, and OPA1 were obtained from Abcam Biotechnology (Cambridge, UK). Antibodies against Drp1, insulin (H-86), α-tubulin, and Tom-20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Autophagy-related gene 5 (Atg5; Genexet, Irvine, CA), LC3-II (MBL, Nagoya, Japan), PINK1 (Cell Signaling Technology, Danvers, MA), and alkaline phosphatase-conjugated secondary antibodies were used to recognize proteins according to a standard protocol. Enhanced chemiluminescence signals were captured on film. Western blot data were normalized to an internal control (α-tubulin) as determined by software (PhotoCaptMW).

**Immunofluorescence and confocal microscopy.** Cells were imaged under oil immersion by immunofluorescence microscopy at ×1,000 or by using the ×63/1.4 oil CS objective on a Leica TCS P55 confocal microscope. The fluorescent plasmids GFP-LC3 or pEYFP-Mito were...
separately transplanted into untreated cells using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. After resting overnight, cells were treated with CML-BSA or BSA for specified times and then washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 20 min at room temperature for visualization.

For protein localization, treated cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then stained with the following primary/secondary antibody combinations: Parkin/Alexa Fluor 488 (anti-rabbit; Invitrogen) or Tom20/Alexa Fluor 568 (anti-mouse; Invitrogen); CML/Alexa Fluor 488 (anti-rabbit; Invitrogen) or LC3/Alexa Fluor 568 (anti-mouse; Invitrogen).

Flow cytometry. All analyses were performed with FACSscan (Becton Dickinson, Immunocytometry Systems, San Jose, CA). A minimum of 10,000 cells/sample were analyzed. Debris was gated out based on light scatter measurements. Data were acquired in list mode, and the relative proportions of cells within different areas of the fluorescence profile were quantified using the LYSYS II software program (Becton-Dickson). For autophagosome quantification, acidic vesicular organelles were visualized by staining with 1 mg/ml acridine orange (Molecular Probes, Carlsbad, CA), as described previously (27). Mitochondrial membrane potentials were measured by detecting the shift in the fluorescence signal of the cationic dye JC-1 (5 μM; Molecular Probes) (31). For ROS quantitation, intracellular ROS were determined with M dichlorodihydrofluorescein diacetate (Molecular Probes).

ATP assay. Mouse pancreatic homogenates and cellular ATP levels were quantified with a luciferin and luciferase-based ATP assay kit (A22066; Molecular Probe) according to the manufacturer’s instructions. After processing, the collected cellular supernatants were read on a Wallac Victor 1420 multilabel counter (Perkin-Elmer). ATP levels were calculated as nanomolars ATP per milligram of protein and normalized to the ATP levels of untreated control cells.

Quantification of lipid peroxides. Lipid peroxide content was determined with MDA. Briefly, a reagent blank, 1,1,3,3-tetraethoxypropane standard working solutions, and samples were assayed based on the phosphoric acid and thiobarbituric acid (TBA) method, as described previously (38). TBA-reacted MDA was detected by high-performance liquid chromatography with a narrow-pore C18 column (4.6 × 250 mm, particle size 5 μm). The eluent was monitored with a fluorescence detector (with excitation at 525 nm and emission at 550 nm).

Long-extension PCR. Large-scale mtDNA deletions were identified by long-extension PCR. Total DNA was extracted from cells with proteinase K digestion of cellular proteins according to a standard protocol. Purified DNA was dissolved in TE buffer. The target mtDNA sequence was amplified from total DNA sample using the following thermal profile: denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 56°C for 40 s, primer extension at 68°C for 8 s, and then primer extension at 68°C for 8 min. The forward primer (5'-TTTCTTCTCCAAACCTTCTT-3') and reverse primer (5'-AGTGAGATAAGGAAGAGCTG-3') were used to generate a 5,293-bp PCR product from wild-type mtDNA.

Insulin secretion assay. Cells maintained at a density of 5 × 10^6 cells/ml were treated with CML-BSA or BSA on the second day following seeding; medium and the treatments were replaced every 24 h. The insulin levels in the cell culture media were measured using a rat insulin RIA kit (Linco, St. Charles, MO) with 125I-labeled insulin according to the manufacturers’ instructions.

Reverse transcription and quantitative real-time PCR. Total RNA was extracted from treated cells using the RNeasy Mini Kit (Qiagen) and reverse transcribed using MMLV reverse transcriptase (Invitrogen). The resulting cDNAs were amplified by quantitative PCR using primers as follows: rat proinsulin gene, forward (5'-ACAGC ACCTTTGTGGTGCTCC-3') and reverse (5'-CCAGTGTTG TAGAGGAGAC-3'); 18S rRNA gene for an internal control, forward (5'-TCAATCTCGGCTGGTAAGC-3') and reverse (5'- GGACCAAGCGAAAAGATT-3'). Real-time PCR conditions were initial denaturation at 95°C for 5 min followed by 40 cycles of 15 s at 95°C, 15 s at 56°C, 40 s at 72°C, and 20 s at 40°C.

Data presentation and statistical analysis. Data are expressed as means ± SE for the indicated number of independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA); P < 0.05 was considered significant.

RESULTS

Increased RAGE expression, mitochondrial aggregation, and autophagy in diabetic mouse pancreas. All mice were of a similar age when examined. The diabetic db/db mice showed a reduced pancreatic weight and significant increases in the levels of blood glucose and HbA1c compared with C57BL/6 mice (Table 1). To determine the effects of antioxidant treatment, the db/db mice were orally administered ALA for 12 wk. The db/db mice treated with ALA had normal pancreatic weight comparable with control mice and had a lighter body weight along with moderate decreases in blood glucose and in HbA1c levels compared with untreated db/db mice (Table 1). In addition, plasma insulin concentrations were decreased significantly in ALA mice compared with db/db mice (P < 0.05; Table 1). We next compared the mitochondrial morphology and autophagy in pancreatic islets of a diabetic db/db mouse with the wild-type C57BL/6 mouse. TEM images of pancreatic

Table 1. Effects of ALA on metabolic parameters of db/db mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>db/db</th>
<th>ALA-db/db</th>
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<tr>
<td>Body weight, g</td>
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<td>56.55 ± 3.42*</td>
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<td>Pancreas weight, g/kg body wt</td>
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<td>Blood glucose, mg/dl</td>
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<td>413.50 ± 39.46*</td>
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<td>HbA1c, %</td>
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<td>8.00 ± 1.47*</td>
<td>6.40 ± 0.36*</td>
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<tr>
<td>HbA1c, mmol/mol</td>
<td>11.26 ± 5.21</td>
<td>50.79 ± 24.22*</td>
<td>46.89 ± 3.93*</td>
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<tr>
<td>FSI, ng/ml</td>
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<td>5.73 ± 2.34*</td>
<td>1.40 ± 0.21#</td>
</tr>
<tr>
<td>ATP, %</td>
<td>166.02 ± 10.12</td>
<td>60.46 ± 16.82*</td>
<td>75.59 ± 11.40*</td>
</tr>
</tbody>
</table>

Data are means ± SE (n = 5/group). ALA, α-lipoic acid; WT, wild type; FSI, fasting serum insulin. ALA was administered orally to 10-wk-old db/db mice for 12 wk. *P < 0.05 compared with C57BL/6 (WT) group; #P < 0.05 compared with db/db group.

Fig. 1. Mitochondrial aggregation and increased autophagy in pancreases of diabetic mice. A, left: transmission electron microscopy images of pancreatic cell organelles isolated from C57BL/6 [wild type (WT)], db/db, and α-lipoic acid (ALA)-treated (200 mg/kg) db/db mice demonstrating autophagosomes engulfing mitochondria. Triangle indicates double membranes characteristic of autophagosomes; white arrows indicate the late autophagic compartment containing a partially degraded, electron-dense digestion body. A, right: statistical analysis of autophagosome number in each group. Autophagosomes were counted in histological sections of pancreatic tissues. Data are presented as means ± SE (n = 5/group). B, left: Western blot analysis of N-(carboxymethyl) lysine (CML), receptor for advanced glycosylated end product (RAGE), mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy 1 (OPA1)-L (long form), OPA1-S (short form), dynamin-related protein 1 (Drp1), and autophagy-related proteins (autophagy-related gene 5 (Atg5), beclin 1, LC3-III, PTEN-induced putative kinase 1 (PINK1), and Parkin) in mouse pancreases. B, right: graph of quantitation of these data adjusted with α-tubulin protein level and expressed as ratio over its own control (WT). Data are presented as means ± SE (n = 5/group). *P < 0.05 compared with WT mice as controls; #P < 0.05 compared with untreated db/db mice. IG, insulin granules; M, mitochondria.
islet tissues from untreated db/db mice showed that most mitochondria exhibited swelling and amorphous mitochondrial cristae as well as some smaller mitochondria in autophagosomes (Fig. 1A). The db/db mice with ALA treatment exhibited less mitochondrial swelling and more well-defined mitochondrial cristae. More autophagosomes with visible bilayer structures were found in pancreases of diabetic db/db mice compared with those of C57BL/6J controls or ALA-treated db/db mice.
CML-induced mitochondrial fission and mitophagy

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CML-BSA induces autophagy. Treatment of RIN-m5F cells with CML-BSA (6 μM) for 24 h significantly increased the number of autophagosomes compared with the vehicle-treated [control (Ctrl)] or BSA-treated group. To confirm the autophagy induction, the cell was cotreated with CML-BSA and chloroquine (CQ). Western blots of mouse pancreatic extracts showed increased expressions of RAGE, Atg5, LC3-II/I, beclin 1, PINK1, Parkin, and Drp1 and decreased expression of fusion proteins Mfn1 and Mfn2 in diabetic db/db mice compared with wild-type C57BL/6J mice (Fig. 1A). The ratio of OPA1-L/S isoforms reveals a continuous decrease in diabetic db/db mice compared with C57BL/6J controls. The detection of LC3-I (cytosolic) to LC3-II (membrane bound) conversion is a useful and sensitive marker for distinguishing autophagy in mammalian cells (17). The ratios between LC3-II and LC3-I are shown in Fig. 1B. ALA treatment significantly decreased the expression of autophagy-related proteins and fission protein and increased mitochondrial fusion protein (Fig. 1B).

CML-BSA induces RAGE expression and mitophagy in pancreatic RIN-m5F cells. We hypothesized that the hyperglycemia-induced increase in AGE levels caused mitochondrial damage and mitophagy. To determine whether AGES could affect autophagy, we applied CML-BSA to the rat insulinoma cell line RIN-m5F in vitro, which expressed GFP-LC3. GFP-LC3 puncta were examined under a confocal microscope to quantify autophagic activity. There were substantially more cells with more than 10 LC3 puncta per cell following CML-BSA treatment for 24 h compared with control cells treated with BSA alone (Fig. 2A). Treatment of the cell with chloroquine, the lysosomotropic agent, prevented the autophagosomal degradation and resulted in accumulation of GFP-LC3 puncta (Fig. 2A). Similarly, flow cytometric analysis demonstrated that cells treated with CML-BSA increased acidic vesicular organelles when stained with acridine orange dye, suggesting that treatment with CML-BSA increased autophagic activity (Fig. 2B). The expressions of RAGE, Atg5, beclin 1, and LC3-II/I protein were also elevated by CML-BSA treatment (Fig. 2C). We next determined the subcellular localization of CML-BSA and LC3. As shown in Fig. 2D, CML-BSA was bound to the surface membrane and then mediated autophagy response. Pretreatment with ALA or 3-MA (an autophagy inhibitor) attenuated the CML-BSA-mediated induction of cell toxicity (Fig. 2E).

To examine whether the CML-BSA-induced autophagy was related to mitochondria damage, the following experiment was conducted. As shown in Fig. 3A, treatment with CML-BSA induced the depolarization of mitochondrial membrane potential. Moreover, the levels of both PINK1 and Parkin were increased with CML-BSA treatment compared with BSA-treated cells (Fig. 3, B and C). The colocalization of Parkin with Tom-20, a mitochondrial outer membrane protein, indicated that Parkin was expressed in the mitochondria (Fig. 3D). Pretreatment with ALA attenuated the CML-BSA-mediated depolarization of mitochondrial membrane potential and minimized the induction of PINK1 and Parkin.

CML-BSA alters mitochondrial dynamics. Impairment of mitochondrial dynamics is associated with mitochondrial dysfunction. To further understand the effects of CML-BSA on mitochondrial dynamics, we examined changes in mitochondrial morphology and expression levels of mitochondrial dynamic proteins. Using EYFP-Mito, we observed a significant decrease in filamentous mitochondria and an increase in fragmented mitochondria in CML-BSA-treated RIN-m5F cells (Fig. 4A). CML-BSA treatment of RIN-m5F cells resulted in increased expression of the fission protein Drp1 and decreased expression of the fusion proteins Mfn1 and Mfn2. The ratio of OPA1-L to OPA1-S isoforms reveals a continuous decrease after CML-BSA treatment in vitro due to a higher expression of OPA1-S. These results suggest that AGES may disrupt mitochondrial network dynamics and trigger mitophragia fragmentation (Fig. 4B).

CML-BSA causes mitochondrial dysfunction. To test whether AGES could cause damages in mitochondria, we tested the effects of CML-BSA on mitochondrial membrane potential and ATP production in RIN-m5F cells. Treatment with CML-BSA for 24 h resulted in a striking decrease in ATP levels (Fig. 5A). Intracellular ROS increased after treatment with 6 μM CML-BSA (Fig. 5B), plateaued after 3 h, and remained elevated for 72 h (data not shown). Furthermore, oxidative damage was analyzed using the lipid peroxidation marker MDA. Our results show increased MDA in CML-BSA-treated cells (Fig. 5C).

We next examined whether CML-BSA treatment could cause mtDNA deletions. Long-extension PCR was used to detect the expected multiple mtDNA rearrangements. In addition to a 5,293-bp fragment representing wild-type mtDNA, shorter mtDNA fragments (i, ii, iii) indicative of mtDNA deletions were detected after treatment with CML-BSA for 48 h (Fig. 5D).

CML-BSA decreases intracellular insulin levels and insulin secretion. Defects in mitochondrial functions impaired the metabolic coupling and resulted in reduced insulin secretion. Treatment of RIN-m5F cells with 6 μM CML-BSA resulted in decreases in insulin secretion and intracellular insulin levels, and these effects lasted for 24 h. Pretreatment with ALA reversed these CML-BSA-associated effects (Fig. 6, A and B). However, proinsulin levels remained unchanged for 24 h after the addition of CML-BSA (Fig. 6C). These results suggest that the conversion of proinsulin to insulin was reduced by CML-BSA. Our results demonstrated that CML-BSA jeopardized insulin secretion and proinsulin processing for 24 h.

Fig. 2. Nε-(carboxymethyl) lysine-conjugated bovine serum albumin (CML-BSA) induces autophagy. Treatment of RIN-m5F cells with CML-BSA (6 μM) for 24 h significantly increased the number of autophagosomes compared with the vehicle-treated [control (Ctrl)] or BSA-treated group. To confirm the autophagy induction, the cell was cotreated with CML-BSA and chloroquine (CQ). A, top: confocal microscopy images of autophagosomes. A, bottom: graph of quantitation of these data. Means of cells containing ≥10 fluorescent GFP-LC3 puncta/cell were counted from 3 visual fields under immunofluorescence microscopy at ×1,000 magnification. Autophagy was scored as %cells containing ≥10 puncta/cell. B: CML-BSA treatment significantly increased the number of RIN-m5F cells with acidic vesicular organelles (AVOs). C: top: Western blot analysis of the RAGE and autophagy-specific proteins Atg5, beclin 1, and LC3-II/I. C, bottom: graph of quantitation of these data adjusted with α-tubulin protein level and expressed as ratio over its own control (untreated cells). D: immunostaining of CML (Alexa Fluor 488, green) and LC3 (Alexa Fluor 568, red-orange) in RIN-m5F cells treated with CML-BSA, ALA/CML-BSA, BSA, or 0.3% alcohol (Alc) or untreated (Ctrl). E: CML-BSA treatment decreased the viability of RIN-m5F cells, and this effect was abolished by cotreatment with ALA or the autophagy inhibitor 3-methyladenine (3-MA). Cell viability was examined using the trypan blue dye exclusion method. Data are expressed as means ± SE (n = 3). *P < 0.05 compared with control; #P < 0.05 compared with CML-BSA group.
Fig. 3. CML-BSA induces mitophagy. A: CML-BSA decreased the mitochondrial membrane potential, and this effect was abolished by cotreatment with ALA. Mitochondrial membrane potential (ΔΨm) was detected with JC-1 fluorescence by flow cytometry. B and C: CML-BSA increased the protein levels of PINK1 (B) and Parkin (C), and these effects were abolished by cotreatment with ALA. Western blot analysis (top) and a graph of quantitation of these data adjusted with α-tubulin protein level and expressed as ratio over control (untreated cells) (bottom) are shown. *P < 0.05 compared with control; #P < 0.05 compared with CML-BSA group. D: colocalization of Parkin with Tom-20, a mitochondrial outer membrane protein. Confocal microscopy demonstrates the immunostaining of Parkin (Alexa Fluor 488, green) and Tom-20 (Alexa Fluor 568, red-orange) in RIN-m5F cells treated with CML-BSA, ALA/CML-BSA, BSA or 0.3% Alc or without treatment (Ctrl).
DISCUSSION

Defects in pancreatic β-cells are associated with diabetes (9). It has been indicated that AGEs can injure β-cells and cause diabetes (7). In this study, we studied the toxic effect of CML-BSA against pancreatic β-cells; CML-BSA reduced insulin secretion and decreased intracellular insulin levels. We observed a significant increase in the accumulation of autophagosomes and vacuole engulfment of mitochondria in pancreatic islets in db/db mice as well as a remarkable increase in autophagy-related proteins in CML-BSA-treated RIN-m5F cells. The increased autophagy may be attributable to the increased number of defective mitochondria. There was unbalanced mitochondrial dynamics with the increase of fission forms over fusion forms in the presence of CML-BSA. Through functional and mtDNA deletion studies, we showed that CML-BSA increased mitochondrial damage. Most importantly, the upregulation of Drp1 expression and the localization of Parkin to mitochondria connected mitochondrial damage to increased autophagy. Our results suggest that AGE-induced autophagy may play a role in the development of diabetes.

Parkin is selectively recruited to impaired mitochondria by PINK1 translocation and promotes their autophagy (32). CML-BSA induced PINK1 and localized Parkin to the mitochondria along with an increase in the autophagy-related proteins Atg5, beclin 1, and LC3-II/I. Furthermore, the expression of mitochondrial fission-related protein Drp1 was also increased in the CML-BAS-treated cells. Drp1 is a potential target of the PINK1/Parkin-mediated pathway (28). Parkin can promote Drp1 ubiquitination, enable Drp1 to be associated with mitochondria, and induce mitochondrial fission. The balance between fission and fusion governs mitochondrial segregation and elimination by autophagy (35). These results suggest that mitochondrial-specific damage induced autophagy and may provide insight into molecular mechanisms underlying CML-BSA-induced autophagy.

Although AGEs induced cell death in β-cells through apoptosis (7), autophagy also has a major impact on cell homeostasis and cell death (11, 15). Observation of enhanced autophagy with massive vacuole overload may contribute to human β-cell loss in type 2 diabetes (24). Increased autophagy contributes to pancreatic β-cell death following nutrient deprivation (11). We observed increased autophagosomes with ingested mitochondria in diabetic db/db mice. CML-BSA induced the autophagy-related proteins beclin 1, Atg 5, and LC3-II/I in RIN-m5F cells. Moreover, blockade of autophagy by 3-MA maintained cell viability. These results suggest that excessive autophagy may cause defects in β-cell functions and cell death.
Both excess and deficit in autophagy can promote cell injury and have been linked to several diseases, including neurodegeneration and diabetes (22, 36). Two research groups used autophagy-deficient, \( \text{H9252} \)-cell-specific Atg7-knockout mice to show increased apoptosis and decreased cell proliferation resulting in decreased \( \text{H9252} \)-cell mass (15, 16) and to show the degeneration of \( \text{H9252} \)-cells and impaired glucose tolerance with reduced insulin secretion as well as poor stress response to high-fat diets (10, 11). Ebato et al. (10) reported increased autophagosome numbers as a compensatory effect of high-fat diet in \( \beta \)-cells of both diabetic \( \text{db/db} \) mice and nondiabetic \( \text{C57BL/6} \) mice.

Mitochondria play a central role in coupling glucose metabolism to insulin secretion in \( \beta \)-cells, and defects in mitochondrial functions impair this metabolic coupling and result in reduced insulin secretion (23). Functionally, AGEs induced glucose-stimulated insulin secretion defects, mitochondrial abnormalities, ROS generation, and a decline in ATP content (7). Studies have reported that increased oxidative stress may affect mitochondrial dynamics (42), induce mitochondrial depolar-
ziation, and then initiate Parkin-dependent mitochondrial degeneration via autophagy (37). We demonstrated functional damage to mitochondria by CML-BSA through depolarization of mitochondrial membrane potential, decreased ATP content, increased ROS, and the depletion of mtDNA. The unbalancing of mitochondrial dynamics toward the fission form along with the increase in fission proteins also indicated poor mitochondrial health that could induce mitophagy. Taken together, these data suggest that CML-BSA could induce mitochondrial damage and increase mitochon-dria-specific autophagy.

ROS generation has been identified in AGE-treated β-cells (7). In the present study, we showed that CML-BSA treatment increased the levels of ROS and MDA. Administration of ALA to diabetic db/db mice ameliorated mitochondrial morphology, increased Drp1, increased insulin sensitivity, and decreased expression of mitochondrial fusion protein and autophagy-related proteins in pancreatic tissue. However, the impaired glucose tolerance was not improved by ALA treatment in db/db diabetic mice. Although the levels of HbA1c and blood glucose were slightly reduced in db/db and ALA mice, they were not statistically significant (Table 1). Our results suggest that although ALA may alleviate mitochondrial dysfunction in pancreatic cells, ALA alone is not enough to ameliorate the severe hyperglycemia at the advanced stage of diabetes in db/db mice.

Pretreatment with ALA negated the effects of CML-BSA in vitro to prevent mitochondrial damage, maintain balanced mitochondrial dynamics, increase insulin secretion, and prevent the induction of excessive autophagy. This is in line with ALA as an antioxidant. Moreover, upregulation of RAGE vent the induction of excessive autophagy. This is in line with ALA as an antioxidant. Moreover, upregulation of RAGE.

CONCLUSION

We have demonstrated a linkage between mitochondrial damage and mitophagy that can lead to insulin deficits in CML-BSA-treated pancreatic β-cells. Although apoptosis may cause cell death, the compensatory effects of autophagy may also be overextended to cause cell death. The mechanisms for the dysregulation of insulin and the decrease in pancreatic β-cells remain to be investigated.

ACKNOWLEDGMENTS

We thank Dr. How Tseng and John Yi-Chung Lin for their graphics technical support and editorial services, respectively. The db/db mice were gifts from the Development Center for Biotechnology of Taiwan.

REFERENCES

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