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Inhibition of the receptor for advanced glycation endproducts (RAGE) protects pancreatic β-cells

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ABSTRACT

Advanced glycation endproducts (AGEs) and the receptor for AGEs (RAGE) have been linked to the pathogenesis of diabetic complications, such as retinopathy, neuropathy, and nephropathy. AGEs may induce β-cell dysfunction and apoptosis, another complication of diabetes. However, the role of AGE-RAGE interaction in AGE-induced pancreatic β-cell failure has not been fully elucidated. In this study, we investigated whether AGE–RAGE interaction could mediate β-cell failure. We explored the potential mechanisms in insulin secreting (INS-1) cells from a pancreatic β-cell line, as well as primary rat islets. We found that glycated serum (GS) induced apoptosis in pancreatic β-cells in a dose- and time-dependent manner. Treatment with GS increased RAGE protein production in cultured INS-1 cells. GS treatment also decreased bcl-2 gene expression, followed by mitochondrial swelling, increased cytochrome *c* release, and caspase activation. RAGE antibody and knockdown of RAGE reversed the β-cell apoptosis and bcl-2 expression. Inhibition of RAGE prevented AGE-induced pancreatic β-cell apoptosis, but could not restore the function of glucose stimulated insulin secretion (GSIS) in rat islets. In summary, the results of the present study demonstrate that AGEs are integrally involved in RAGE-mediated apoptosis and impaired GSIS dysfunction in pancreatic β-cells. Inhibition of RAGE can effectively protect β-cells against AGE-induced apoptosis, but cannot reverse islet dysfunction in GSIS.

1. Introduction

Diabetes mellitus is a disorder characterized by hyperglycemia due to an absolute or relative deficiency of insulin. Patients with diabetes are prone to complications such as nephropathy, atherosclerosis, neuropathy, retinopathy, and cataracts. Though hyperglycemia plays a role in the pathogenesis of diabetic complications, the mechanisms by which it does so have yet to be clarified. One potential mechanism that has been suggested is non-enzymatic protein glycation [1]. Proteins glycated early in hyperglycemic states will be further modified by the formation of advanced glycation end products (AGE).

As a result of chronically elevated blood glucose in diabetes, AGEs are generated and accumulated. Both AGEs and their receptors have been shown to play a key role in the pathogenesis of diabetic complications [2–7]. The receptor for AGE (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules [8]. AGE–RAGE interaction triggers the activation of critical cell signaling pathways, such as p21ras, mitogen-activated protein kinases (MAPKs), and nuclear factor-κB (NF-κB), leading to the activation of proinflammatory responses and the cellular damage that underlies the complications of diabetes [9–12]. Beta-cell failure is one of the many complications of diabetes [13]. However, it is not known to what extent AGE directly affects pancreatic β-cell viability and function.

In this study, we utilize isolated islets and a rat insulin-secreting β-cell line (INS-1) to test the hypothesis that AGEs contribute to β-cell apoptosis via the interaction of AGE–RAGE, which reduces bcl-2 family gene expression and activates the caspase signaling cascade.

2. Materials and methods

2.1. AGE-fetal bovine serum preparation

GS was prepared and the concentration of AGEs within the GS was measured as described in the electronic supplementary material (ESM).

2.2. Cell culture

INS-1 cells were cultured to near confluence in RPMI-1640 medium (Invitrogen, NY) with 11.1 mmol/l D-glucose supplemented

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with 10% fetal bovine serum (FBS) and 50 μmol/l β-mercaptoethanol (Sigma–Aldrich, MO). All tissue culturing was performed in a thermo tissue-culture incubator that provided an environment of 95% O_2/5% CO_2 gas. GS or NG was added in the appropriate experiments and cells were incubated for an additional 24, 48, 72, or 96 h. The cells were harvested for flow cytometry and DNA laddering experiments by using 1 ml 0.25% trypsin-0.02% EDTA solution.

2.3. Pancreatic islet isolation

All animal studies were performed according to guidelines established by the Research Animal Care Committee of Nanjing Medical University. Male Sprague–Dawley rats (200–250 g) were purchased from Shanghai Laboratory Animal Centre (Chinese Academy of Sciences, China). Islet isolation and culturing techniques have been described previously [14]. Freshly isolated islets were transferred to sterile 6 cm dishes and cultured in RPMI 1640 containing 11.1 mmol/l glucose supplemented with 10% FBS, 10 mmol/l HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The islets were allowed to equilibrate for 3 h, at which point they were counted and moved into a 48-well plate (10 islets/well). They were cultured overnight at 37 °C. The next morning, the islets were treated with NG or 10% GS in the depletion medium. GSIS studies were performed 48 h later.

2.4. Flow cytometry analysis

INS-1 cells (2×10^6 cells per well) were cultured in 6-well plates and treated with NG or GS for 24 h, 48 h, 72 h, or 96 h (time gradient) or with 10%NG, 1%, 2%, 5% or 10% GS for 72 h (dose gradient). The cells were then harvested and fixed with 1 ml 75% ice-cold ethanol at −20 °C overnight. After fixation, the cells were washed in PBS and stained with 500 μl propidium iodide solution (50 μg/ml, Sigma) containing 25 μg/ml RNase. The cells were incubated at room temperature for 0.5 h in the dark, and analysed using a FACS Calibur flow cytometer and Cellquest Pro software (Becton Dickinson Immunocytometry Systems, CA). For determination of RAGE expression on the cell surface, INS-1 cells were treated with GS or NG control for 24 h, then suspended in PBS (1×10^6) and incubated with 1 μg RAGE antibody (Santa Cruz, CA) for 1 h at room temperature. After incubation with FITC conjugated goat anti-mouse secondary antibody (Chemicon, CA) for 45 min at room temperature, cells were analyzed with flow cytometer.

2.5. DNA laddering assay

INS-1 cells were treated with NG or GS, as described above. Cellular DNA isolation and ladder detection were performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). DNA laddering was run on 2% agarose gels along with a molecular weight marker.

2.6. Adenovirus-mediated RNAi

The sequences of DNA nucleotides used to create rat small interfering RNA (siRNA) are shown in Supplement data (Table 1). The sequences were synthesized, annealed and subcloned into pShuttle-H1 according to the method of Shen et al. [15]. To allow infection efficiency to be conveniently monitored, the H1 siRNA fragments were cut from pShuttle-H1 and ligated into the pAdTrack plasmid upstream of the CMV-green fluorescent protein (GFP) cassette. The AdTrack-H1 siRNA plasmid was recombined with back-bone pAdEasy-1 in BJ5183 bacteria. Adenovirus generation, amplification, and titration were performed as described previously [16]. INS-1 cells were infected with adenovirus at a multiplicity of infection of 50 at 37 °C. Two hours after infection, the cells were cultured in fresh medium for another 24 h before being treated with GS for 48 h.

2.7. Real-time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA synthesis was performed using 1 μg of total RNA and an avian myeloblastosis virus reverse transcription system. Real-time quantitative PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7000 Sequence Detection System. All data were analyzed using the expression of β-actin as a reference. The sequences of the primers used are available in Supplement data (Table 2).

2.8. GSIS assay

Isolated rat islets were moved to 48-well plates (10 islets/well) and treated with NG or GS for 48 h. The islets were pre-incubated for 1 h in HEPES-balanced Krebs–Ringer bicarbonate buffer (KRBH) containing 3.3 mmol/l glucose and 1 g/l bovine serum albumin (BSA). The islets were incubated for 1 h in KRBH containing basal (3.3 mmol/l) or stimulatory (16.7 mmol/l) concentrations of glucose. After the static incubation, the supernatants were obtained and frozen at −70 °C for subsequent determination of insulin concentration. The insulin levels were measured using RIA as described previously [17].

2.9. Caspase-9 assay

Caspase-9 activity in the cytosolic fraction was determined as described in the ESM.

2.10. Mitochondrial fraction preparation

INS-1 cells were cultured in 10 cm dishes at 60% confluence for 24 h and treated with NG or GS for 48 h. The cells were harvested and then mitochondrial and cytosolic fractions were isolated using a mitochondrial fractionation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol [18]. Protein concentration was measured with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.11. Transmission electron microscopy

We used transmission electron microscopy to monitor the mitochondrial morphology as described in the ESM.

2.12. Western blot analysis

INS-1 cells were cultured and treated as described above, and lysed with ice-cold lysis buffer. After protein content determination, western blotting was performed as described [17]. Individual immunoblots were probed with antibodies to mouse anti-RAGE monoclonal antibody, mouse anti-cytochrome *c* monoclonal antibody, rabbit anti-PARP-1 polyclonal antibody, or mouse anti-Bcl-2 monoclonal antibody. Target protein levels were quantified relative to levels of control protein, mouse anti-β-actin monoclonal antibody.

2.13. Statistical analysis

Comparisons were performed using Student's *t* test between pairs of groups, or ANOVA for multiple group comparison. Results are presented as means ± SEM. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Apoptosis in GS treated INS-1 cells

AGE-induced apoptosis in INS-1 cells increased in a time-dependent manner after GS treatment (Fig. 1A). NG treated Control INS-1 cells demonstrated 0.9 ± 0.09% apoptosis (Fig. 1B), while GS treated INS-1 cells exhibited a dose-dependent increase of apoptosis at 72 h (1–10%). The induction of apoptosis by GS was associated with significant genomic DNA fragmentation (Fig. 1C). GS was more toxic to the pancreatic β-cells than comparable concentrations of glucose (45 g/l). Specifically, 10% GS induced 58.3 ± 1.9% cell apoptosis, while 25 mmol/l glucose induced only 19.1 ± 1.2% apoptosis (Fig. 1D).

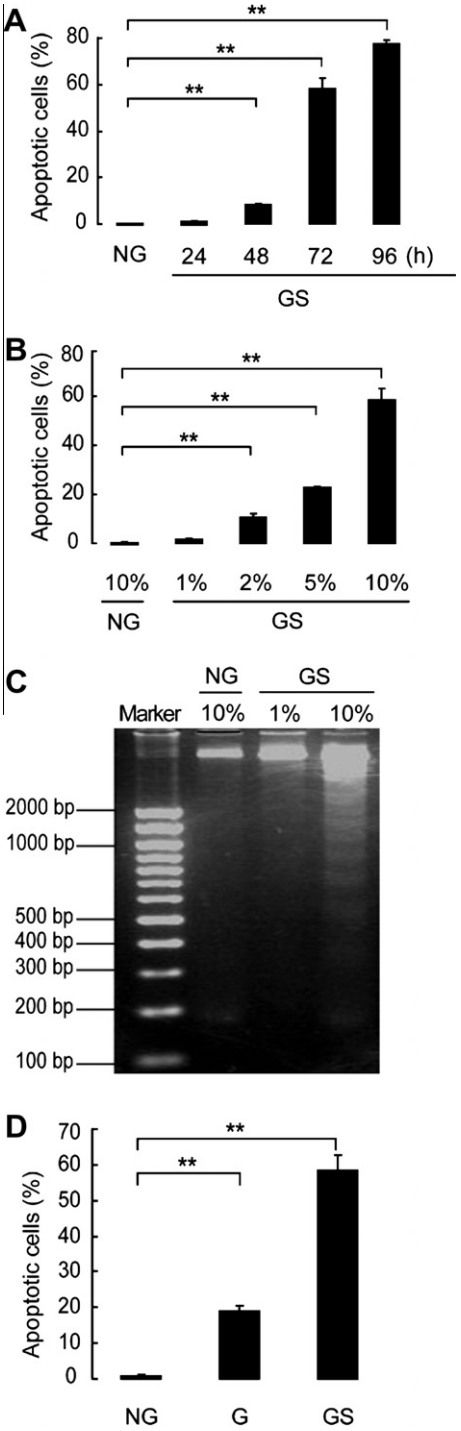

Fig. 1. AGE induces pancreatic β-cell apoptosis. INS-1 cells were treated at different time point or with different concentrations of AGE and high concentration of glucose, followed by flow cytometry using Propidium Iodide dye assays to evaluate the cell apoptosis (A, B, D). Values are means ± SEM of more than three individual experiments. INS-1 cells were treated with NG or 1% GS and 10% GS and total DNA was isolated thereafter (C). Lanes 3 and 4 demonstrate classical DNA laddering when INS-1 cells are treated with 1% and 10% GS. NG treated cells (lane 2) show no DNA laddering. Lane 1 shows molecular-weight marker. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

3.2. RAGE expression

Western blot assays showed that RAGE expression was twofold higher in GS treated cells than in the NG treated control cells (Fig. 2A, B). Flow cytometry findings confirmed the western blot result, revealing a 40% increase in RAGE positive cells after GS treatment relative to the NG treatment (Fig. 2C).

3.3. RAGE blockade attenuates apoptosis

Exposure of INS-1 cells to GS induced a marked increase in apoptosis to 65.86 ± 3.75%. Pre-treatment with anti-RAGE monoclonal antibody decreased the GS-induced cell apoptosis to 7.23 ± 1.12% ($p < 0.0005$ vs. GS treated). Antibody (2 μg/ml) treatment alone did not significantly affect the percentage of cell apoptosis (4.30 ± 1.01%) (Fig. 2D).

3.4. RNAi knockdown of RAGE

The RNAi knockdown efficiency was confirmed by western blot analysis (data not shown). Expression of RAGE was suppressed by approximately 57% by infection of INS-1 cells with adenoviruses expressing RAGE siRNA compared with a control siRNA (Fig. 2F, G). Apoptosis in control siRNA-transduced INS-1 cells was 8.44 ± 0.29% while the INS-1 cells expressing a specific RAGE siRNA showed 2.39 ± 0.08% apoptosis when treated with GS ($p < 0.01$, Fig. 2E). NG treated INS-1 cells transduced with the control adenovirus showed no significant difference in apoptosis compared with that of NG treated INS-1 cells infected with the RAGE siRNA adenovirus ($p > 0.05$).

3.5. GS treatment induces morphological changes in mitochondria

To monitor the morphological change of mitochondria in INS-1 cells after GS treatment, we used Mitotrack, a fluorescent dye that specifically accumulates in mitochondria. Normal mitochondria appeared as thin filaments [20]. When the cells were exposed to GS, their mitochondria swelled, changing from filamentous to spherical and enlarged diameter shape. We counted 200 cells in each treatment group to quantify the mitochondrial morphological changes. Ninety percent of GS treated cells showed mitochondrial swelling shown in Fig. 3A, whereas none of the NG treated control cells showed such swelling. Using electron microscopy, we confirmed that the majority of GS treated INS-1 cells mitochondria displayed swollen. Under control conditions, mitochondria appeared as typical elongated rods with normal sizes (Fig. 3B).

3.6. Mitochondrial dysfunction in GS treated cells

Mitochondria associated cytochrome *c* was decreased and cytosolic cytochrome *c* was increased in INS-1 cells exposed to GS for 48 h (Fig. 3C). Direct determination of caspase-9 activity by colorimetric assay showed a significant increase in caspase-9 activity after 48 h GS treatment (Fig. 3D). Addition of anti-RAGE antibody resulted in a significant decrease in caspase-9 activity after 48 h (Fig. 3D). To investigate whether caspase-3 activation was also involved in apoptosis of GS treated INS-1 cells, we used

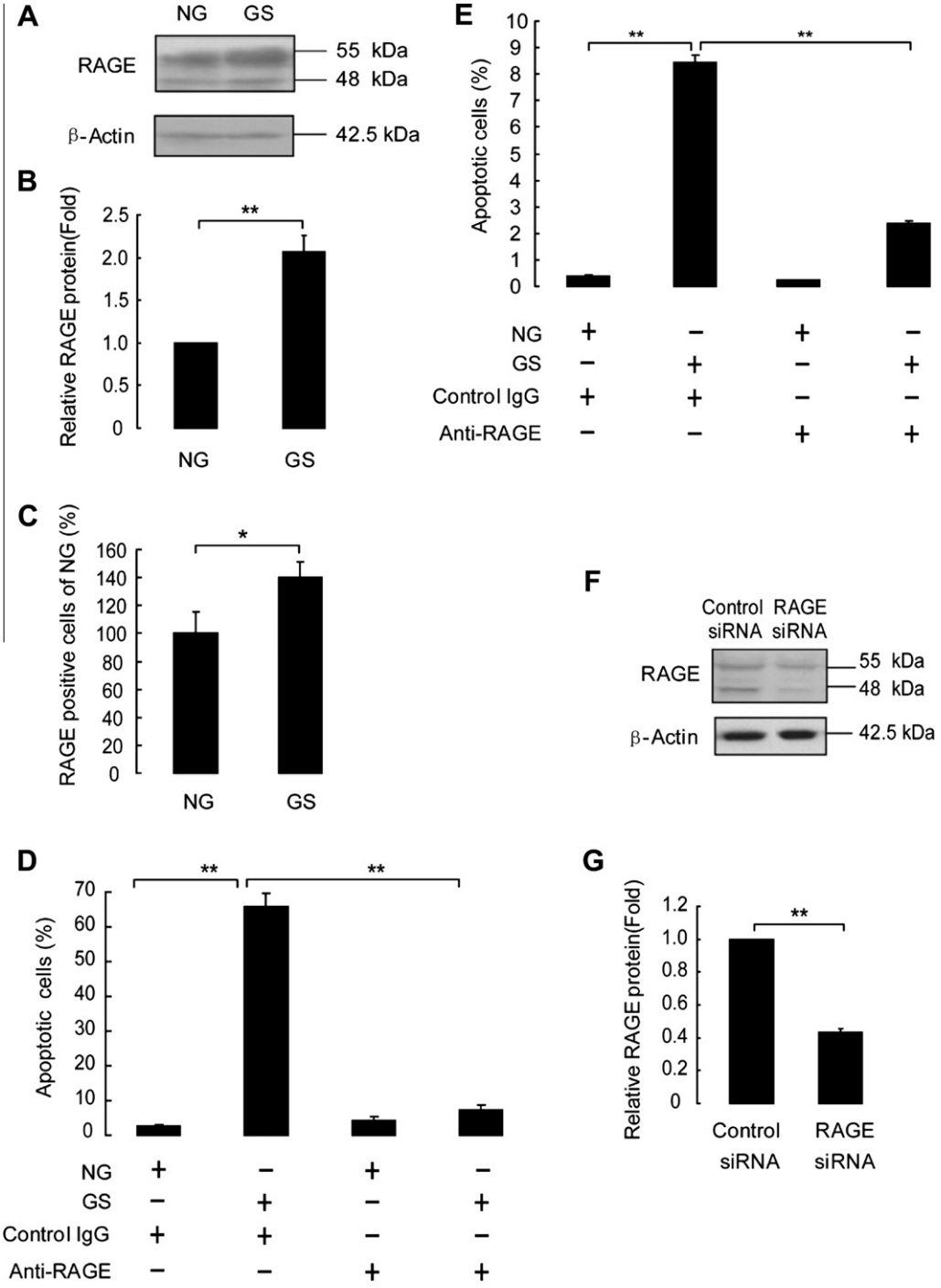

Fig. 2. AGE induces cell surface RAGE expression in pancreatic β-cells and anti-RAGE antibody or RAGE knockdown prevents GS-induced apoptosis. INS-1 cells were treated with NG or with GS for 24 h. All the cells were collected for total protein extraction and western blot to assess the protein levels of RAGE. The representative immunoblots and the relative ratios of RAGE to β-actin levels in three separate experiments are shown (A, B). After treating with NG or GS for 24 h, RAGE positive cells were counted using flow cytometry (C). Pre-incubation with anti-RAGE antibody prevents GS induced INS-1 cell apoptosis lasting 72 h (D). INS-1 cells were infected with adenoviruses expressing control-siRNA or RAGE-siRNA, and treated with NG or GS for 48 h. RAGE-RNAi reduced the GS- induced INS-1 cell apoptosis (E). The protein levels of RAGE were determined by western blot analyses to assess the knockdown efficiency of these adenoviral-based RNAi, relative to β-actin (F, G). The data shown are means ± SEM of three separate experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

western blot analysis for PARP-1, a marker for caspase-3 activity. We found that GS treated cells, but not NG treated cells, produced two bands in the correct molecular mass range for PARP-1 (89–116 kDa). Challenge of INS-1 cells with GS resulted in appearance increased cleavage of the nuclear PARP-1 protein starting from 48 h (Fig. 3E). Further prolonged treatment with GS (1–10%) at 72 h induced a even more pronounced and dose-dependent increase in the cleavage of PARP-1 protein (Fig. 3F).

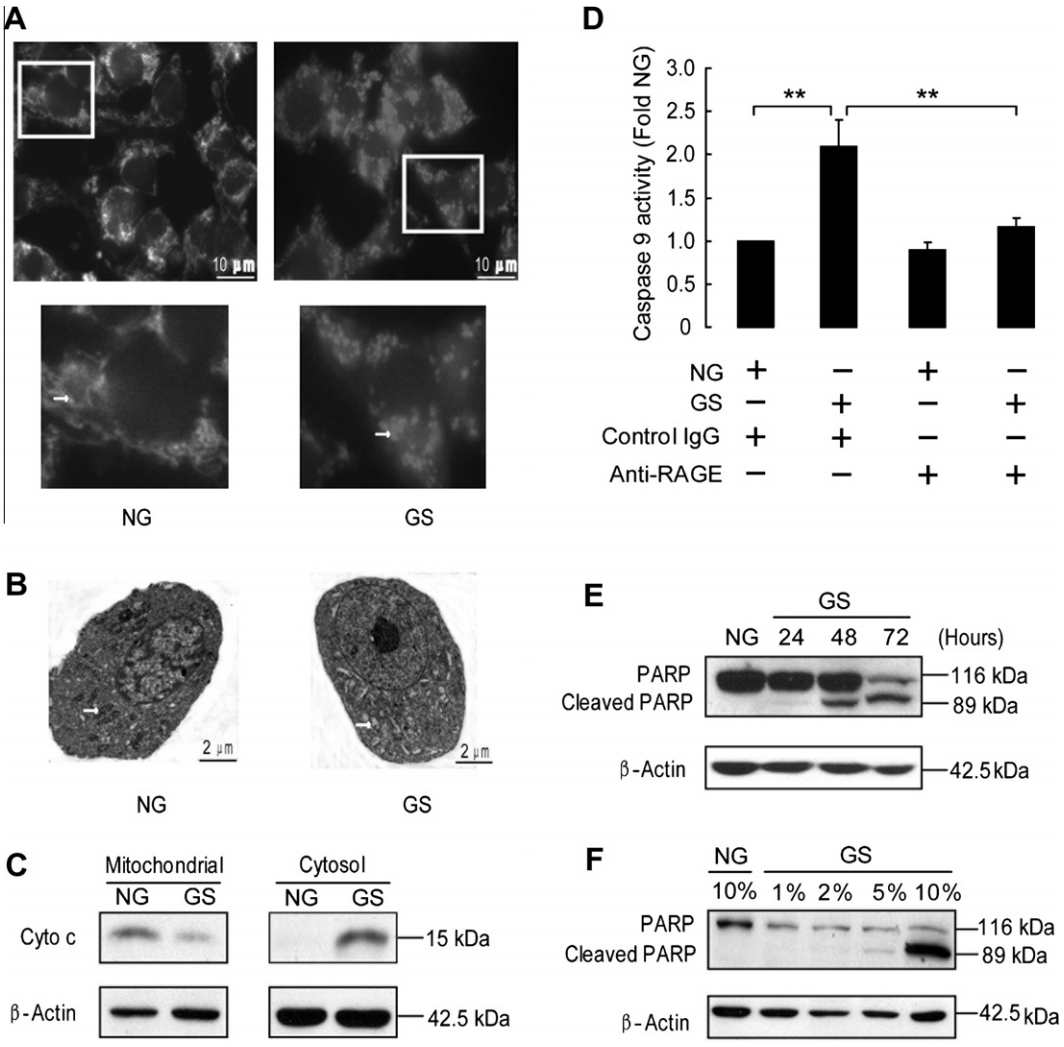

Fig. 3. Mitochondria are induced to swell, cytochrome *c* is released from mitochondria and effector caspases are activated in GS treated β-cells. Mitochondria within an INS-1 cell were stained by Mitotracker and imaged using a fluorescence microscope. Under the normal condition, mitochondria appeared as filamentous structures (A, left). After mitochondria were induced to swell by exposing the cell to GS for 48 h, mitochondria were found to change into spherical shapes (A, right). Scale bar: 10 mm. Mitochondria within an INS-1 cell were imaged using an electron microscope (B). Left: NG treated INS-1 cells. Right: GS treated INS-1 cells. We calculated changes in volume and diameter when a mitochondrion swelled without stretching its surface. The length/diameter ratio was assumed to be five before swelling [25]. Scale bar: 2 μm. INS-1 cells were treated with NG or GS for 48 h. The protein levels of cytochrome *c* in mitochondrial and cytosol shifted after GS exposure (C). Caspase-9 activity in INS-1 cells measured via colorimetric immunosorbent enzyme assay after treatment with GS or NG and anti-RAGE antibodies for 48 h (D). Effect of GS on cleaved PARP-1 protein expression after 24, 48 and 72 h of treatment in INS-1 cells (E). Effect of different concentrations of GS on cleaved PARP-1 protein expression after 72 h of treatment in INS-1 cells. The band at 89 kDa corresponds to the cleaved PARP-1 protein (F). One representative western blot of three independent experiments is shown for each target protein. $^{**}p < 0.01$.

3.7. Anti-RAGE antibody reverses GS effects on apoptotic markers

We next considered how GS might lead to the release of cytochrome *c* from mitochondria. Since the anti-apoptotic protein Bcl-2 is a known inhibitor of cytochrome *c* release, we investigated the impact of GS treatment on bcl-2 gene expression via western blot and RT-PCR assays. Bcl-2 mRNA and protein levels were decreased moderately in the mitochondrial fraction from GS treated INS-1 cells (Fig. 4A, C, D). Treatment with anti-RAGE antibody reversed the GS-induced inhibition of bcl-2 mRNA and protein expression. We also found that the levels of bcl-xL mRNA, another protective member of bcl-2 family, was decreased by AGE exposure and recovered by adding anti-RAGE antibody (Fig. 4B). We did not find any significant changes in the expression of other members of bcl-2 family.

3.8. Insulin expression in GS treated INS-1 cells

Using one-step RT-PCR, we demonstrated that treating INS-1 cells with GS for 24 h leads to a decrease in insulin-1 and insulin-2 mRNA expression (Fig. 4E, F). Addition of anti-RAGE antibody partially reversed the effects of GS (Fig. 4E, F), indicating that GS could impair insulin mRNA synthesis.

3.9. Insulin secretion in GS treated islets

To evaluate the involvement of GS in pancreatic β-cell dysfunction, we treated the isolated rat islets with GS then conducted GSIS assays. As shown in Fig. 4G, GS treatment diminished insulin secretion from rat islets stimulated with 16.7 mmol/l glucose relative to NG treated controls ($p < 0.01$). This negative effect of GS was not reversed by anti-RAGE antibody treatment.

4. Discussion

In the present study, we demonstrated that AGE could dramatically induce apoptosis in INS-1 cells in a dose and time-dependent manner after GS treatment. GS elevated RAGE expression, implying that AGE–RAGE interaction is necessary to the illicit effects of GS

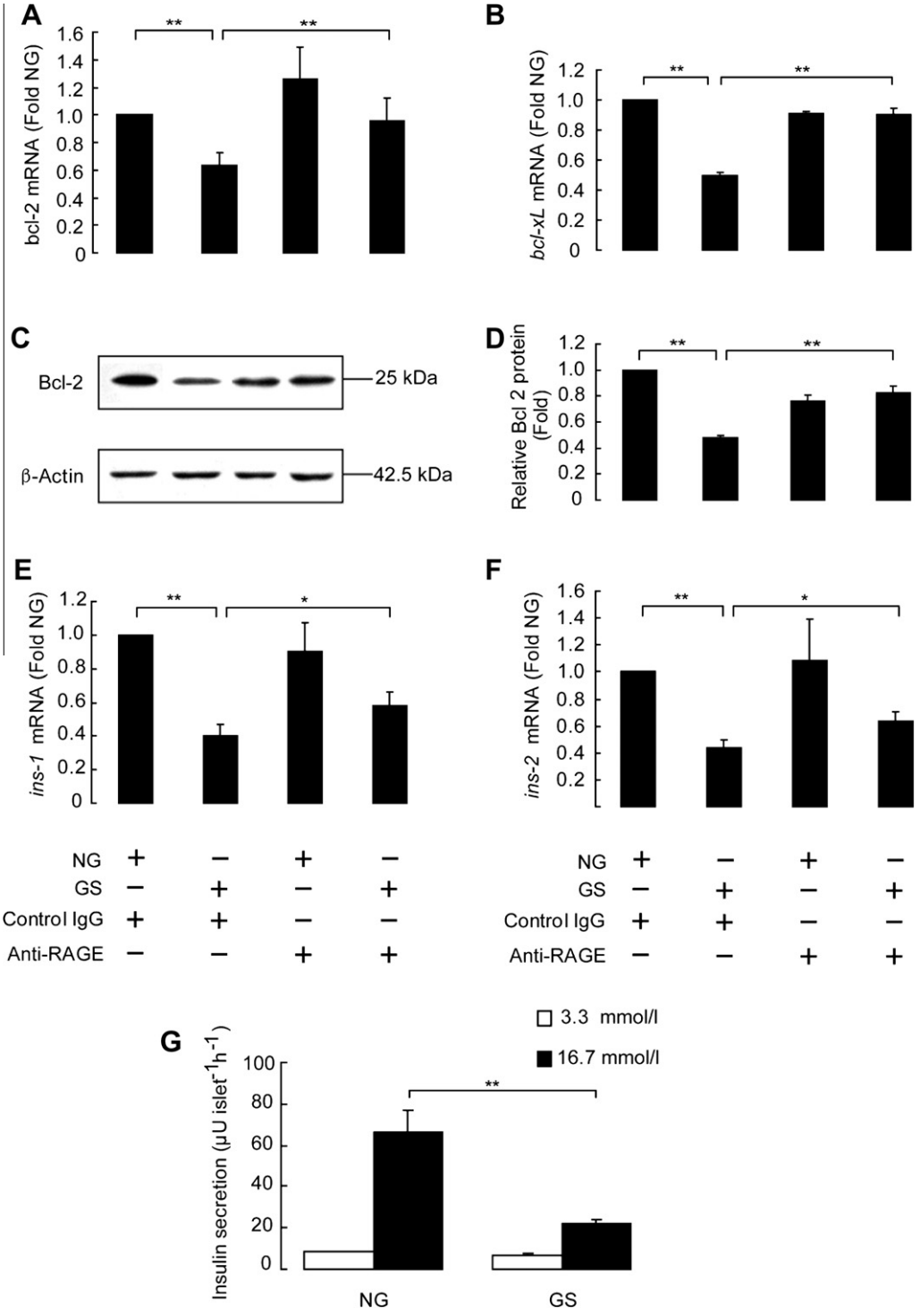

Fig. 4. Anti-RAGE antibody reverses GS-induced decrease of Bcl-2, Bcl-xL and insulin expression but fails to reverse the inhibited GSIS. Treatment with GS for 24 h induced a decrease in Bcl-2 and Bcl-xL mRNA, which was blocked by anti-RAGE (A, B). Protein levels were similarly affected (C). The relative ratios of Bcl-2 to β-actin levels in three separate experiments are shown (D). Insulin-1 mRNA was reduced after 48 h treatment with GS, reversible with anti-RAGE (E). Insulin-2 showed similar effects (F). GSIS was reduced by 1 h treatment with GS (G). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

on β-cell viability. GS stimulated cyctochrome c release, inhibited the expression of some anti-apoptotic bcl-2 proteins, and promoted caspase activation. Anti-RAGE monoclonal antibody blocked cytochrome *c* release, restored bcl-2 gene expression, and inhibited caspase activity. Finally, anti-RAGE antibody or knockdown of RAGE by RNAi prevented AGE-mediated β-cell apoptosis. It is clear from these data that AGE-RAGE interaction plays a critical role in GS-induced β-cell death.

AGE is known to play a critical role in the complications of both type 1 and type 2 diabetes. The interaction of AGE and RAGE has been suggested to cause apoptosis by inducing oxidative stress on various cell types [19]. However, there is still a lack of

mechanistic insight about how AGE might induce β-cell death. We have shown that GS can induce apoptosis in pancreatic β-cells through increased caspase activation, which is likely mediated by AGE–RAGE interaction. However, we do not know the full spectrum of intracellular events regulated by AGE and cannot exclude additional oxidative stress as a contributing mechanism.

Some recent studies have shown that AGE or glycated serum (GS) increase apoptosis *in vitro* and *in vivo*. Luciano et al. (2008) reported that GS-treatment significantly decreased proliferation rate in HIT-T15 cells, a change that was accompanied by an increase in necrosis and apoptosis rates. This cell death was related to AGE-induced oxidative stress [20]. Similarly, Zhao et al. (2009) found that AGE-treatment in mice or in isolated islets impaired glucose-stimulated insulin secretion (GSIS) by inducing nitric oxide, eventually leading to inhibition of cytochrome *c* oxidase and ATP synthesis [21]. Lim et al. (2008) reported that apoptosis increased in INS-1 cells or primary cultured islets after treatment with AGE, observing increased RAGE expression, as well [22].

Of particular interest in the present study is the finding that GS-treatment induced a decrease in bcl-2 gene expression and protein production relative to the NG control treatment. Members of bcl-2 family are known to play protective roles against apopotosis in many cellular models [23]. The function of bcl-2 proteins in pancreatic β-cells is still unclear, although a recent study using an adenovirally-transduced bcl-2 expression did report a decrease in β-cell death [24]. The reduction of bcl-2 and bcl-xL expression by GS-treatment is consistent with the observations of GS-induced INS-1 cell death, and provides a novel mechanism by which GS contributes to β-cell death and dysfunction. The full pathway by which GS induces this decrease in bcl-2 and bcl-xL expression, are still unclear, but we here produce evidence of RAGE involvement.

5. Conclusion

It is important to determine how AGE lead to impaired β-cell function and apoptosis. We provide data that demonstrate that the AGE–RAGE interaction is an important mechanism by which GS induces β-cell impairment and apoptosis. As the signaling pathways of β-cell failure are increasingly clarified, the essential modulators of these processes can be identified, providing potential novel therapeutic targets, such as RAGE.

Conflict of interest

The authors have no conflict of interest.

Disclosure summary

The authors have nothing to disclose.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.085.

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