SPANDIDOS PUBLICATIONS

RAGE ligands induce apoptotic cell death of pancreatic ß-cells via oxidative stress

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Received June 9, 2010; Accepted August 12, 2010

DOI: 10.3892/ijmm_00000529

Abstract. Activation of the receptor for advanced glycation endproducts (RAGE) by its ligands leads to cellular damage contributing to diabetic complications. It is not clearly known whether RAGE ligands influence pancreatic ß-cells. In this study, we investigated the expression of RAGE in islet cells and the effect of RAGE ligands, S100b and HMG-1, on islet cells. RAGE was expressed in INS-1 cells and isolated rat and human islets at mRNA and protein levels. RAGE and its ligand, S100b, were detected on islet cells in 28-week-old diabetic OLETF rats. Both S100b and HMG-1 induced apoptotic cell death of INS-1 and islet cells. This INS-1 cell apoptosis was accompanied by increased intracellular oxidative stress and inhibited by antioxidants or a NADPH oxidase inhibitor. Our results showing S100b/RAGE expression on islets of diabetic rat model and RAGE ligands-induced islet cell apoptosis via NADPH oxidase-mediated ROS generation suggest that RAGE ligands-RAGE interaction may contribute not only to the development of diabetic complications but also to the progressive ß-cell loss in type 2 diabetes by inducing oxidative stress.

Introduction

Chronic hyperglycemia may damage the tissues by multiple pathways; among these is the nonenzymatic glycation and oxidation of proteins and lipids, resulting in the formation of advanced glycation endproducts (AGEs) (1). One of the principal means by which AGEs exert their effects is via interaction with cellular receptors, such as the receptor for AGE (RAGE) (2,3). This receptor is normally expressed at low levels on the surface of most cell types, including vascular and inflammatory cells, Müller cells of the retina, podocytes of the kidney, neurons and microglial cells. RAGE, a member of the immunoglobulin superfamily, is a multiligand receptor which, in addition to AGEs, binds other ligands, such as proinflammatory, calcium-binding S100 proteins and a non-histone DNA binding nuclear protein, high mobility group protein box-1 (HMG-1) (4,5). In pathological conditions, especially in diabetic vascular diseases, RAGE expression is often upregulated with increased expression of its ligands as well (6,7). Engagement of RAGE by its ligands was shown to generate reactive oxygen species (ROS) via receptor-dependent signaling and to activate inflammatory signaling cascades in vascular and inflammatory cells (8-11).

The decline in ß-cell function and mass is known to contribute to progressive nature of type 2 diabetes. The ß-cell loss in type 2 diabetes is accompanied by a marked increase in ß-cell apoptosis, as shown in human pancreas autopsy specimens and in isolated islets (12,13). Chronic oxidative stress is considered as one of central mechanisms for glucose and lipid toxicity in pancreatic ß-cells in diabetes (14,15). Much evidence now links AGE-RAGE interactions to the micro- and macrovascular complications of diabetes. However, there is only scanty information on either the expression of RAGE and its ligands or the effect of RAGE ligands on pancreatic ß-cells. Both RAGE and its ligand, S100b, have been observed by immunohistochemistry on islet cells with an inflammatory infiltrate in diabetic NOD mouse but not on islet cells without insulitis (16). A recent study showed that a RAGE ligand, S100b, directly upregulated the expression of inflammatory cyclooxygenase-2 gene in human islets *in vitro* via RAGE activation (17).

Based on these observations, we hypothesized that AGE/ligands-RAGE interaction might contribute to the progressive ß-cell loss in type 2 diabetes via induction of intracellular ROS. In this study, we established that RAGE was expressed on islet cells and investigated the effect of RAGE ligands, S100b and HMG-1, on INS-1 and isolated islet cells.

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Key words: receptor for AGE ligands, apoptosis, pancreatic ß-cells, diabetes

Materials and methods

Cell culture and islet isolations. INS-1 cells were cultured in RPMI-1640 with 10% FCS at 37˚C and 5% CO_2. Rat vascular smooth muscle cells (VSMCs) cultured in DMEM with 10% FCS. Rat islets were isolated from male SD rats by collagenase digestion and Ficoll gradient purification. Pig islets were isolated from adult market pigs as previously described (18) and maintained in Medium 199 with 10% FCS for 24 h before the experiment. Human islets were isolated from the brain-dead donors at the Innovative Research Institute for Cell Therapy of Seoul National University Hospital, according to the previously described method (19) and cultured for 24-48 h in complete CMRL-1066 medium with 10% FCS at 37˚C.

Real-time RT-PCR. Total RNA was extracted from INS-1 cells, VSMCs, and isolated rat and human islets, using TRIzol reagent (Invitrogen, Carlsbad, CA). Samples of cDNA were PCR-amplified using the Exicycler™ Quantitative Thermal Block (Bioneer, Daejeon, Korea). PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. For relative quantification of RAGE expression, a comparative threshold cycle (CT) method was used after normalization with ß-actin. Rat RAGE gene specific primer (Forward: 5'-GGAATTGTCGATGAGGG GAC-3', Reverse: 5'-CAACAGCTGAATGCCCTCTG-3') was designed using registered gene bank sequences. DNA sequencing confirmed this amplification product to be identical with the corresponding nucleotide sequence of rat RAGE. For human RAGE gene, previously published pirmer sequences were used (20).

Western blot analysis. Protein was extracted with Complete Lysis-M (Roche, Indianapolis, IN) with protease and phosphatase cocktails. Protein samples (50 μg) were separated by electrophoresis through 7-10% polyacrylamide/0.1% SDS gels, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA), and then immunoblotted with goat polyclonal anti-RAGE (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-actin (Sigma, St. Louis, MO) antibodies. Immunodetection was performed with enhanced chemiluminescent detection technique (Amersham Biosciences, Uppsala, Sweden).

Immunohistochemical staining. Paraffin-embedded pancreas samples retrieved from 28-week-old OLETF and Long-Evans Tokushima Otsuka (LETO) rats were cut to 4 μm-thick consecutive sections. After deparaffinization, dehydration, and blocking, the sections were incubated with anti-rat insulin (1:100 dilution, Santa Cruz Biotechnology), anti-S100 (1:100 dilution, Sigma), anti-RAGE (1:100 dilution, Santa Cruz Biotechnology) antibodies. Sections were then incubated in biotinylated secondary antibody followed by avidin-biotin peroxidase (ABC Elite kit; Vector laboratories, Burlingame, CA), for 30 min each. The tissue-bound peroxidase was visualized by a diaminobenzidine reaction (Vector Laboratories) and counterstained with hematoxylin.

Measurement of cellular DNA content by flow cytometry. After treatment with RAGE-ligands, INS-1 cells were harvested and fixed with 70% ethanol at -20˚C. Cells were incubated in 0.75 μmol/l propidium iodide (PI) for 5 min. The cellular DNA content was measured by flow cytometry on a BD FACS Calibur (Franklin Lakes, NJ).

Detection of apoptosis. For flow cytometric detection of Annexin-V (AV)-labeling of phosphatidylserine, INS-1 cells were harvested and incubated in buffer containing AV (BD Biosciences, San Diego, CA) and PI for 15 min at room temperature. For detection of cytoplasmic DNA fragments, RAGE ligands-treated INS-1 cells were lysed and the cytoplasmic oligonucleosomes were measured by an enzyme immunoassay using; Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany). In both assays, the experiment was performed 3 times under identical conditions. Apoptosis in isolated islet cells was detected in each set of 50 islets using APOPercentage™ kit (Biocolor, Belfast, Northern Ireland) as described previously (21).

Measurement of mitochondrial membrane potential. Mitochondrial membrane potentials (MMP) were determined by staining with JC-1 dye (Molecular Probes, Eugene, OR) (22). Briefly, rat islets were incubated in culture medium containing 2 μmol/l JC-1 for 30 min at 37˚C. The islets were washed in PBS and observed immediately under a fluorescent microscope.

Detection of reactive oxygen species (ROS). To measure intracellular ROS generation, INS-1 cells were stained with 1 μmol/l dihydroethidium (H&E, Molecular Probes) at 37˚C for 10 min and examined under a fluorescent microscope. Intracellular ROS was also quantified by flow cytometry following staining with 10 μmol/l 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes) for 30 min at 37˚C.

Statistical analysis. Statistical analysis was performed using the PRISM (GraphPad Software Inc., San Diego, CA). Results are expressed as a mean ± SD. Statistical significance was defined as $p<0.05$.

Results

Expression of RAGE in islet cells and detection of RAGE and S100b in islets of OLETF rats. Using the rat ß-cell line INS-1 cells, rat islets and human islets, we detected significant endogenous RAGE expression by RT-PCR confirming that RAGE is expressed in islet ß-cells (Fig. 1A). In real-time RT-PCR, the relative abundance of RAGE mRNA normalized to ß-actin, compared with rat VSMCs, was higher in INS-1 cells (32.9±7.6-fold, $p<0.01$) and rat islets (51.6±14.8-fold, $p<0.001$). In Western blot analysis, a major protein band of RAGE (~46 kDa) was detected both in INS-1 cells and rat islets (Fig. 1B). In human and pig islets, RAGE protein of the same mass was also expressed.

In immunohistochemical staining, the islets of 28-week-old OLETF rats were disorganized with uneven distribution of insulin-positive cells and septation by fibrosis compared with the islets of LETO rats (Fig. 1C). RAGE and its ligand S100b were not detected in the islets of LETO rats. In contrast, the islets of OLETF rats showed strong expression of RAGE and

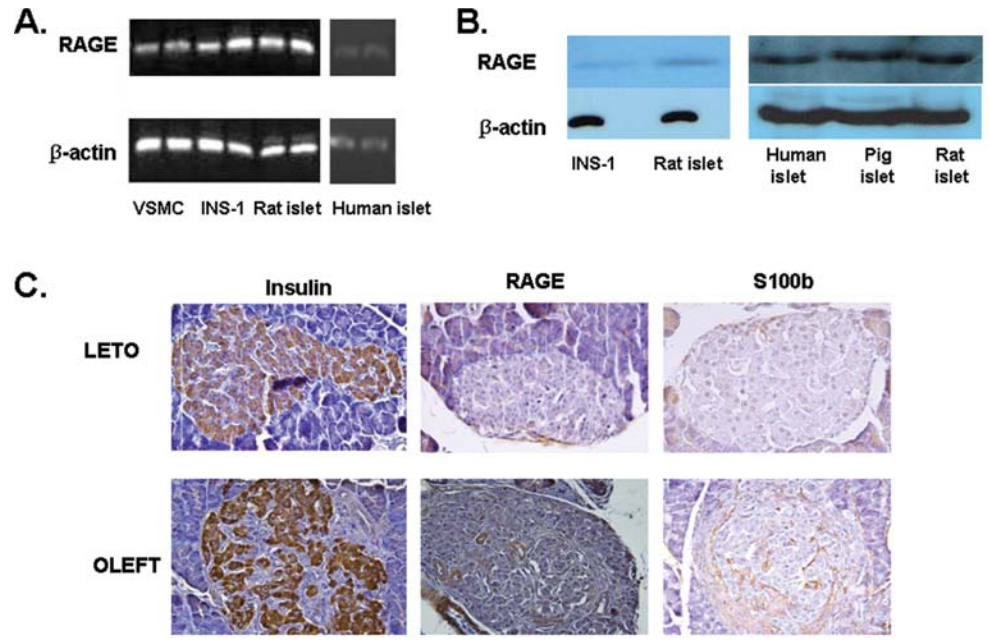

Figure 1. RAGE and S100b expression in islet cells. (A) RT-PCR of RAGE. The gels show rat and human RAGE fragments after 35 cycles of amplification of RNAs isolated from the VSMCs, INS-1 cells, rat islets and human islets. (B) Western blot detection of RAGE. A major band of RAGE (~46 kDa) was detected both in INS-1 cells and rat islets. In human and pig islets, RAGE protein of the same mass was also detected. (C) Immunohistochemical staining for RAGE and its ligand S100b in pancreatic islets of OLETF rats. OLETF and control LETO rat pancreatic sections (28-week-old) were stained with antibodies to insulin, RAGE, and S100b, respectively. The depicted islets are representative of islets from three rats in each group. The islets of OLETF rats were disorganized with uneven distribution of insulin-positive cells and showed strong expression of RAGE and S100b in the fibrotic and degenerative regions of islets. Expression of S100b and RAGE was not detected in islet cells from LETO rats.

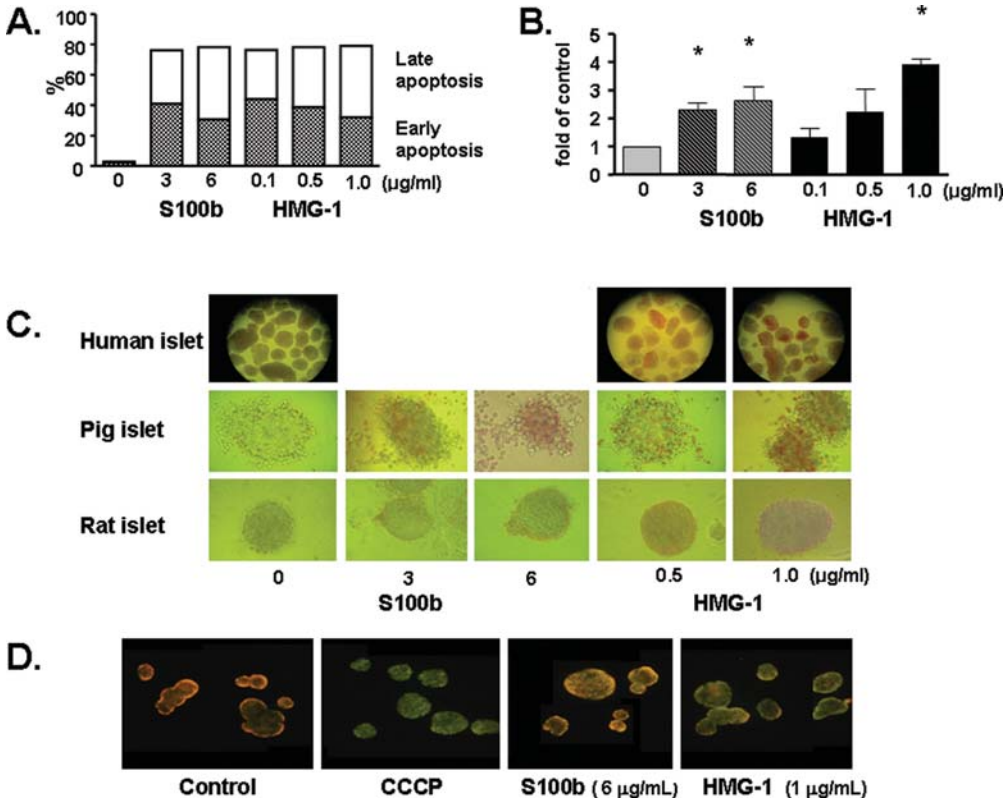

Figure 2. Induction of apoptosis by RAGE ligands in islet cells. (A and B) Cultured INS-1 cells were treated with S100b or HMG-1 at indicated concentrations for 6 h. (A) In AV-PI FACS analysis, a significant increase of apoptosis was observed and the percentage of late apoptotic (AV- and PI-positive) cells increased in a dose-dependent manner. Results are means of 3 experiments. (B) Cytoplasmic fragmented DNA measured by ELISA also increased dose-dependently in INS-1 cells by S100b or HMG-1. Results are shown as means ± SD; $^{*}p<0.05$. (C) Isolated human, pig and rat islets were treated with S100b or HMG-1 at indicated concentrations for 24 h. In APOPercentage™ staining, red pink colored apoptotic cells increased by S100b or HMG-1 compared with control islets. (D) In JC-1 staining of rat islets, red aggregates were consistently found in control islet cells, whereas the homogeneous green fluorescence was observed in islet cells treated with 100 μmol/l CCCP indicating MMP disruption. The green fluorescence was increased in islet cells treated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 24 h compared with control islet cells.

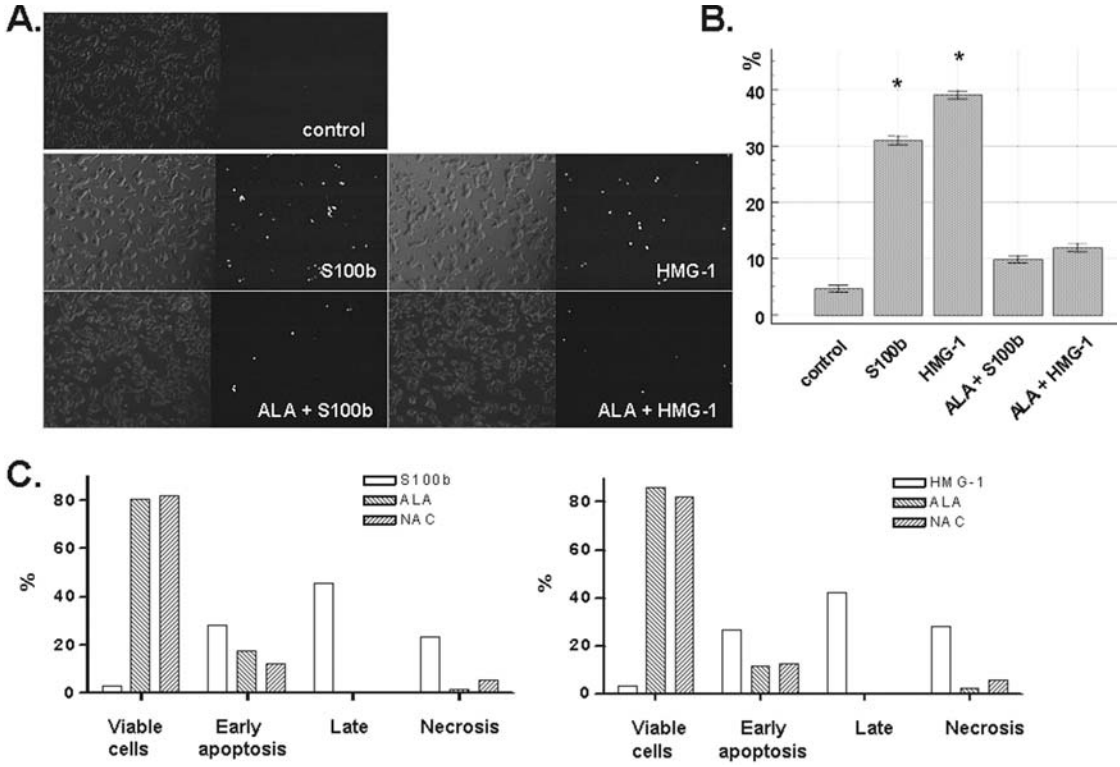

Figure 3. Involvement of ROS in RAGE ligands-induced INS-1 cell apoptosis. (A) Intracellular ROS detected by H&E oxidation to fluorescent ethidium bromide increased in INS-1 cells treated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 6 h. Pretreatment with 150 μmol/l ALA for 24 h before exposure to RAGE ligands decreased the intracellular ROS. (B) In flow cytometry assay with H_2DCFDA staining, INS-1 cells treated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 6 h exhibited a significant increase in the percentage of ROS-positive cells compared with control cells. Pretreatment with ALA for 24 h before exposure to RAGE ligands significantly decreased the percentage of ROS-positive cells. Results are shown as means ± SD; $^*p<0.001$ vs. control and ALA pretreatment group. (C) In AV-PI FACS analysis, pretreatment with 150 μmol/l ALA and 1mmol/l NAC for 24 h before exposure to 6 μg/ml S100b or 1 μg/ml HMG-1 for 6 h effectively reduced the S100b or HMG-1-induced INS-1 cell apoptosis. Results are means of 3 experiments.

S100b in the fibrotic and degenerative regions of islets. Both endocrine and nonendocrine (vascular and inflammatory) cells expressed RAGE or S100b.

Induction of apoptotic cell death in INS-1 and islet cells by RAGE-ligands. INS-1 cells incubated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 24 h were stained with PI and sorted according to DNA content by FACS analysis. The subdiploid population increased from 3.2±1.3% in control cells to 18.5±0.9% ($P<0.001$) in S100b-treted cells and 21.1±0.9% ($P<0.001$) in HMG-1-treated cells indicating that both ligands over 24-h period induces a cell death. By AV and PI flow cytometry, a significant increase in apoptosis in INS-1 cells was detected after RAGE-ligand treatment for 6 h at indicated concentrations and the percentage of late apoptotic (AV- and PI-positive) cells increased in a dose-dependent manner (Fig. 2A). As shown in Fig. 2B, the cytoplasmic fragmented DNA increased dose-dependently in INS-1 cells treated with S100b or HMG-1 for 6 h.

In situ APOPercentage staining, in which apoptotic islet cells showed a red pink color, S100b or HMG-1 treatment for 24 h induced apoptosis in human, pig and rat islet cells (Fig. 2C). In JC-1 staining, red aggregates were consistently found in control islet cells, whereas the homogenous green fluorescence was observed in islet cells treated with 100 μmol/l CCCP indicating MMP disruption. The green fluorescence was increased in islet cells treated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 24 h compared with control islet cells, indicating reduced MMP (Fig. 2D).

Involvement of intracellular ROS in RAGE ligand-induced apoptosis in INS-1 cells. The fluorescence due to H&E oxidation to ethidium bromide by ROS increased in INS-1 cells stimulated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 6 h (Fig. 3A). In flow cytometry assay with H_2DCFDA staining, RAGE ligand-treated INS-1 cells also exhibited a significant increase in ROS-positive cells (Fig. 3B).

Pretreatment with 150 μmol/l alpha-lipoic acid (ALA) and 1mmol/l N-acetyl cysteine (NAC) for 24 h before exposure to RAGE ligands effectively reduced the S100b or HMG-1-induced ROS generation (Fig. 3A and B) and INS-1 cell apoptosis (Fig. 3C). None of protein kinase inhibitors, LY-294002, SB 203580, and SP600125, inhibited the RAGE-ligands-induced apoptosis.

Involvement of NADPH oxidase in RAGE ligand-induced ROS production leading to apoptosis. The generation of ROS by S100b or HMG-1 was attenuated by 30 min pre-incubation with a NADPH oxidase inhibitor, 4-(2-aminoethyl)benzene-sulfonyl fluoride (AEBSF, 20 μmol/l) (Fig. 4A). Pretreatment with AEBSF also partially but significantly reduced the S100b or HMG-1-induced INS-1 cell apoptosis (Fig. 4B and C).

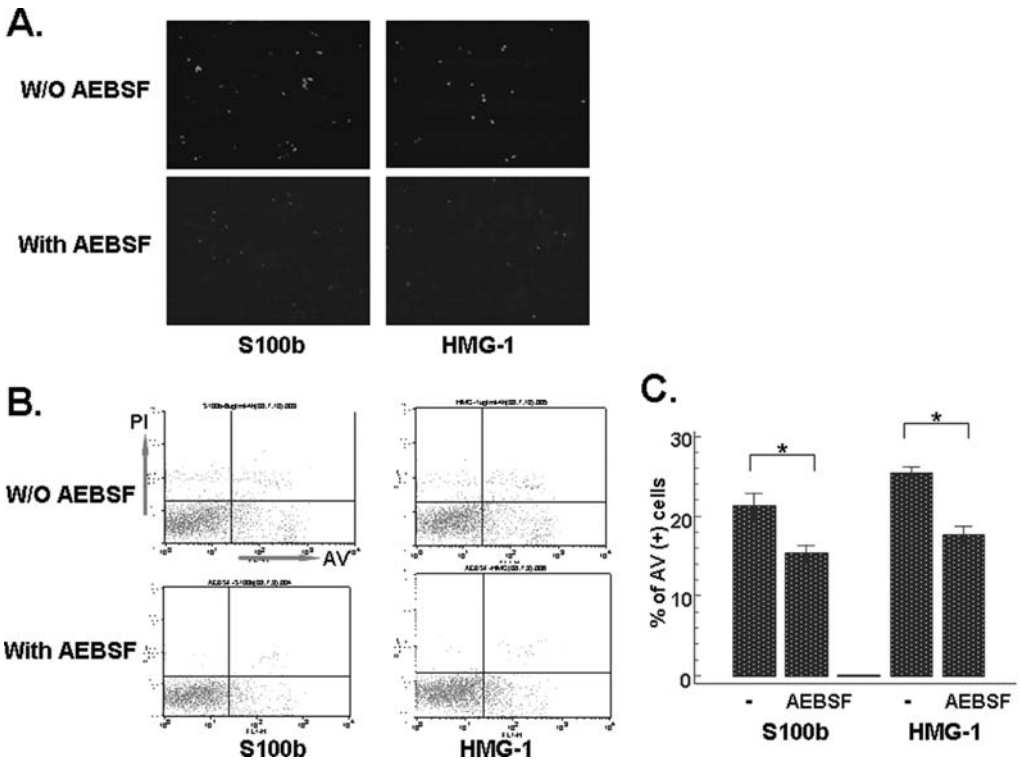

Figure 4. Involvement of NADPH oxidase in ROS-mediated INS-1 cell apoptosis by RAGE ligands. (A) Increased intracellular ROS detected by H&E oxidation in INS-1 cells treated with 6 μg/ml S100b or 1 μg/ml HMG-1 for 6 h was attenuated by pretreatment with a NADPH oxidase inhibitor, 20 μmol/l AEBSF for 30 min. (B and C) In AV-PI FACS analysis, pretreatment with 20 μmol/l AEBSF for 30 min also partially but significantly reduced the apoptosis in INS-1 cells induced by exposure to 6 μg/ml S100b or 1 μg/ml HMG-1 for 4 h. Results are shown as means ± SD; $^{*}p<0.01$.

Discussion

RAGE has been shown to be expressed by diverse cell types, including macrophages, endothelial cells, VSMCs, and neuronal cells (3). Pancreatic islet cells have been assumed to express RAGE. As a first step, we investigated the expression of RAGE on islet cells. Our study demonstrates that not only INS-1 ß-cell line but also rat and human islets express RAGE. Interestingly, the relative mRNA level of INS-1 and rat islet cells was higher than that of VSMCs. On VSMCs, AGE-RAGE interaction has been extensively studied and is shown to stimulate cellular migration and production of proinfammatory factors (23-25).

RAGE is not solely a receptor for AGEs and binds pro-inflammatory S100 proteins and HMG-1. Recent evidence suggests that the RAGE ligand-RAGE interaction may be promoted by inflammatory processes and oxidative cellular injury. In pathological conditions in which RAGE ligands accumulate, especially in a proinflammatory environment such as diabetes mellitus, atherosclerosis and neurodegenerative disorders, RAGE expression is often upregulated (6,7). S100 proteins and RAGE were increased in diabetic macrovessels and renal cortex in murine models, thus suggesting that these molecules may contribute to the acceleration of diabetic complications (26,27). In the case of pancreatic islets of type 1 diabetes, a recent study showed that both RAGE and its ligand, S100b, were detected by immunohistochemistry only on islet cells with an inflammatory infiltrate in diabetic NOD mouse but not on islet cells without insulitis (16). In concordance with this finding, we observed that both RAGE and S100b were expressed on islet cells of 28-week-old OLETF rat which is a rat model of type 2 diabetes but not on islet cells of age-matched control LETO rat.

RAGE stimulates ROS generation via receptor-dependent signaling and RAGE activation by its ligands has been shown to induce oxidative stress-mediated apoptosis in neuronal cells, pericytes and corneal endothelial cells (10,28,29). Oxidative stress is the subject of intense research in diabetes mellitus, due to their possible involvement in the pathogenesis of micro- and macrovascular complications and pancreatic ß-cell death. In this study, we show for the first time that specific inflammatory ligands of RAGE, S100b and HMG-1, can induce ROS-mediated apoptosis in pancreatic ß-cells. Both S100b and HMG-1 induced apoptotic cell death of INS-1 cells and rat, pig and human islet cells. This INS-1 cell apoptosis was accompanied by increased intracellular oxidative stress and effectively blocked by pretreatment with anti-oxidants such as ALA and NAC.

Engagement of RAGE by its ligands generates ROS, in part via superoxide-producing enzyme NADPH oxidase and also, possibly, via mitochondrial pathways in both vascular and neuronal cells affected by diabetes (8-10). The source of the ROS in ß-cells also remains controversial with two proposed candidates, i.e., leakage from the mitochondrial electron transport chain and NADPH oxidase (30). In our results, AEBSF, a pharmacological inhibitor of NADPH oxidase, clearly abrogated ROS generation by RAGE-ligands in INS-1 cells and attenuated the apoptosis. This result

indicates that RAGE ligands induce oxidative stress, at least in part via NADPH oxidase, resulting in cell injury and apoptotic death in pancreatic ß-cells.

In the present study, we show that RAGE and its ligand, S100b, express in pancreatic islets of rat model of type 2 diabetes and RAGE ligands, S100b and HMG-1, induce pancreatic ß-cell apoptosis *in vitro*, via NADPH oxidase-mediated ROS generation. Collectively, these results suggest that RAGE ligands-RAGE interaction may contribute not only to the development of chronic diabetic complications but also to the progressive pancreatic ß-cell loss in type 2 diabetes by inducing intracellular oxidative stress. Our findings are expected to initiate further detailed elucidation of the role of RAGE ligands/RAGE in pancreatic islets.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-313-E00267) and a research grant from the Innovative Research Institute for Cell Therapy, Republic of Korea (A062260) to S.I.

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