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Receptor for advanced glycation endproducts signaling cascades are activated in pancreatic fibroblasts, but not in the INS1E insulinoma cell line: Are mesenchymal cells major players in chronic inflammation?

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ABSTRACT

The receptor for advanced glycation endproducts (RAGE) is a pattern recognition receptor that plays an important role in natural immunity. It is suggested that mesenchymal cells are the major players during inflammation. Previously, we reported that advanced glycation end products (AGE), known to be one of the ligands of RAGE, inhibited glucose-induced insulin secretion from *ex vivo* pancreatic islets, although the mechanism responsible remains largely unknown. In the present study, we examined the cascades operating downstream from RAGE using the insulinoma cell line INS1E and primary-cultured pancreatic fibroblasts as *in vitro* models for parenchymal (β) cells and mesenchymal cells, respectively. Phosphorylation of c-jun N-terminal kinase, inhibitor of nuclear factor κB kinase, and nuclear factor κB was stimulated by AGE or high mobility group binding 1 (HMGB1) in pancreatic fibroblasts, whereas no such effect was observed in INS1E cells. Expression of the *Ccl5, Il-6*, and *Il-1b* genes was increased by AGE/HMGB1 in fibroblasts, but not in INS1E cells. On the other hand, AGE inhibited the secretion of insulin from *ex vivo* pancreatic islets, and this effect was ameliorated by MK615, a Japanese apricot extract used as an anti-inflammatory agent. Glucose-induced insulin secretion from INS1E cells was not affected by direct administration of AGE/HMGB1, but was inhibited by fibroblast-conditioned medium. These results suggest that AGE suppresses glucose-induced insulin secretion from pancreatic islets through indirect mesenchymal RAGE signaling.

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Chronic inflammation; Insulin secretion; Mesenchymal signaling; Receptor for advanced glycation end products

Introduction

The toll-like receptor (TLR), nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-related (NLR) gene family plays integral roles in natural immunity and chronic inflammation.[1,2] TLR activation leads to production of inflammatory cytokines through intracellular signaling molecules such as inhibitor of nuclear factor κB kinase (IKK)-nuclear factor κB (NF-κB), whereas NLR acts via the inflammasome protein complex.[3,4] Sakaguchi et al. have shown that the receptor for advanced glycation endproducts (RAGE), a pattern-recognition receptor, shares ligands and adaptor proteins with the TLR.[5] Subsequent studies have supported the hypothesis that, similarly to TLR and NLR, RAGE plays an important role in natural immunity.[6,7]

Previously, we reported that advanced glycation end products (AGE) [8], known to be one of the ligands of RAGE, inhibited glucose-induced insulin secretion from *ex vivo* rat pancreatic islets.[9] We used 2 different AGE compounds – glucose-AGE (AGE1) and glyceraldehyde-AGE (AGE2) – but their inhibitory effects were almost identical.[9] This was unexpected because AGE2 has stronger cytotoxicity than AGE1.[10] Thus, the inhibitory effects of AGE1 or AGE2 on insulin secretion could be explained by specific cellular

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signaling, rather than general cytotoxicity such as oxidative stress or endoplasmic reticulum stress.[9,11] One candidate signaling molecule is RAGE, originally identified as a receptor for AGE.[12] However, the involvement of RAGE does not necessarily mean that RAGE signaling occurs autonomously in β-cells. *In vivo*, pancreatic islets contain not only parenchymal endocrine cells but also mesenchymal fibroblasts and macrophages. In heart tissue, inflammasome signaling is conveyed through (mesenchymal) fibroblasts, instead through (parenchymal) cardiomyocytes,[13] raising the possibility that signals downstream of RAGE could be conveyed through pancreatic fibroblasts, rather than through β-cells *per se*.

In the present study, we investigated the mechanism whereby AGE suppresses insulin secretion from *ex vivo* pancreatic islets. While RAGE ligands activated signals downstream of RAGE in rat pancreatic fibroblasts, these responses were negligible in an insulinoma cell line, INS1E. Consistently, RAGE ligands induced the expression of genes for inflammatory cytokines in pancreatic fibroblasts, but not in INS1E cells. All of these effects of AGE were nullified by the anti-inflammatory agent MK615. Fibroblast-conditioned medium, but not the RAGE ligands *per se*, suppressed glucose-induced insulin secretion from INS1E cells. Collectively, our findings suggest that AGE inhibits glucose-induced insulin secretion through indirect mesenchymal signaling.

Materials and methods

Animals

All animal experiments were designed according to the guidelines of the animal facility at Dokkyo Medical University and approved by the institutional ethics committee. Male Wistar rats (aged 9–10 weeks) or pregnant female Wistar rats were purchased from Japan SLC and housed under standard conditions. Pancreases from the male rats were used for primary culture of pancreatic islets,[9] whereas neonatal pancreases were used for primary culture of fibroblasts. Immediately after parturition, the neonates were euthanized by decapitation and their pancreases were collected.

Cell culture

The INS1E cell line, a gift from Prof. Pierre Maechler,[14] was maintained in RPMI-1640 (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco), 0.5 mM monothioglycerol (Antioxidant, Wako, Osaka, Japan), 2 mM L-alanyl-L-glutamine (Wako), and 50 μg/ml gentamicin sulfate (Wako). Rat pancreatic fibroblasts were cultured under the same conditions as INS1E cells. Rat neonatal pancreases were collected and enzymatically digested in a collagenase/proteinase cocktail (0.1% collagenase L, Nitta Gelatin, Osaka, Japan, 0.2% dispase II, Gibco in HBSS, Gibco) for 60 mins in a reciprocating water bath shaker at 37°C. Undigested debris was removed using a nylon mesh and the cells were cultured as described below. Attached fibroblasts proliferated continuously and were sequentially passaged (sub-cultured in another dish), effectively diluting any contaminating islets and blood cells. Pancreatic fibroblasts exhibited cellular senescence after 3 to 6 passages, and were used before cell division had slowed down.

Reagents

AGE1 and AGE2 were gifts from Prof. Masayoshi Takeuchi. Briefly, bovine serum albumin (BSA) at 25 g/ml was incubated under sterile conditions with 0.5 mol/L d-glucose for 8 weeks (AGE1), or with 0.1 mol/L d-glyceraldehyde for 7 d (AGE2), at 37°C. Then, low-molecular-weight reactants and sugars were removed using a PD-10 column (GE Healthcare UK Ltd., Buckinghamshire, UK) and dialysis against phosphate-buffered saline. Control unglycated BSA was incubated under the same conditions except for absence of reducing sugar. Preparations were tested for endotoxin using an Endospecy ES-20S system (Seikagaku, Tokyo, Japan). AGE1 and AGE2 were used for stimulation of insulin secretion from *ex vivo* islets (Fig. 1). For other experiments (after Fig. 2), commercially available AGE1 (AGE-BSA, Merck Millipore, Billerica, MA, USA) was used. Unless otherwise stated, 'AGE' denotes the purchased AGE-BSA. Although we initially used unglycated BSA as a negative control, BSA itself had only a negligible influence on insulin secretion from *ex vivo* islets (data not shown). Therefore, we omitted the procedure and used the 'untreated' control (represented as ϕ or w/o ligands in Figs. 2–4). Human recombinant high mobility group box 1 (HMGB1) was purchased from R&D systems (Minneapolis, MN, USA). We used HMGB1 as an alternative ligand for RAGE (Supplementary Figure 1).[5] MK615 is a boiled extract of the Japanese

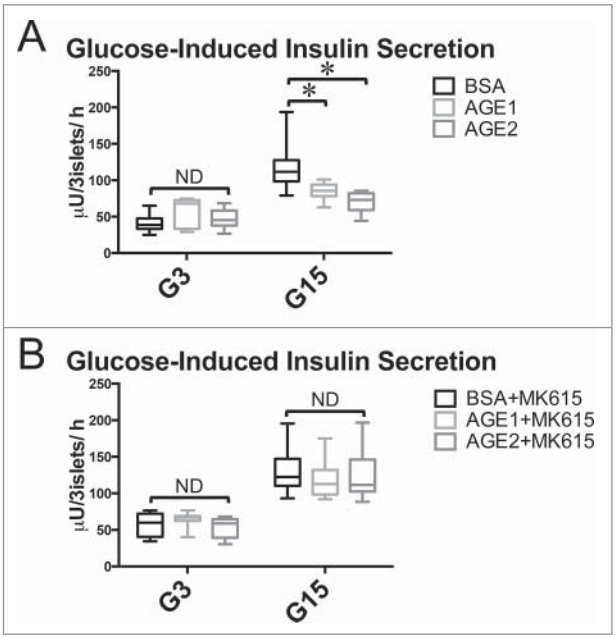

Figure 1. Insulin secretion from *ex vivo* pancreatic islets. (A) Rat pancreatic islets were treated with BSA (0.1 mg/ml, black box-plot), AGE1 (0.1 mg/ml, light gray box-plot), and AGE2 (0.1 mg/ml, dark gray box-plot) for 24 h, then insulin secretion was measured under the G3 (fasting blood glucose) or G15 (blood glucose after a meal) condition. (B) MK615 was added with or without AGE1/2. Box plot represents the quartile deviation ($n = 11–25$). Asterisk: statistically significant difference ($p < 0.05$ by Dunnett's test). ND: no difference.

apricot (*Prunus mume*), and has been reported to exert anti-inflammatory effects.[15] MK615 has multiple inhibitory functions against downstream substrates of RAGE, facilitating more synergistic output than specific RAGE inhibitors. In this study, we used MK615 as an anti-inflammatory reagent to abrogate RAGE downstream cascades. MK615 was provided by Ada-Bio Co., Ltd. (Gunma, Japan). AGE1, AGE2, AGE, HMGB1 and MK615 were used at the following concentrations: AGE1/AGE2/AGE (0.1 mg/ml), HMGB1 (100 ng/ml) and MK615 (diluted with 100-fold)

Insulin secretion assay

The procedures for primary culture of whole-mount islets have been described elsewhere.[9] For experiments using conditioned medium, the following procedures were employed: After pancreatic fibroblasts had been treated with AGE or HMGB1 for 24 h, the conditioned medium was collected and stored in a freezer until use. INS1E cells were seeded at 1×10^5 cells/ml in 24-well plates. After attachment, the cells were grown stably for 48 h, and subsequently the culture medium was replaced with each of the conditioned media (control, AGE or HMGB1 with or without MK615) from fibroblasts. After 24 h of pre-incubation with conditioned medium, high-glucose DMEM (Wako) was replaced with conditioned medium and the cells were incubated for another 2 h (high glucose). This replacement of the medium (glucose concentration in the conditioned media and high-glucose DMEM, < 11 mM and 25 mM, respectively) was able to mimic the conventional glucose-stimulated insulin assay. A rat insulin assay kit (Morinaga, Kanagawa, Japan) was used to quantify the amount of secreted insulin, and the colorimetric signal was detected with a plate reader (InfinitePro200, Tecan, Männedorf, Switzerland). The concentration was calculated based on a standard curve.

Immunoblotting and enzyme-linked immunosorbent assay

We used immunoblotting to observe activation of signaling downstream of RAGE. RAGE shares signaling substrates with TLR[5] (Supplementary Figure 1). Downstream protein myeloid differentiation primary response gene 88 (MyD88) activates phosphorylation of c-jun N-terminal kinase (JNK), p38, protein kinase B (AKT) and the IκB kinase (IKK) complex. The phosphorylated IKK complex subsequently transmits a signal to inhibitor of nuclear factor κB (NK-κB), leading to nuclear translocation of NK-κB subunit (RelA/p65)[5] (Supplementary Figure 1). To quantify the RAGE downstream signals, we used primary antibodies (Cell Signaling Technology, Danvers, MA, USA) against the following proteins: phospho-JNK (cat#4668), JNK2 (#9258), phospho-p38 (#4511), p38 (#8690), phospho-AKT (#4060), AKT (#4691), phospho-IKKα/β (#2697), IKKα (#2682), phopho-NF-κB (RelA/p65, #3033) and NF-κB (#8242). The procedures for immunoblotting have been described elsewhere.[16] Briefly, cultured cells were fixed with 10% trichloroacetic acid (Wako) and whole-cell proteins were dissolved in a solubilizer (7 M urea, 2 M thiourea, 2% Triton X-100, Wako). Each band intensity was quantified using an image analyzer (IQTL version 8.1, GE Healthcare, Little Chalfont, United Kingdom) and the index of phosphorylation was calculated as the ratio: (band of phosphorylated form)/(band of total protein). Secreted IL-6 was quantified with

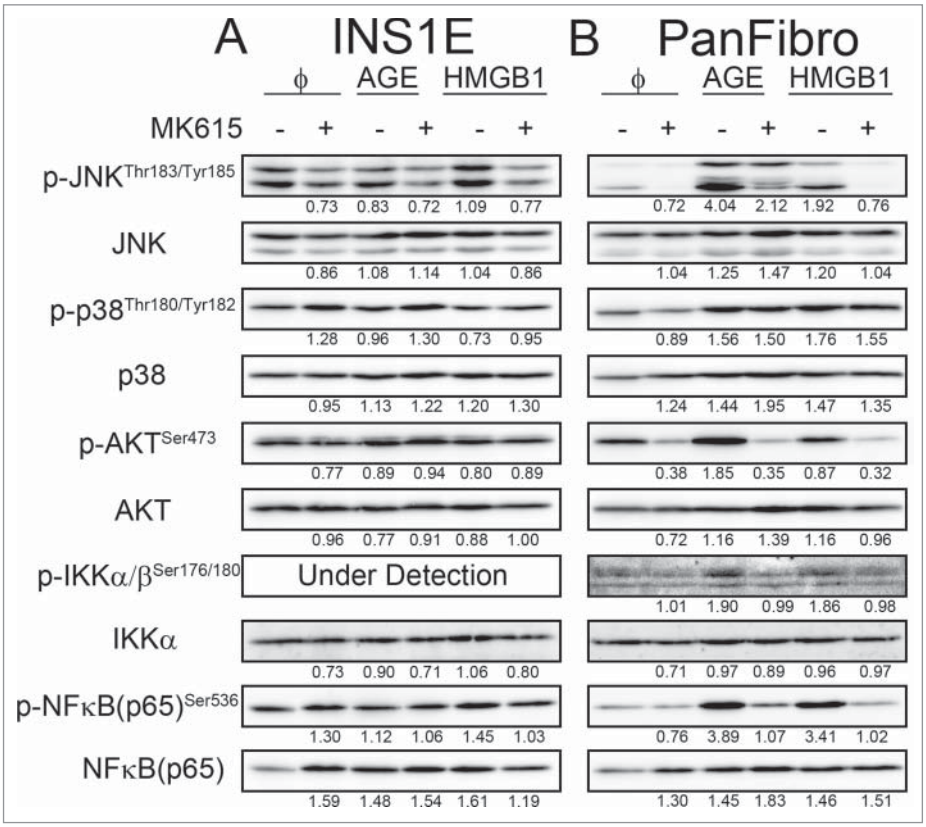

Figure 2. RAGE downstream signaling in INS1E insulinoma cells and pancreatic fibroblasts. (A) INS1E insulinoma (B) Pancreatic fibroblasts: Whole-cell lysates were extracted after each drug treatment (AGE: 0.1 mg/ml, HMGB1: 100 ng/ml, MK615: diluted 100-fold). Incubation time was 6 h (for NF-κB) or 2 h (for other proteins). Each well was loaded with 30μg protein, and phosphorylation of the designated proteins was examined by Western blotting. The band intensity was quantified using an image analyzer, and the (drug-treated)/(non-treated control) ratios are presented below. Note that INS1E cells responded poorly to AGE or HMGB1, whereas pancreatic fibroblasts responded well.

commercial ELISA kits (Abfrontier, Seoul, South Korea) and a plate reader.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was purified using RNAiso Plus (Takara, Osaka, Japan). Reverse transcription was performed using PrimeScript with gDNA eraser (Takara). For quantifying the copy number of each mRNA, we used a StepOne Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) and the Taqman method. Data were calculated according to the delta-cycle of threshold (ΔC_T) method. For normalization of each sample, the C_T of 18S rRNA (rRNA, a housekeeping gene) was determined. Thus, the normalized expression index was ΔC_T = (C_T of a gene of interest) – (C_T of 18S rRNA). We used this ΔC_T parameter for statistical analysis because mRNA expression follows a log-normal distribution, whereas ΔC_T follows a normal distribution. Accordingly, in our figures, we present each value as (20-ΔC_T) = Log_2(relative expression). A gene-specific fluorescent probe was designed using a web-based program (https://qpcr.probefinder.com/organism.jsp). The primer sequences and corresponding universal probes employed were as follows: rat chemokine (C-C motif) ligand 5 (Ccl5, a gene encoding regulated on activation, normal T cell expressed and secreted (RANTES), 5′-CTCACCGTCATCCTCGTTG-3′, 5′ - GAGTGGTGTCCGAGCCATA- 3′ and universal probe ID#16), rat Il1b (IL-1β, 5′ - TGTGATGAAAGACGGCACAC-3′, 5′ - CTTCTTCTTTGGGTATTGTTTGG-3′ and universal probe ID#78) and rat Il6 (IL-6, 5′ -CCCTTCAGGAACAGCTATGAA-3′, 5′ - ACAACATCAGTCCCAAGAAGG-3′ and universal probe ID#20). For eukaryotic 18S rRNA and rat Ager quantification, Taqman probes were used (4352930E and Rn01525753_g1, respectively; Life Technologies, Waltham, MA, USA). For PCR, Thunderbird Probe qPCR mix (Toyobo, Osaka, Japan) was used and the PCR

conditions consisted of 40 cycles of 2 steps (95°C for 15 s and 60°C for 50 s).

Statistical analysis

The statistical significance of differences was assessed as follows:

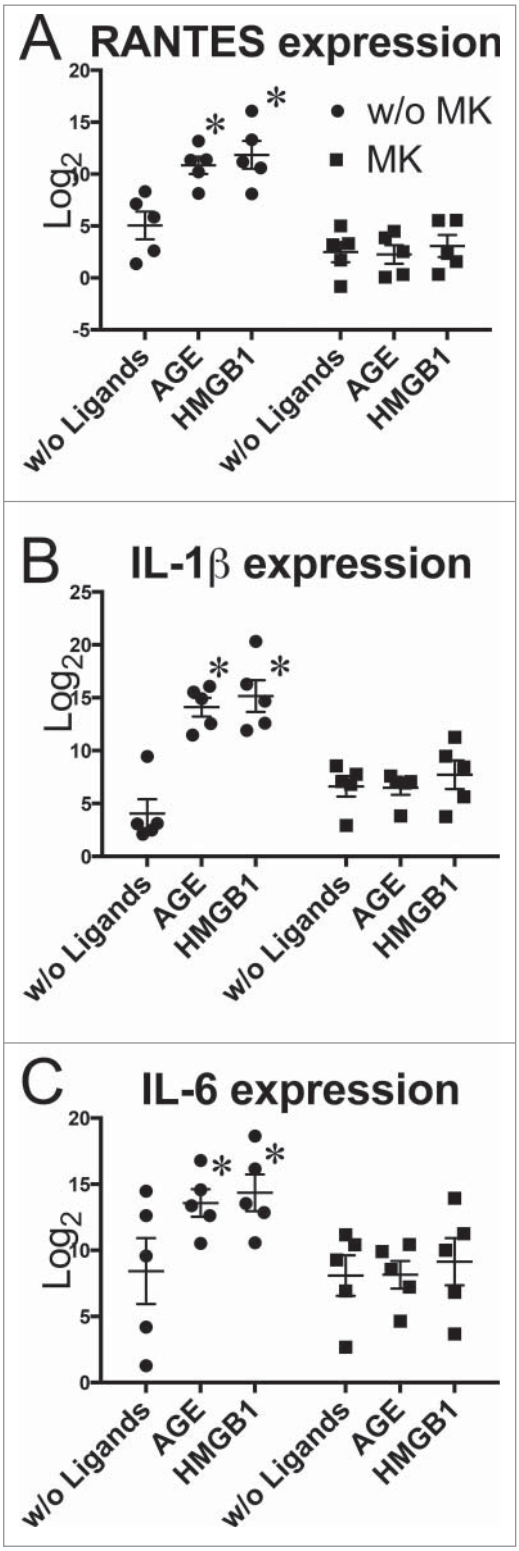

Figure 1: We analyzed differences among 12 different treatment groups by 3-way analysis of variance (ANOVA) for glucose concentration, AGE1/2 and MK615 treatments. Since we detected mutual interaction between glucose, AGE1/2 and MK615, we split the data groups and analyzed them again with or without an increase in glucose/MK615 by Dunnett's test (for AGE1/2).

Figures 3 and 4 and Supplementary Figures 2, 4 and 6: First we analyzed differences among 6 different treatment groups by 2-way repeated measures ANOVA (for RAGE ligands and MK615 treatments). Since we detected mutual interaction between RAGE ligands and MK615, we split the data groups and analyzed them again with or without MK615 by one-way repeated measures ANOVA (for RAGE ligands). Subsequently, the data were *post hoc* analyzed by paired *t* test to determine the p-value. Analyses were performed using SPSS software (IBM, Armonk, NY, USA).

Results

Suppression of glucose-induced insulin secretion from ex vivo rat islets by AGE1 and AGE2, and amelioration of the inhibition by MK615

First we isolated rat pancreatic islets and measured their insulin secretion *ex vivo*. For G3 (glucose 3 mM: blood glucose concentration before a meal), insulin secretion in the presence of bovine serum albumin (BSA), AGE1-BSA, and AGE2-BSA was 40.8 ± 2.15, 55.8 ± 4.23 and 47.8 ± 2.47 (no difference by Dunnett's test) μU/3 islets/h, respectively (Fig. 1). For G15 (glucose 15 mM: blood glucose concentration after a meal), these values increased to 118 ± 7.25, 85.7 ± 2.94 ($p = 0.001$) and 70.0 ± 3.39 ($p = 0.001$) μU/3 islets/h respectively

Figure 3. Expression of mRNAs for cytokine genes in rat pancreatic fibroblasts. After 24 h of treatment with each agent (AGE: 0.1 mg/ml, HMGB1: 100 ng/ml, MK615: diluted 100-fold), mRNA was purified and reverse-transcribed for quantitative polymerase chain reaction. AGE or HMGB1 increased the Log_2(relative expression) of RANTES (A, circle), IL-1β (B, circle) and IL-6 (C, circle). In contrast, AGE or HMGB1 did not change the Log_2(relative expression) of RANTES (A, square), IL-1β (B, square), and IL-6 (C, square) in the presence of MK615, suggesting that MK615 nullified the effects of AGE/HMGB1. Scatter plot shows the experimental data ($n = 5$: different numbers of passages, from 2 independently established fibroblast cultures). Asterisks: statistically significant difference from control ($p < 0.05$ by rmANOVA and post hoc paired *t* test).

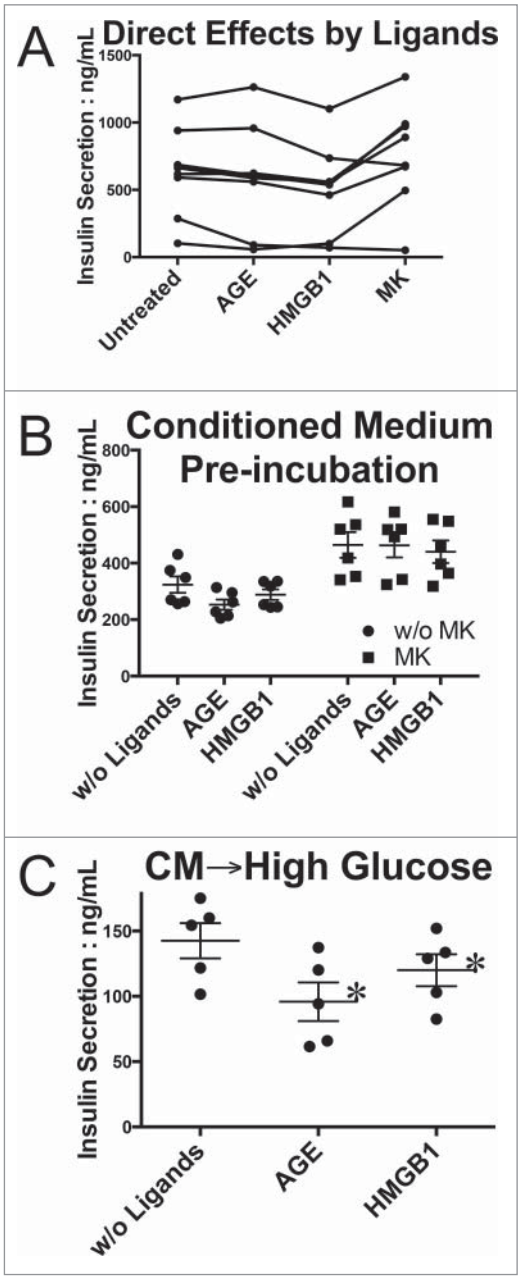

Figure 4. Insulin secretion from INS1E cells. (A) Measurement of insulin secretion and accumulated insulin from INS1E cells incubated with AGE1, HMGB1 or MK615 for 24 h. Line graph represents the experimental data (n = 8: different numbers of INS1E cell passages). (B) Conditioned media were collected from pancreatic fibroblasts (treated with AGE, HMGB1 and/or MK615). INS1E cells were incubated with these conditioned media for 24 h (pre-incubation) and accumulated insulin was measured. Conditioned medium from fibroblasts treated with AGE or HMGB1 modestly inhibited insulin secretion from INS1E cells. C. Afterpre-incubation for 24 h (glucose concentration < 11 mM), INS1E cells were incubated with high-glucose DMEM (25 mM) for 2 h, and insulin secreted during the brief increase in glucose concentration was measured. Scatter plot (circles and squares: without and with MK, respectively) shows the experimental data (n = 5–6: 4 independently established fibroblasts were used for distinct passages of INS1E cells). Asterisks: statistically significant difference from control ($p < 0.05$ by rmANOVA and post hoc paired *t* test).

(Fig. 1). Thus, AGE1 and AGE2 significantly inhibited insulin secretion in response to glucose. Next we added MK615 with or without AGE1/AGE2: for G3 in the presence of BSA+MK615, AGE1+MK615 and AGE2+MK615, insulin levels were 56.6 ± 3.49, 64.1 ± 3.11 and 52.7 ± 2.6 (no significant difference) μU/3 islets/h, respectively (Fig. 1). For G15, these values were 129.9 ± 5.47, 118.1 ± 6.91 and 125.2 ± 8.98 (no significant difference) μU/3 islets/h, respectively (Fig. 1). Thus, MK615 nullified the inhibitory effect of AGE1 or AGE2.

RAGE downstream phosphorylation cascades in INS1E cells treated with AGE or HMGB1

We next examined the phosphorylation of c-jun N-terminal kinase (JNK), p38, protein kinase B (AKT), IKKα/β, and NF-κB (RelA/p65) in INS1E cells treated with AGE or high mobility group box 1 (HMGB1, an alternative ligand for RAGE, Supplementary Figure 1). JNK, p38 and AKT were constitutively phosphorylated, whereas neither AGE nor HMGB1 showed a change in phosphorylation (quantified as phosphorylated/total and analyzed by one-way repeated measures ANOVA (rmANOVA), no significant difference, Fig. 2A, Supplementary Figure 2). Phosphorylation of IKKα/β protein was below the limit of detection under all of the experimental conditions. NF-κB protein was also constitutively phosphorylated, and the modification was not affected by either AGE or HMGB1 (no significant difference, Fig. 2A, Supplementary Fig. 2).

AGE or HMGB1 induces phosphorylation of RAGE downstream cascades in rat pancreatic fibroblasts

We next examined the phosphorylation of JNK, p38, AKT, IKKα/β, and NF-κB (p65) in rat pancreatic fibroblasts treated with AGE or HMGB1. The basal phosphorylation level of JNK was not as high as that of INS1E cells (Fig. 2A and B). AGE or HMGB1 significantly increased the phosphorylation of JNK (AGE: $p = 0.045$ by rmANOVA and *post hoc* paired *t* test, HMGB1: $p = 0.038$, Fig. 2B and Supplementary Figure 2). Co-treatment with MK615, on the other hand, nullified these effects (in the presence of MK615, no significant difference by rmANOVA, Fig. 2B and Supplementary Fig. 2). p38 was constitutively phosphorylated, and HMGB1, but not AGE, further increased the phosphorylation ($p = 0.037$, Fig. 2B and Supplementary Fig. 2). Constitutive

phosphorylation of AKT was also high, and the effects of AGE and HMGB1 were also negligible (no significant difference, Fig. 2B and Supplementary Fig. 2). Although phosphorylation of IKKα was around the lowest limit of detection, AGE or HMGB1 modestly increased the phosphorylation level (Fig. 2B) whereas MK615 nullified these effects (Fig. 2B). AGE and HMGB1 increased the phosphorylation of NF-κB (p65) (AGE: $p = 0.019$ by rmANOVA and *post hoc* paired *t* test, HMGB1: $p = 0.019$, Fig. 2B and Supplementary Fig. 2) and MK615 nullified these effects (no significant difference in the presence of MK615, Fig. 2B and Supplementary Fig. 2).

AGE or HMGB1 induces expression of genes for inflammatory cytokines in rat pancreatic fibroblasts, but not in INS1E cells

To investigate whether AGE or HMGB1 influences the expression of genes for inflammatory cytokines, we measured mRNA in rat pancreatic fibroblasts. We employed real-time RT-PCR and obtained the Log_2(relative expression) value (20-ΔC_T: see Materials and Methods) for each experimental condition. Changes in Log_2(relative expression) elicited by AGE in rat pancreatic fibroblasts were: from 5.1 ± 2.96 to 10.85 ± 1.85 for RANTES ($p = 0.036$ by rmANOVA and post hoc paired *t* test, Fig. 3A), from 8.1 ± 5.46 to 14.11 ± 1.99 for interleukin-1β (IL-1β, $p = 0.024$, Fig. 3B), and from 9.1 ± 6.26 to 13.58 ± 2.32 for IL-6 ($p = 0.061$, Fig. 3C). Changes in Log_2(relative expression) elicited by HMGB1 were: from 5.1 ± 2.96 to 11.45 ± 3.69 for RANTES ($p = 0.041$, Fig. 3A), from 8.1 ± 5.46 to 15.16 ± 3.37 for IL-1β ($p = 0.010$, Fig. 3B), and from 9.1 ± 6.26 to 14.35 ± 3.12 for IL-6 ($p = 0.037$, Fig. 3C). Together, AGE or HMGB1 increased the relative expression for RANTES, IL-1β, and IL-6 in pancreatic fibroblasts. In contrast, neither AGE nor HMGB1 changed the PCR amplification of cytokine genes in INS1E cells (Supplementary Fig. 3). When MK615 and AGE/HMGB1 were added together in culture with pancreatic fibroblasts, the Log_2(relative expression) values for each gene were as follows: MK615: 2.5 ± 2.18, MK615+AGE: 2.3 ± 2.01, MK615+HMGB1: 3.1 ± 2.37 (for RANTES, no significant difference by ANOVA, Fig. 3A), MK615: 6.6 ± 2.18, MK615+AGE: 6.5 ± 1.51, MK615+HMGB1: 7.7 ± 3.01 (for IL-1β, no significant difference, Fig. 3B), MK615: 8.1 ± 3.42, MK615+AGE: 8.2 ± 2.33, MK615+HMGB1: 9.1 ± 3.99 (for IL-6, no significant difference, Fig. 3C). Thus, MK615 nullified the effect of AGE or HMGB1 in increasing the Log_2(relative expression) value for RANTES, IL-1β, and IL-6 (Fig. 3). MK615 did not affect PCR amplification of the above genes in the INS1E cell line (Supplementary Fig. 3). When we measured the amount of IL-6 protein by ELISA, the changes in protein secretion were consistent with the mRNA expression data (in the absence of MK615, control: 13.73 ± 14.82 pg/mL, AGE: 262.16 ± 80.39 pg/mL, $p = 0.003$, HMGB1: 164.5 ± 44.44, $p = 0.001$, in the presence of MK615, MK615: 35.39 ± 20.82, MK615+AGE: 35.68 ± 14.69, MK615+HMGB1: 40.39 ± 19.73, no significant difference, Supplementary Fig. 4)

AGE or HMGB1 inhibits insulin secretion from INS1E cells via factors secreted from fibroblasts

We measured insulin secretion from INS1E cells and estimated the effects of AGE or HMGB1. As expected, AGE or HMGB1 alone did not influence insulin secretion (Fig. 4A). Data from immunoblotting, RT-PCR and ELISA suggested that AGE influenced insulin secretion indirectly through fibroblast RAGE signaling. Therefore, insulin secretion was measured after treatment with fibroblast-conditioned medium (see Materials and Methods). Insulin secretion during pre-incubation (fibroblast-conditioned medium treated with control, AGE, or HMGB1) was 323.96 ± 65.46, 252.99 ± 40.94 (AGE, no significant difference by rmANOVA and post hoc paired *t* test) and 288.43 ± 41.76 (HMGB1, no significant difference), respectively (Fig. 4B). Insulin secretion during pre-incubation (fibroblast-conditioned medium treated with MK615, MK615+AGE or MK615+HMGB1) was 464.33 ± 101.18, 463.25 ± 95.95 and 440.33 ± 89.25 (no significant difference by rmANOVA, Fig. 4B), respectively. Subsequently, each medium was replaced with high-glucose DMEM to mimic glucose-stimulated insulin secretion (see Methods). However, INS1E cells underwent massive cell death upon sequential change from MK615 to high glucose (data not shown), presumably because of prolonged inhibition of JNK phosphorylation (Fig. 2A). Without MK615 treatment, insulin secretion in the presence of high-glucose DMEM was 142.59 ± 30.07, 95.86 ± 33.13 (AGE, $p = 0.031$ by rmANOVA and post hoc paired *t* test) and 120.07 ± 27.34 (HMGB1, $p = 0.015$), respectively (Fig. 4C).

Thus, AGE/HMGB1 suppressed glucose-induced insulin secretion through fibroblast conditioning.

Discussion

Previously we reported that AGE1 or AGE2 inhibits insulin secretion from *ex vivo* islets.[9] So far, AGE has been considered to inhibit insulin secretion in a cell-autonomous manner.[17-20] Forbes and colleagues reported that MIN6N8, another insulinoma cell line, responded to AGE within a concentration range similar to that used in our study.[19] Another group reported that INS1 cells (the parental cell line from which INS1E was derived[14]) synthesize a substantial amount of RAGE protein, and that knockdown of this endogenous RAGE protects the cells from glycated serum-induced apoptosis.[20] It is probably pertinent to discuss these findings in terms of "sensitivity." In the experiments using MIN6N8, the cells were incubated with AGE for a much longer period (i.e. 28 d) than in our study (less than one day), whereas Forbes et al. indicated that incubation for one day was enough for *ex vivo* islets.[19] We observed a similar discrepancy with regard to incubation time (Figs. 1 and 4) and accordingly hypothesized that RAGE ligands influence islets through "more sensitive" mesenchymal cells. In a study of cardiac ischemia/reperfusion injury, Kawaguchi et al. demonstrated that inflammasome signaling is activated not in heart parenchymal cells (cardiomyocytes), but in resident mesenchymal cells (cardiac fibroblasts).[13] Furthermore, recent evidence suggests that the fibroblast should be considered a sentinel cell, and a center of chemokine synthesis during inflammation.[21-23] Our data indicated that AGE or HMGB1 activated RAGE in pancreatic fibroblasts, and that subsequently secreted cytokines (including others that we did not measure) inhibited glucose-stimulated insulin secretion from INS1E cells (Fig. 4). Consistent with our hypothesis, RAGE protein is not detected in pancreatic islets in control rat pancreas, whereas RAGE is reportedly localized exclusively in fibrous (mesenchymal) tissue in diabetic OLETF islets.[18] Therefore, we propose that mesenchymal cells, rather than parenchymal cells, play more significant roles in RAGE signaling (Supplementary Fig. 5).

Previously, we have shown that MK615 suppressed the expression of RAGE in a hepatocellular carcinoma cell line.[24] Consistent with this, we confirmed that MK615 decreased RAGE expression in INS1E cells (Supplementary Fig. 6A), but conversely increased RAGE expression in pancreatic fibroblasts (Supplementary Fig. 6A and B). On the other hand, AGE or HMGB1 decreased the expression of RAGE mRNA (Supplementary Fig. 6B). Athough the reason for these totally opposite effects of MK615 remains unclear, the changes observed in fibroblasts could result from a feedback mechanism mediated by RAGE signaling. Although we tried to measure the level of RAGE protein by immunoblotting, the titer of the antibody was weak and we found no convincing evidence for any increase/decrease in protein (data not shown). MK615 is a boiled extract of Japanese apricot, and the boiling procedure changes the chemical structure of the original constituents. One possibility is that MK615 includes some form of AGE, which acts as an inhibitory ligand of RAGE. However, comparison of the dissociation constants with recombinant RAGE was difficult because of the specific requirement of oligomerization for pattern recognition, and the relatively low affinity ($\sim$10 μM).[25]

Finally, with regard to AKT and p38 phosphorylation, our present Fig. 2 is partly inconsistent with Sakaguchi et al.,[5] who used human umbilical vein endothelial cells to estimate the phosphorylation cascade initiated by RAGE. This difference in cellular contexts could explain the lack of consistency in the downstream cascades. Hitherto, RAGE has been considered to be ubiquitous, but in order to validate its function, an appropriate choice of experimental materials will be necessary.

Conclusion

AGE or HMGB1 was shown to activate the phosphorylation cascades downstream of RAGE, and induced the expression of genes for inflammatory cytokines in pancreatic fibroblasts, but not in INS1E insulinoma cells. AGE1/AGE2 suppressed glucose-induced insulin secretion from *ex vivo* pancreatic islets through indirect mesenchymal RAGE signaling.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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