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Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Extracellular superoxide released from mitochondria mediates mast cell death by advanced glycation end products

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ARTICLE INFO

Article history:

Received 16 April 2008

Received in revised form 12 August 2008

Accepted 25 August 2008

Available online xxxx

Keywords:

Superoxide

Mitochondria

Cell death

Mast cell

ABSTRACT

Advanced glycation end products (AGEs) accumulate during aging and to higher extents under pathological conditions such as diabetes. Since we previously showed that mast cells expressed the AGE-binding protein, receptor for AGEs (RAGE) on their cell surface, we examined whether AGE affected mast cell survival. Herein, we demonstrate that mast cells undergo apoptosis in response to AGE. Glycated albumin (GA), an AGE, but not stimulation with the high-affinity IgE receptor (FcεRI), can induce mast cell death, as measured by annexin V/propidium iodide double-staining. GA (≥0.1 mg/ml) exhibited this pro-apoptotic activity in a concentration-dependent manner. GA and FcεRI stimulation increased the cytosolic Ca²⁺ levels to a similar extent, whereas GA, but not FcεRI stimulation, caused mitochondrial Ca²⁺ overload and membrane potential collapse, resulting in mitochondrial integrity disruption, cytochrome c release and caspase-3/7 activation. In addition, GA, but not FcεRI stimulation, induced extracellular release of superoxide from mitochondria and that this release played a key role in the disruption of Ca²⁺ homeostasis. Knockdown of RAGE expression using small interfering RNA abolished GA-induced apoptosis, mitochondrial Ca²⁺ overload, and superoxide release, demonstrating that RAGE mediates the GA-induced mitochondrial death pathway. AGE-induced mast cell apoptosis may contribute to the immunocompromised and inflammatory conditions.

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1. Introduction

Advanced glycation end products (AGEs) are formed by non-enzymatic reactions between carbonyl groups of reducing sugars and free amino groups of macromolecules such as proteins, lipoproteins and nucleic acids [1–3]. This heterogeneous group of glycated structures is found in the plasma and accumulates in vessel walls and tissues during aging and at an accelerated rate in diabetes [4,5]. AGE formation is considered to be one of the major etiologic factors in aging- and diabetes-related chronic inflammatory diseases, by promoting cellular dysfunction and breaking immune tolerance. Diabetes mellitus is characterized by hyperglycemia that facilitates AGE formation in both blood and tissues [6,7]. However, the mechanisms by which AGEs cause cellular dysfunction are not fully understood. One potential mechanism is through the induction of cell death by apoptosis. AGEs are pro-apoptotic for cultured microvascular cells, neuronal cells, fibroblasts and renal mesangial cells [8–13], and these apoptotic effects are associated with aging and diabetic complications, such as atherosclerosis, nephropathy, neuropathy, defective wound healing and retinopathy.

Recent studies focusing on their interactions with many cell types have led to the isolation and characterization of a number of AGE cell surface receptors/binding proteins. They include receptor for AGEs (RAGE), oligosaccharyl transferase-48 (OST-48; AGE-R1), 80K-H phosphoprotein (AGE-R2), galectin-3 (AGE-R3), and the scavenger receptors class A type II (MSR-AII), and class B type I (MSR-BI; CD36) [14–17]. These receptors have been shown to play distinct functional roles in AGE toxicity as well as AGE removal. The best-characterized receptor is RAGE [18,19], a member of immunoglobulin superfamily of cell surface receptors that binds many ligands including those of the S100/calgranulin family. RAGE is known to play key roles in cell dysfunction and the mechanisms of inflammatory diseases through the activation of the Ras-MAPK pathway, while some of the other receptors may have other functions such as AGE detoxification and AGE removal [20–23].

Immune cells are other potential targets of AGEs, since diabetes and aging are accompanied by altered immunological reactions. It is widely accepted that similar to aged individuals, uncontrolled type I diabetic patients are more susceptible to bacterial and fungal infections than normal subjects, although the cause has not yet been determined [24]. The higher susceptibility to and severity of infections and the increased frequency of inflammatory diseases, 70 such as atherosclerosis and periodontitis, in diabetic and aging 71 individuals, suggest AGE-induced dysregulation of the immune 72

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system. Indeed, several functional alterations including altered superoxide ($O_2^{\cdot-}$) production, defective chemotaxis and phagocytosis have been demonstrated in diabetic mononuclear phagocytes [25]. Oxidative stress has been shown to play a role in the pathogenesis of diabetic complications. It has also been shown that diabetic monocytes produce significantly more $O_2^{\cdot-}$ than non-diabetic cells in the resting state or in response to divergent stimuli, including AGE and S100B, and that the increased $O_2^{\cdot-}$ generation is correlated with glycated hemoglobin levels in diabetic patients [26]. On the other hand, AGE albumin impairs human neutrophil functions including transendothelial migration, *Staphylococcus aureus*-induced $O_2^{\cdot-}$ generation, and bacterial killing [27]. However, the mechanisms by which oxidative stress modulates biological effects of AGE are poorly understood. In addition, effects of AGEs on immune cell survival are unknown.

Mast cells play key roles in allergic and inflammatory responses as well as in innate and acquired immunity [28,29]. We recently showed that mast cells express both RAGE and galectin-3 on their cell surface and that exogenous galectin-3 can exhibit strong pro-apoptotic activity toward these cells via RAGE [30]. These observations suggest that RAGE and galectin-3 are key regulators of mast cell death. To establish this view, we examined whether exogenous AGE induced mast cell death. In the current study we report for the first time that an AGE, glycated albumin (GA) induces mast cell death by apoptosis through binding to RAGE. Our results indicate that GA specifically evokes mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) overload, resulting in mitochondrial integrity disruption and the caspase-dependent cell death. We further showed that extracellular superoxide ($O_2^{\cdot-}$) released from mitochondria played a key role in $[Ca^{2+}]_m$ overload.

2. Materials and methods

2.1. Materials

Bongkreic acid dissolved in water was obtained from BioMol (Plymouth Meeting, PA, USA). Atractyloside, thapsigargin (TG) and superoxide dismutase (SOD) were obtained from Sigma (St. Louis, MO, USA). Atractyloside and TG were dissolved in dimethyl sulfoxide (DMSO) and diluted with Hank's balanced salt solution (HBSS) to a final concentration of <0.1% before use. At a concentration of 0.1%, DMSO had no effects on its own throughout the present study. 2,4,6-Trinitrophenyl (TNP)-conjugated bovine serum albumin (TNP-BSA, TNP:BSA conjugation ratio of 25:1) was purchased from Cosmo Bio (Tokyo, Japan). A neutralizing antibody against receptor for AGEs (RAGE) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fluo3-acetoxymethyl ester (Fluo3/AM) was obtained from Dojindo Laboratories (Kumamoto, Japan). Rhod-2-acetoxymethyl ester (rhod-2/AM), MitoSOX Red (MitoSOX) [3,8-phenanthridinediamine, 5-(6'-triphenyl-phosphoniumhexyl)-5,6-dihydro-6-phenyl] and JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenz-imidazolocarbocyanine iodide) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). All other chemicals were of analytical grade.

2.2. Preparation of glycated albumin (GA)

GA were aseptically prepared by incubating 10 mg/ml BSA (RIA grade fraction V; Sigma) with 1 M glucose in phosphate-buffered saline (PBS) containing 0.1% NaN_3 for 13 weeks at 37 °C. Control unmodified albumin was prepared in a similar way in the absence of glucose. The GA and unmodified albumin samples were desalted by Sephadex G-25 (fine grade; 1.8×30 cm) column gel filtration with 0.05 M NH_4HCO_3 and freeze-dried to remove the NH_4HCO_3 . Amino acid analysis of aliquots of GA and unmodified albumin was performed using a System 6300E Amino Acid Analyzer (Beckman, Fullerton, CA) as previously described [31]. The amino acid composition of GA were

similar to that of unmodified albumin, except for the following amino acids: Lys (GA, 33.2 residues; unmodified albumin, 59 residues), Arg (GA, 13.8 residues; unmodified albumin, 23 residues) and His (GA, 14.3 residues; unmodified albumin, 17 residues). The relative electrophoretic mobility (Re) of GA to methylene blue was 0.78 on a cellulose acetate membrane with Veronal buffer (pH8.6), whereas the Re of unmodified albumin was 0.50, indicating that GA possess fewer positive charges, consistent with the data for the amino acid analysis. GA (4 µg/ml) but not unmodified albumin reacted with a peroxidase conjugated anti-AGE monoclonal antibody (Wako Pure Chemicals, Osaka, Japan). Throughout the current study, unmodified albumin was used as a control.

2.3. RBL-2H3 cells

RBL-2H3 cells were obtained from the National Institute of Health Sciences (Japanese Collection of Research Bioresources (JCRB), Tokyo Japan; Cell number, JCRB0023) and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Tokyo, Japan) in a 5% CO_2 -containing atmosphere. The cells were harvested by incubation in HBSS containing 1 mM ethylenediamine tetraacetate and 0.25% trypsin for 5 min at 37 °C. Cells suspended in supplemented DMEM were plated on 100-mm culture dishes (1×10^7 cells/10 ml) or in 6-well plates (3×10^6 cells/well) and cultured at 37 °C.

2.4. Bone marrow-derived mast cells (BMMCs)

BMMCs were prepared from the femurs of 4–8-week-old C57BL/6 J and C3HeB/FeJ mice as previously described [32]. All animal experiments were performed according to Nihon University guidelines. The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% FBS, 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 5×10^{-5} M β -mercaptoethanol (Wako), 100 µg/ml sodium pyruvate (Invitrogen), 1% minimal essential medium (MEM) non-essential amino acid solution (Invitrogen) and 5 ng/ml of recombinant IL-3 (PeproTech Inc., Rocky Hill, NJ, USA) in a 5% CO_2 -containing atmosphere at 37 °C. After 4–6 weeks of culture, the cells were stained for their cell surface expression of Fc ϵ RI. BMMCs were used for experiments after 4–8 weeks of culture (>95% mast cells).

2.5. HMC-1 cells

HMC-1 cells (a human mast cell line) were cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin in a 5% CO_2 -containing atmosphere as previously described [33].

2.6. Evaluation of cell death

Apoptotic cell death and overall cell death were evaluated by double-staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) as previously described [30]. Briefly, cells adhered to 24-well plates (5×10^5 cells/well) were treated with the inhibitor tested in 10% FBS/DMEM and then stimulated with GA, TNP-BSA, or TG for 18–22 h. Next, phosphatidylserine that translocated from the inner leaflet of the plasma membrane to the outer leaflet was detected by annexin V staining using a commercially available kit (Annexin V FITC Apoptosis Detection Kit I; BD PharMingen, San Diego, CA, USA), according to the manufacturer's instructions. By double-staining the cells with annexin V-FITC and PI, subsets of cells that were annexin V-positive and PI-negative (apoptotic) or annexin V-positive and PI-positive (necrotic and/or cells in advanced apoptosis) were determined. Double-negative cells were considered to be living cells. The stained cells were evaluated in a FACSCalibur and analyzed using the CellQuest software (Becton-Dickinson, San Jose, CA, USA).

2.7. Cell surface expression of RAGE

Cell surface expression of RAGE was determined by flow cytometry as previously described [30]. RBL-2H3 cells or BMMCs (5×10^5 cells/20 μ l) were incubated with anti-RAGE antibody for 30 min at 4 °C. The cells were then centrifuged into a pellet, resuspended in PBS and incubated with FITC-conjugated anti-goat IgG (Santa Cruz Biotechnology) for 30 min at 4 °C. The expression of RAGE on these cells was evaluated in a FACScalibur and analyzed using the CellQuest software.

2.8. Measurement of $[Ca^{2+}]_i$

The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the Ca^{2+} -reactive fluorescent probe Fluo3/AM as previously described [34]. Briefly, a cell suspension (1×10^6 cells/ml) was incubated with 4 μ M Fluo3/AM for 30 min at 37 °C, washed with HBSS, and resuspended in Ca^{2+} -containing medium (HBSS supplemented with 1 mM $CaCl_2$) or Ca^{2+} -free medium (HBSS supplemented with 1 mM ethylene glycol tetraacetic acid (EGTA) instead of 1 mM $CaCl_2$). Fluorescence was monitored at 5-s intervals for up to 3 min using a microplate fluorometer (Fluoroskan Ascent CF; Labsystems, Helsinki, Finland) with excitation and emission at 485 nm and 527 nm, respectively.

2.9. Measurement of $[Ca^{2+}]_m$

The mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) was measured using the mitochondria-localizing Ca^{2+} -reactive fluorescence probe rhod-2/AM as previously described [36]. Briefly, to improve the discrimination between cytosolic and mitochondria-localized dye [35], 5 μ M rhod-2/AM was reduced to the colorless nonfluorescent dihydrorhod-2/AM by sodium borohydride according to the manufacturer's protocol. A cell suspension (1×10^6 cells/ml) was incubated with the dye for 30 min at 37 °C, washed with HBSS and resuspended in HBSS supplemented with 1 mM $CaCl_2$. After stimulation of the cells, fluorescence was monitored at 5-s intervals for up to 3 min using the above described microplate fluorometer with excitation and emission at 544 nm and 590 nm, respectively using 5 μ M A23187 and 5 μ M A23187 plus 10 mM EGTA, respectively, as positive and negative controls.

2.10. Cytochrome c release assay

Cytochrome c release was measured as previously described [30]. Briefly, cells adhered to 6-well plates (3×10^6 cells/well) were washed, treated with the inhibitor tested in 10% FBS/DMEM and stimulated with GA for 18 h. To assess the cytochrome c content in the mitochondria, the cells were fractionated into mitochondrial and cytosolic fractions by differential centrifugation using a mitochondria isolation kit (Pierce, Rockford, IL), and the mitochondrial fraction was lysed with 1% Triton X-100. The cytochrome c content in each fraction was determined using a commercially available a solid-phase sandwich ELISA kit (Cytochrome c, Mouse/Rat, ELISA Kit, Quantikine M[®], R&D Systems) according to the manufacturer's protocol.

2.11. Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$) with JC-1

Changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) were measured using the lipophilic cation JC-1 as described previously [34]. Cells (5×10^5 /500 μ l) were loaded with 2 μ M JC-1 for 15 min in a CO_2 incubator, washed, and resuspended in HBSS. After cell stimulation, the green fluorescence (the monomeric JC-1) and red fluorescence (J-aggregates) were measured using the FL-1 and FL-2 channels, respectively, with a FACScalibur (Bectone-Dickinson).

2.12. Caspase-3/7 activation and mitochondrial membrane potential measurements

Caspase-3/7 activation and mitochondrial membrane potential were evaluated as previously described [30]. Briefly, cells adhered to 24-well plates (5×10^5 cells/well) were treated with the inhibitor tested in 10% FBS/DMEM and then stimulated with GA for 18 h. Caspase-3/7 activation and changes in the mitochondrial membrane potential were determined using a Dual Sensor: MitoCasp[™] (Cell Technology Inc., Mountain View, CA), according to the manufacturer's protocol. Stained cells were evaluated in a FACScalibur and analyzed using the CellQuest software.

2.13. $O_2^{\cdot -}$ production assay

$O_2^{\cdot -}$ production was measured using a SOD-inhibitable cytochrome c reduction assay as previously described [32]. Briefly, RBL-2H3 cells (1×10^6 cells/ml) were stimulated with GA, and 20 μ M cytochrome c was added. The reduction in absorbance at 540 nm (ΔOD_{540}) was measured immediately in a Model 550 Microplate Reader (Nippon Bio-Rad Laboratories, Osaka, Japan). To measure the SOD-dependent responses, SOD was added to the reaction mixture in separate samples at a final concentration of 10 U/ml, and the reduction in absorbance at 540 nm (ΔOD_{540} [SOD]) was measured in the same way. The data are shown as SOD-inhibitable cytochrome c reduction, corresponding to $\Delta OD_{540} - \Delta OD_{540}$ [SOD].

Mitochondrial $O_2^{\cdot -}$ generation was measured using the mitochondria-targeting probe MitoSOX Red as previously described [36]. RBL-2H3 cells (1×10^6 cells/ml) were incubated with 5 μ M MitoSOX Red for 15 min at 37 °C in a final volume of 450 μ l. Then 50 μ l of stimulants ($10\times$) was added and incubated at 37 °C for the time indicated before harvest. Cells were then washed, resuspended in HBSS on ice, centrifuged at 4 °C. After stimulation, the red fluorescence was measured using the FL-2 channel, with a FACScalibur (Becton Dickinson). In inhibition experiments, the agents tested were added to cells just before stimulation.

2.14. RT-PCR

Total RNA was isolated from 1×10^6 cells using ISOGEN (Nippon Gene Corporation, Tokyo, Japan) and reverse transcribed to cDNA using SuperScript[™] II reverse transcriptase (Invitrogen), oligo dT primer (Invitrogen) and 0.5 mM dNTP Mixture (Invitrogen). RAGE and GAPDH mRNA were measured by RT-PCR using a Model PC-802 thermal cycler (Astec Corporation, Fukuoka, Japan). Primers were for RAGE, 5'-CTACCTATTCTGCAGCTTC-3' and 5'-CTGATGTTGACAGGAGGGCTTCC-3'; for GAPDH: 5'-ACCACAGTC-CATGCCATCAC and 5'-TCCACCACCCTGTGCTGTA-3'. The PCR was performed for 30 cycles, each consisting of 1 min at 95 °C for denaturation, 1 min at 60 °C for annealing and 1 min at 72 °C for extension.

2.15. siRNA knockdown of RAGE expression

The target siRNA for RAGE and a negative control siRNA with an irrelevant sequence were purchased from Ambion (Austin, Texas, USA). Adherent cells plated in 6-well plates (2.5×10^5 cells/well) or in 24-well plates (5.0×10^4 cells/well) were transfected for 48 h with RAGE siRNA (final concentration, 5 nM) using siPORT[™] NeoFX transfection agent (Ambion), according to the manufacturer's instruction. The efficacy of knockdown for RAGE gene expression was evaluated using RT-PCR analysis as described above. Transfected cells were then treated with GA, and apoptotic cell death, $[Ca^{2+}]_m$ levels, and $O_2^{\cdot -}$ production were assessed as described above.

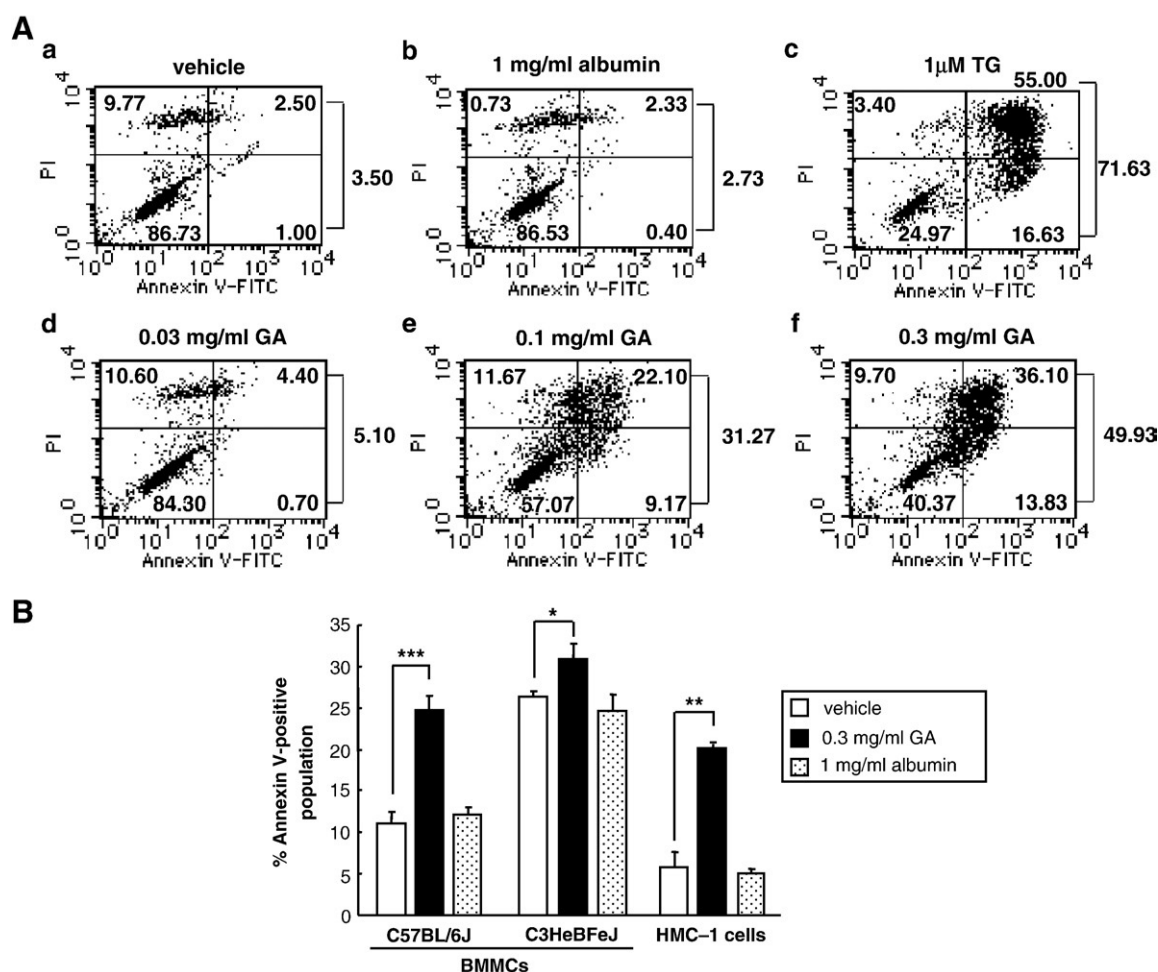


Fig. 1. Mast cells undergo apoptosis upon exposure to GA. (A) RBL-2H3 cells adhered to 24-well plates (5×10^5 cells/200 μ l) were stimulated with GA at the indicated concentrations (d–f), 1 mg/ml albumin (b) or 1 μ M TG (c) for 18 h at 37 $^{\circ}$ C, and then harvested. (B) BMMCs and HMC-1 cells suspended in 24-well plates (5×10^5 cells/200 μ l) were stimulated with 0.3 mg/ml GA or 1 mg/ml albumin for 18 h at 37 $^{\circ}$ C. Cells were then stained with FITC-conjugated annexin V and PI, and analyzed by flow cytometry. Double-negative cells are considered to be living cells. The data are representative of at least four independent experiments.

2.16. Statistical analysis

Student's *t*-test was used to determine the statistical significance of differences among the experimental groups. Values of $p < 0.05$ were considered significant.

3. Results

3.1. GA induces mast cell apoptosis

First, we tested the ability of GA to induce cell death. Adherent RBL-2H3 cells in serum-containing medium were exposed to varying concentrations of GA or 1 μ M TG (positive control) for 18–22 h, and phosphatidylserine exposure and PI permeability were analyzed by flow cytometry. Clear increases in the annexin V- and/or PI-positive cell populations were observed for GA-treated cells compared with vehicle- (Fig. 1A, panel a) or unmodified albumin-treated control cells (Fig. 1A, panel b), indicating that GA can induce cell death by both apoptosis and non-apoptosis/necrosis. GA exhibited this proapoptotic activity in a concentration-dependent manner with a minimum effective concentration of 0.1 mg/ml (Fig. 1A, panels d–f). The percent annexin V-positive populations indicative of apoptotic cells for vehicle- and 0.3 mg/ml GA-treated cells were $4.4 \pm 0.9\%$ and $49.5 \pm 5.2\%$, respectively ($n=7$). To exclude the possibility that the induction of apoptosis was an aberrant special case for tumor mast

cells like RBL-2H3, we tested the effects of GA on BMMC survival. BMMCs prepared from two different strains of mice (C57BL/6 J and C3HeBFeJ) were examined for their susceptibility to apoptosis after treatment with GA for 18 h. Both BMMCs displayed sizable apoptosis,

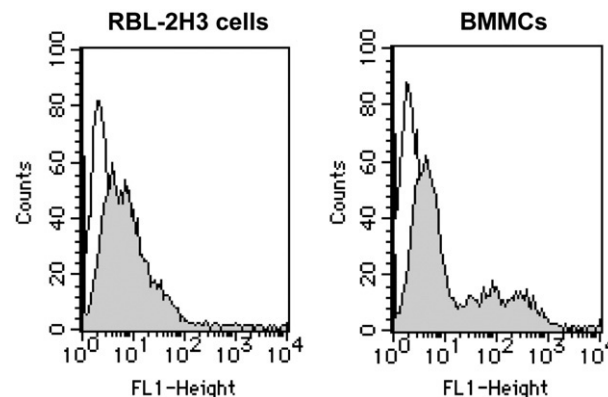


Fig. 2. Mast cells express RAGE on their cell surface. RBL-2H3 cells or BMMCs in PBS (5×10^5 cells/20 μ l) were stained with anti-RAGE antibody, and the expression of each molecule on the cells was evaluated by flow cytometry. The filled histograms represent specific staining and the solid lines represent the non-stained controls. The data are the representative of three independent experiments.

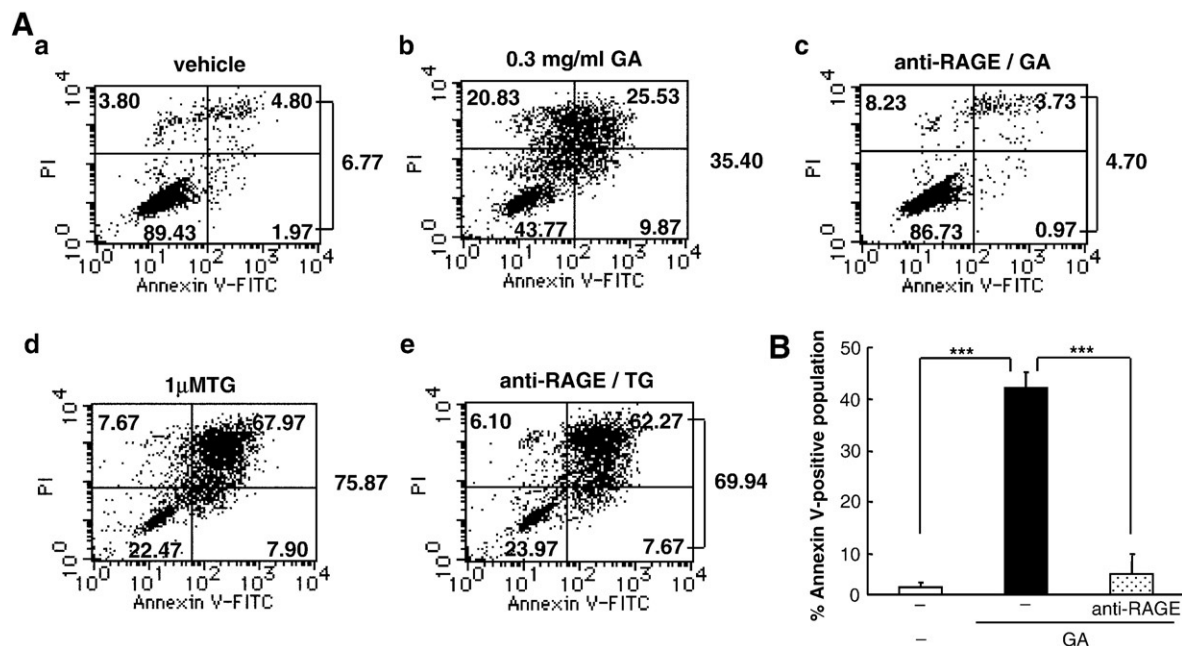


Fig. 3. GA elicits a pro-apoptotic effect through binding to a putative surface receptor via RAGE. (A) RBL-2H3 cells (5×10^5 cells/200 μ l) were incubated with 0.3 mg/ml GA (b, c) or 1 μ M TG (d, e) for 18 h at 37 $^{\circ}$ C in the presence of 1 μ g/ml anti-RAGE antibody (c, e), and cell death was analyzed by annexin V/PI double-staining. (B) Summarized data of the percentages of the annexin V-positive cell populations relative to the where whole cell population set at 100%. The data represent the mean $\pm$ SE of five independent experiments. The statistical significance was determined using an unpaired Student's *t*-test. ****p* < 0.001.

although their sensitivity to GA treatment was apparently different. Furthermore, we obtained essentially similar results with the human mast cell line, HMC-1 (Fig. 1B). On the other hand, unmodified albumin had cytotoxicity at concentrations up to 1 mg/ml. Collectively, these results demonstrate that GA provokes mast cell death by apoptosis.

3.2. Expression of RAGE on mast cells

Since AGEs are a heterogeneous group of glycosylated proteins, the actions of AGEs are mediated by various receptors. RAGE, a member of the immunoglobulin superfamily, is the best-characterized cellular receptor for AGEs [18,19]. Similar to those used in our previous study [30], flow cytometric analyses showed that both the RBL-2H3 cells and the BMMCs used throughout the present study clearly expressed RAGE on their cell surfaces (Fig. 2).

3.3. GA-induced apoptosis is mediated by RAGE

Since RBL-2H3 cells were more susceptible to GA-induced apoptosis than BMMCs and HMC-1 cells, we analyzed the GA-induced apoptosis in more detail using RBL-2H3 cells. We initially elucidated the roles of RAGE in the pro-apoptotic activity of GA by using blocking antibodies against the molecule. The anti-RAGE antibody completely blocked GA-induced apoptosis (Fig. 3A, panel c). On the other hand, this blocking antibody had a minimal effect on TG-induced apoptosis (Fig. 3A, panel e). Fig. 3B shows a statistical analysis of the data for cells treated with 0.3 mg/ml GA for 18 h. Taken together, these data indicate that GA induce mast cell death through binding to putative surface receptor including RAGE.

3.4. GA induces a Ca^{2+} flux and mitochondrial Ca^{2+} overload via RAGE

Since Ca^{2+} is thought to be an important mediator of cell death, we next examined whether GA could affect Ca^{2+} signaling. RBL-2H3 cells preloaded with the Ca^{2+} -sensitive fluorescent dye Fluo3/AM were treated with varying concentrations of GA, and fluorescence

was measured for 3 min using a microplate fluorescence reader. GA induced a concentration-dependent increase in $[\text{Ca}^{2+}]_i$ with a minimum effective concentration of 0.1 mg/ml (Fig. 4A), whereas unmodified albumin had no effect at concentrations up to 1 mg/ml. The amplitude of the increase was comparable to that observed upon stimulation with TNP-BSA (Fc ϵ RI stimulation), although GA, but not TNP-BSA induced substantial cell death (see Fig. 5B). Thus, GA can induce Ca^{2+} mobilization and apoptosis at comparable concentrations. The effect was still observed for cells presensitized with IgE, indicating that this effect is independent of Fc ϵ RI (data not shown). The GA-induced Ca^{2+} flux was substantially reduced but still observed in nominally Ca^{2+} -free medium (in the presence of 1 mM EGTA; Fig. 4B), suggesting that GA stimulates Ca^{2+} mobilization mainly from intracellular stores and to a lesser extent, from the extracellular milieu. Furthermore, the anti-RAGE antibody substantially reduced GA-induced Ca^{2+} flux (Fig. 4C). We also measured mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_m$) using the mitochondrial membrane potential ($\Delta\Psi_m$)-dependent Ca^{2+} -sensitive fluorescent dye rhod-2/AM. GA caused a concentration-dependent increase in $[\text{Ca}^{2+}]_m$, which was observed immediately after stimulation (Fig. 5A). Moreover, even at the minimum effective concentration (0.1 mg/ml), the effect of GA was higher than that of TNP-BSA stimulation, which had a minimal effect on cell survival (Fig. 5B). In addition, blocking antibodies against RAGE blocked GA-induced apoptosis (see Fig. 3), reduced the $[\text{Ca}^{2+}]_m$ increase comparable to that seen after TNP-BSA stimulation (Fig. 5C). These results indicate that GA evokes an extensive $[\text{Ca}^{2+}]_m$ load via RAGE, which may play a role in GA-induced apoptosis.

3.5. GA induces apoptosis in a permeability transition pore (PTP)-dependent manner

We recently reported that exogenous galectin-3 evokes mast cell death through a mitochondrial PTP-dependent pathway [30]. We therefore examined whether GA also utilize similar death pathway. As shown in Fig. 6, the PTP antagonist bongkrekic acid completely blocked GA-induced cell death (Fig. 6A, panel c), whereas the PTP

agonist atryctyloside apparently had minimal effects on the cell death, although it increased the necrotic cell (annexin V- and PI-positive) population and decreased the apoptotic (annexin V-positive but PI-negative) population (Fig. 6A, panel d). In contrast, the two agents had no effect on TG-induced cell death (Fig. 6B). These results indicate that GA, but not TG, induces mast cell death in a PTP-dependent manner.

3.6. GA induces mitochondrial cytochrome c release, caspase-3/7 activation and mitochondrial membrane potential reduction in a PTP-dependent manner

Mitochondria are now recognized to act as central gateway controllers in the cell death pathway through their release of various pro-apoptotic substances, including cytochrome c, into the cytoplasm, which stimulate the caspase-dependent death pathway. Moreover, PTPs are thought to be the principal route of cytochrome c release. We

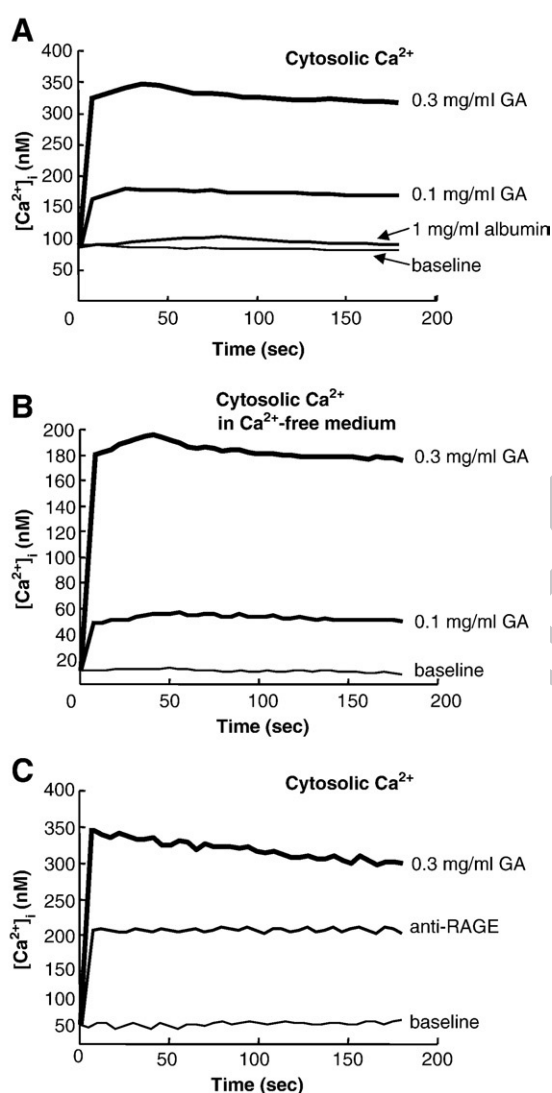


Fig. 4. GA induces a Ca^{2+} influx via RAGE. (A–C) RBL-2H3 cells (1×10^6 cells/ml) were incubated with $4 \mu\text{M}$ fluo3/AM at 37°C for 30 min and then washed with HBSS. The fluo3-loaded cells were suspended in (A) Ca^{2+} -containing medium (HBSS supplemented with 1 mM CaCl_2) or (B) Ca^{2+} -free medium (HBSS supplemented with 1 mM EGTA instead of 1 mM CaCl_2), and stimulated with GA at the indicated concentrations or 1 mg/ml albumin. In (C), the cells suspended in Ca^{2+} -containing medium were treated with $1 \mu\text{g/ml}$ anti-RAGE antibody, and then immediately stimulated with 0.3 mg/ml GA. Fluorescence was monitored for up to 3 min in a microplate fluorometer. The data shown as $[\text{Ca}^{2+}]_i$ (nM) are representative of at least five independent experiments.

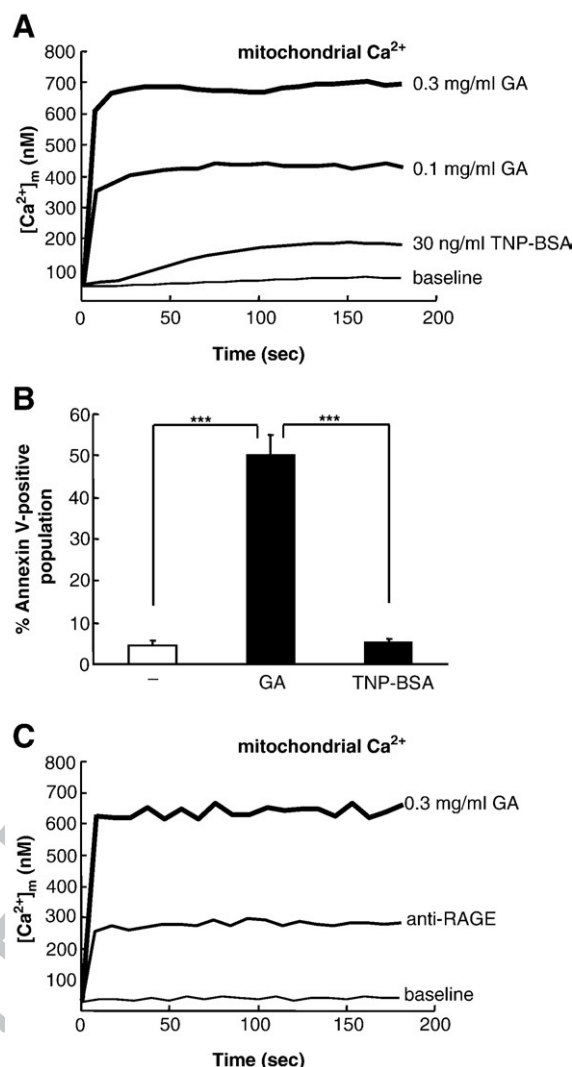


Fig. 5. GA induces mitochondrial Ca^{2+} overload via RAGE. (A, B) RBL-2H3 cells (1×10^6 cells/ml) were incubated with $5 \mu\text{M}$ rhod-2/AM at 37°C for 30 min and then washed with HBSS. The rhod2-loaded cells were stimulated with (A) GA at the indicated concentrations or 30 ng/ml TNP-BSA. Fluorescence was monitored for up to 3 min in a microplate fluorometer. (B) RBL-2H3 cells grown in 24-well plates (5×10^5 cells/200 μl) were incubated with 0.3 mg/ml GA or 30 ng/ml TNP-BSA for 18 h at 37°C , and cell death was analyzed by annexin V/PI double-staining. The data were calculated as the percentages of annexin V-positive cell populations relative to the whole cell population set at 100%, and represent the mean $\pm$ SE of six independent experiments. The statistical significance of differences versus GA stimulation alone was determined using an unpaired Student's t -test. $***p < 0.001$. (C) The rhod2-loaded cells were stimulated with 0.3 mg/ml GA in the presence or absence of $1 \mu\text{g/ml}$ anti-RAGE antibody. Fluorescence was monitored for up to 3 min in a microplate fluorometer. These data shown as $[\text{Ca}^{2+}]_m$ are representative of at least five independent experiments.

therefore determined whether GA affected the mitochondrial integrity. GA caused a substantial increase in the cytosolic cytochrome c content and decrease in the mitochondrial cytochrome c content (Fig. 7A and B), indicating induction of cytochrome c release. This release was significantly reduced by preincubating the cells with antibodies against RAGE. Moreover, bongkreikic acid significantly inhibited GA-induced cytochrome c release, while atryctyloside had a marginal effect (Fig. 7B). Cytochrome c released into the cytosol forms multimeric complexes with apoptotic protease factor-1 and pro-caspase-9 leading to the activation of caspase-9 and downstream caspases such as caspase-3/7, which play critical roles in the apoptotic cascade. In addition, the collapse of $\Delta\Psi_m$ is an important characteristic of cell death. We therefore examined the effects of GA on these events.

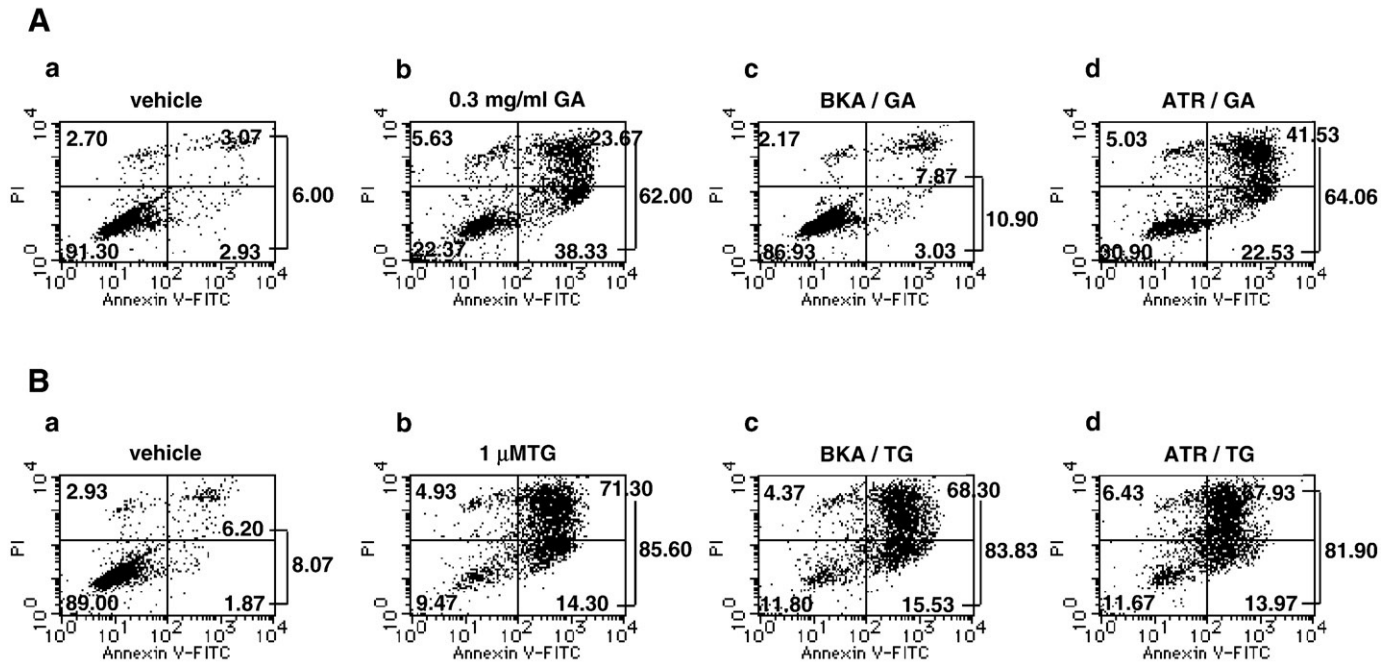


Fig. 6. GA induces apoptosis in a PTP-dependent manner. (A, B) RBL-2H3 cells (5×10^5 cells/200 μ l) were incubated with 0.3 mg/ml GA (A, b–d) or 1 μ M TG (B, b–d) for 18 h at 37 °C in the presence of 1 μ M bongkreikic acid (BKA, c) or 10 μ M atractyloside (ATR, d). Cell death was analyzed by annexin V/PI double-staining. The data are representative of at least three independent experiments.

To evaluate the collapse of $\Delta\Psi_m$ under these conditions, we measured $\Delta\Psi_m$ using JC-1. Treatment with GA and antimycin (the selective inhibitor for complex III of the electron transport chain) caused almost complete loss of $\Delta\Psi_m$ (~20% and >95% decrease, respectively), whereas TNP-BSA and NOR-3 (nitric oxide donor) had no such effect (Fig. 7C). As shown in Fig. 7D, GA but not TNP-BSA, also induced caspase-3/7 activation, and these effects were both blocked by bongkreikic acid and marginally enhanced by atractyloside. These data indicate that GA induces mitochondrial integrity collapse and caspase activation in a PTP-dependent manner.

3.7. GA, but not FcεRI stimulation, stimulates $O_2^{\cdot-}$ release from mitochondria

Reactive oxygen species (ROS) are intimately associated with both apoptotic and non-apoptotic/necrotic cell death. We previously showed that galectin-3-induced mast cell apoptosis was preceded by production and release of $O_2^{\cdot-}$ into the extracellular fluid [30]. We therefore examined whether GA induced $O_2^{\cdot-}$ production in mast cells. As shown in Fig. 8A, GA induced substantial $O_2^{\cdot-}$ release into the extracellular fluid, as evidenced by the SOD-inhibitable reduction of cytochrome c. This $O_2^{\cdot-}$ release was seen immediately, reached its maximum within a few minutes, and remained at that level for up to 5 min. The kinetics and the amplitude were similar to those observed upon TNP-BSA stimulation (Fig. 8B). We recently demonstrated that both a NADPH oxidase and mitochondria were involved in ROS production in mast cells and their proinflammatory responses [36]. Therefore, we next tested the roles of NADPH oxidase and mitochondria in GA-induced $O_2^{\cdot-}$ release. Apocynin acts as a NADPH oxidase-specific inhibitor, since it blocks the assembly of the membrane-associated flavocytochrome gp91^{phox} with regulatory subunit p47^{phox} and p67^{phox} [37,38]. Apocynin marginally reduced GA-induced $O_2^{\cdot-}$ release (Fig. 8A and C), whereas it substantially inhibited TNP-BSA-induced $O_2^{\cdot-}$ release (Fig. 8B and D). On the other hand, bongkreikic acid strongly reduced GA-induced $O_2^{\cdot-}$ release (Fig. 8A and C), whereas it had a minimal effect on TNP-BSA-induced $O_2^{\cdot-}$ release (Fig. 8B and D). These results suggested that GA, but not TNP-BSA stimulation, induced

$O_2^{\cdot-}$ release in a PTP-dependent manner. To examine this further, we directly measured mitochondrial $O_2^{\cdot-}$ levels using MitoSOX, a fluoroprobe for selective detection of $O_2^{\cdot-}$ in the mitochondria [39,40]. As shown in Fig. 9A and B, substantial increases in fluorescence intensity of MitoSOX were reproducibly observed after TNP-BSA stimulation, whereas the MitoSOX signal was usually decreased after GA exposure. Moreover, we found that pretreatment of the cells with BKA abolished GA-induced decrease in the MitoSOX signal, and apocynin had a lesser effect (Fig. 9A). On the other hand, BKA exhibited a minimal effect on TNP-BSA-induced increase in MitoSOX signal (Fig. 9B). Collectively, these results demonstrate that GA, but not TNP-BSA stimulation, stimulates $O_2^{\cdot-}$ release from the mitochondria.

3.8. Extracellular $O_2^{\cdot-}$ mediates GA-induced apoptosis and mitochondrial Ca^{2+} dysregulation

We previously showed that exogenous SOD, a cell-impermeable $O_2^{\cdot-}$ -scavenging enzyme completely protected mast cells from galectin-3-induced apoptosis, which was blocked by anti-RAGE antibody [30]. These observations suggest that $O_2^{\cdot-}$ plays key roles in RAGE-mediated cell death. To clarify the roles further, we examined the effects of exogenous SOD on GA-induced cell death. As shown in Fig. 10A, exogenous SOD completely blocked GA-induced cell death. Moreover, SOD significantly suppressed all of the GA-induced cytochrome c release, caspase-3/7 activation, and decrease in $\Delta\Psi_m$ (Fig. 10B and C). In addition, SOD reduced GA-induced $[Ca^{2+}]_m$ leading to a level near to that observed upon TNP-BSA stimulation (Fig. 10D). Taken together, these data indicate that extracellular $O_2^{\cdot-}$ mediates GA-induced mitochondrial Ca^{2+} dysregulation and cell death.

3.9. RAGE mediates GA-induced mitochondrial death pathway

To demonstrate the role of RAGE in GA-induced mast cell apoptosis more directly, we examined the effects of knocking down RAGE expression on the apoptosis. As shown in Fig. 11A, by using siRNA we succeeded in knocking down the expression of RAGE

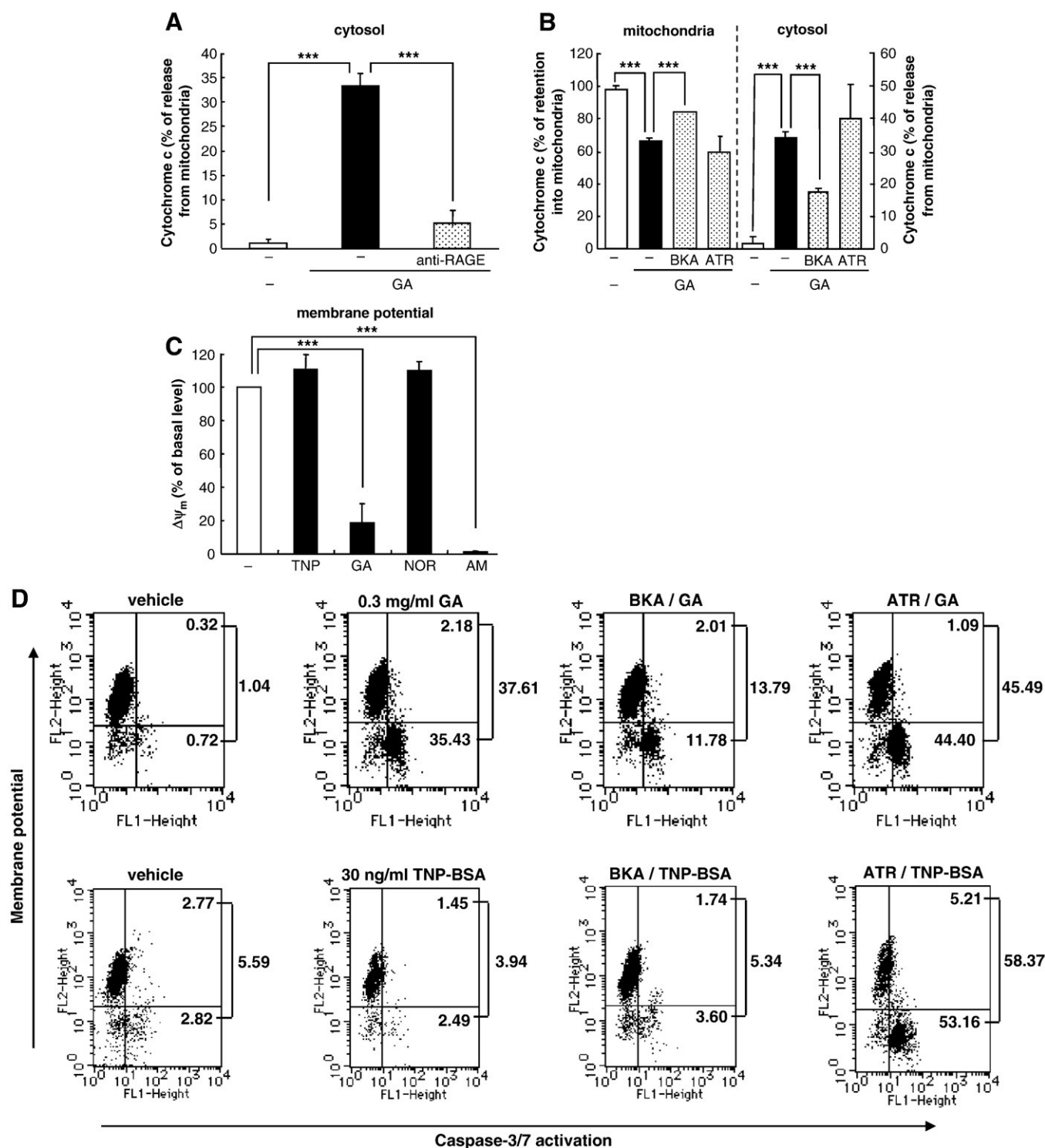


Fig. 7. GA induces disruption of mitochondrial integrity and caspase activation in a PTP-dependent manner. (A, B) RBL-2H3 cells (3×10^6 cells/ml) were (A) stimulated with 0.3 mg/ml GA for 18 h at 37 °C in the presence or absence of 1 μ M anti-RAGE antibody or (B) treated with 1 μ M bongkreik acid (BKA) or 10 μ M atractyloside (ATR) and then immediately stimulated with 0.3 mg/ml GA. The cells were fractionated into mitochondrial and cytosolic fractions by differential centrifugation, and the cytochrome c content in each fraction was determined using an ELISA kit. The data represent the mean $\pm$ SE of five independent experiments. The statistical significance of differences versus GA stimulation in each fraction was determined using an unpaired Student's *t*-test. ** $p < 0.05$; *** $p < 0.001$. (C) RBL-2H3 cells (5×10^5 cells/500 μ l) were loaded with JC-1, and the JC-1-loaded cells suspended in Ca^{2+} -containing medium were treated with 30 ng/ml TNP-BSA, 0.3 mg/ml GA, 100 μ M NOR-3 (NOR), or 5 μ M antimycin A (AM) for 18 h. The green and red dye fluorescence was then measured by flow cytometry. The data are shown as percentages of the basal level and represent the mean $\pm$ SE of three independent experiments. The statistical significance of differences versus untreated control was determined using an unpaired Student's *t*-test. *** $p < 0.001$. (D) RBL-2H3 cells (4×10^5 cells/well) were stimulated with 0.3 mg/ml GA or 30 ng/ml TNP-BSA at 37 °C in the presence or absence of 1 μ M BKA or 10 μ M ATR. Subsequently, caspase-3/7 activation and changes in the mitochondrial membrane potential were determined by flow cytometry. The data are representative of at least three independent experiments.

selectively. Unlike control cells, the RAGE knocked down cells did not undergo apoptosis in response to GA (Fig. 11B). Moreover, we observed that in the RAGE knocked down cells GA had minimal

effects on $O_2^{\cdot -}$ production and $[Ca^{2+}]_m$ overload (Fig. 11C and D). These data clearly demonstrate that RAGE mediates GA-induced mitochondrial death pathway.

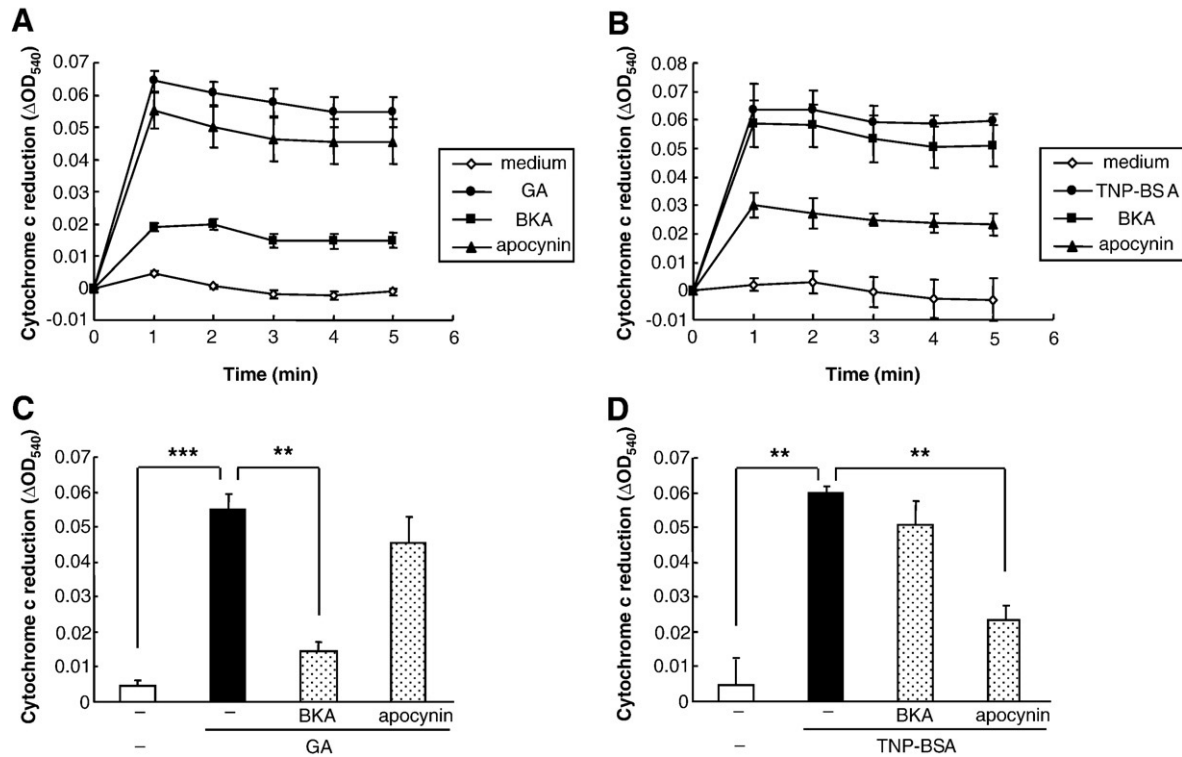


Fig. 8. GA, but not TNP-BSA stimulation, induces $O_2^{\cdot -}$ release in a PTP-dependent manner. (A, B) RBL-2H3 cells (1×10^6 cells/ml) were stimulated with (A) 0.3 mg/ml GA or (B) 30 ng/ml TNP-BSA in the absence or presence of 1 μ M BKA or 0.3 mM apocynin, and $O_2^{\cdot -}$ generation was measured by monitoring cytochrome c reduction (ΔOD_{540}) using a microplate reader. (C, D) Statistical analysis data of the ΔOD_{540} following 5 min of exposure to (C) GA or (D) TNP-BSA. The data represent the mean $\pm$ SE of six independent experiments. The statistical significance versus GA stimulation in each fraction was determined using an unpaired Student's *t*-test. ***p* < 0.05; ****p* < 0.001.

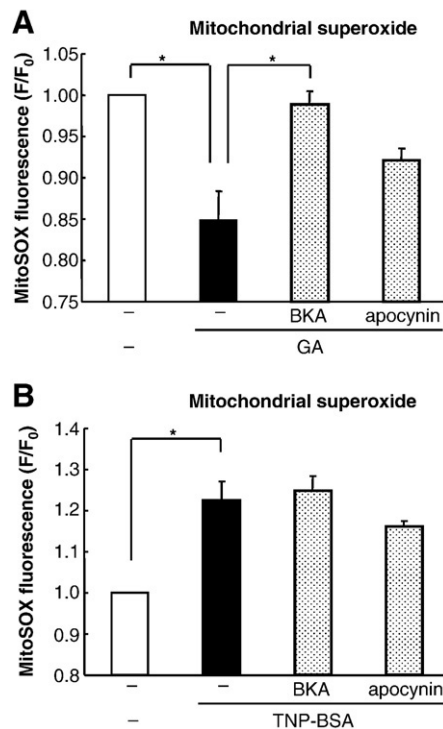


Fig. 9. GA induces $O_2^{\cdot -}$ release from the mitochondria. RBL-2H3 cells (1×10^6 /ml) suspended in HBSS were incubated with 5 μ M MitoSOX Red for 15 min at 37 °C. Then the cells were stimulated with (A) 0.3 mg/ml GA or (B) 30 ng/ml TNP-BSA in the absence or presence of 1 μ M BKA or 0.3 mM apocynin at 37 °C and allowed to stand on ice immediately. After centrifugation at 4 °C, the cells were analyzed for MitoSOX signal in a flow cytometer. The data are shown as F/F_0 , where F_0 represents fluorescence in unstimulated cells and F is fluorescence in stimulated cells, and represent mean $\pm$ SE of three independent experiments. The statistical significance versus GA stimulation in each fraction was determined using an unpaired Student's *t*-test. **p* < 0.01.

4. Discussion

In the current study, we demonstrated that GA, an AGE provokes mast cell death by apoptosis and that RAGE, an AGE-binding protein mediates the pro-apoptotic activity. These findings are consistent with the view that RAGE mediates AGE toxicity [8–13]. Moreover, we demonstrated that activation of RAGE causes the mitochondrial death pathway in which $[Ca^{2+}]_m$ overload and release of mitochondrial $O_2^{\cdot -}$ may play critical roles. ROS have also been found to play roles in the pathogenesis of diabetic complications, and normalization of mitochondrial ROS has been shown to prevent glucose-induced metabolic abnormalities [41,42]. A number of studies have suggested that ROS generation plays a role in AGE-induced apoptosis [41–45]. However, the conclusions of most of these studies were solely based on findings that various nonspecific antioxidants attenuate apoptosis without direct measurement of ROS. As a result, the oxidant species involved and their sources remain unidentified. On the other hand, a few investigators have directly shown that AGE signaling leads to ROS production, via mitochondria and NADPH oxidase [25,45,46]. Unfortunately however, these reports have provided no evidence that the ROS examined did indeed mediate AGE toxicity. Our results clearly show that GA-induced mast cell apoptosis was associated with elevated $O_2^{\cdot -}$ generation in mitochondria and release into the extracellular fluid. Furthermore, the cell-impermeable $O_2^{\cdot -}$ -selective scavenging enzyme SOD completely blocked the apoptosis, indicating that extracellular $O_2^{\cdot -}$ is a mediator of the pro-apoptotic activity of GA. Our data are in accordance with an early report indicating that activation of RAGE induces elevated $O_2^{\cdot -}$ generation in the mitochondria [45]. Another important finding in the current study is that GA, but not TNP-BSA stimulation, induces $O_2^{\cdot -}$ release from mitochondria into the extracellular fluid, although both stimuli evoke $O_2^{\cdot -}$ generation in the mitochondria. Given that GA, but not TNP-BSA stimulation, induce substantial apoptosis, this finding suggests that extracellular $O_2^{\cdot -}$ release from mitochondria rather

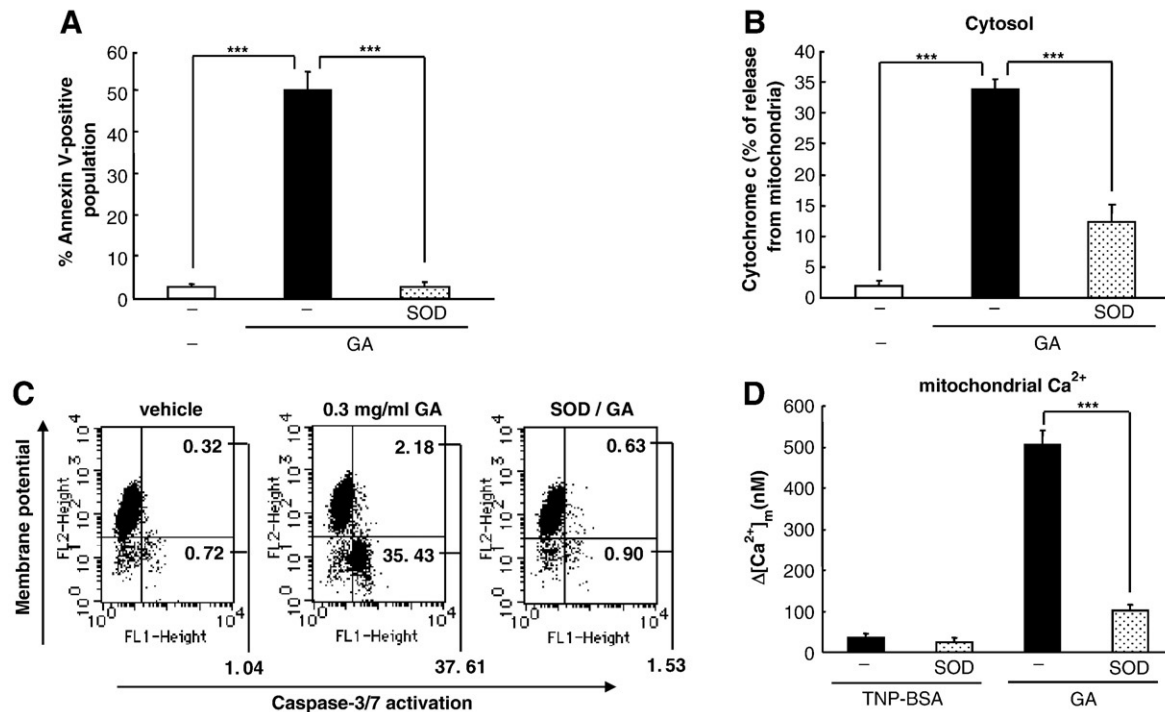


Fig. 10. Extracellular O_2^- mediates GA-induced mast cell apoptosis. (A) RBL-2H3 cells grown in 24-well plates (5×10^5 cells/200 μ l) were incubated with 0.3 mg/ml GA in the absence or presence of 10 U/ml SOD for 18 h at 37 °C, and cell death was analyzed by annexin V/PI double-staining. The data were calculated as the percentages of annexin V-positive cell populations relative to the whole cell population set at 100%, and represent the mean $\pm$ SE of at least three independent experiments. (B) RBL-2H3 cells (3×10^6 cells/ml) were stimulated with 0.3 mg/ml GA in the absence or presence of 10 U/ml SOD for 18 h at 37 °C. The cytochrome c contents in the cytosolic fractions were determined using an ELISA kit as described in the legend to Fig. 7. The data represent the mean $\pm$ SE of five independent experiments. (C) RBL-2H3 cells (3×10^6 cells/ml) were stimulated with 0.3 mg/ml GA at 37 °C in the presence or absence of 10 U/ml SOD for 18 h at 37 °C. Next, caspase-3/7 activation and changes in the mitochondrial membrane potential were determined by flow cytometry. The data are representative of five independent experiments. (D) Rhod2-loaded cells (1×10^6 cells/ml) were treated with 10 U/ml SOD, and immediately stimulated with 0.3 mg/ml GA or 30 ng/ml TNP-BSA. Fluorescence was monitored for up to 3 min in a microplate fluorometer. The data are shown as the increase in $[Ca^{2+}]_m$ ($\Delta[Ca^{2+}]_m$) and represent the mean $\pm$ SE of three independent experiments. The statistical significance of differences versus GA stimulation alone was determined using an unpaired Student's *t*-test. ****p* < 0.001.

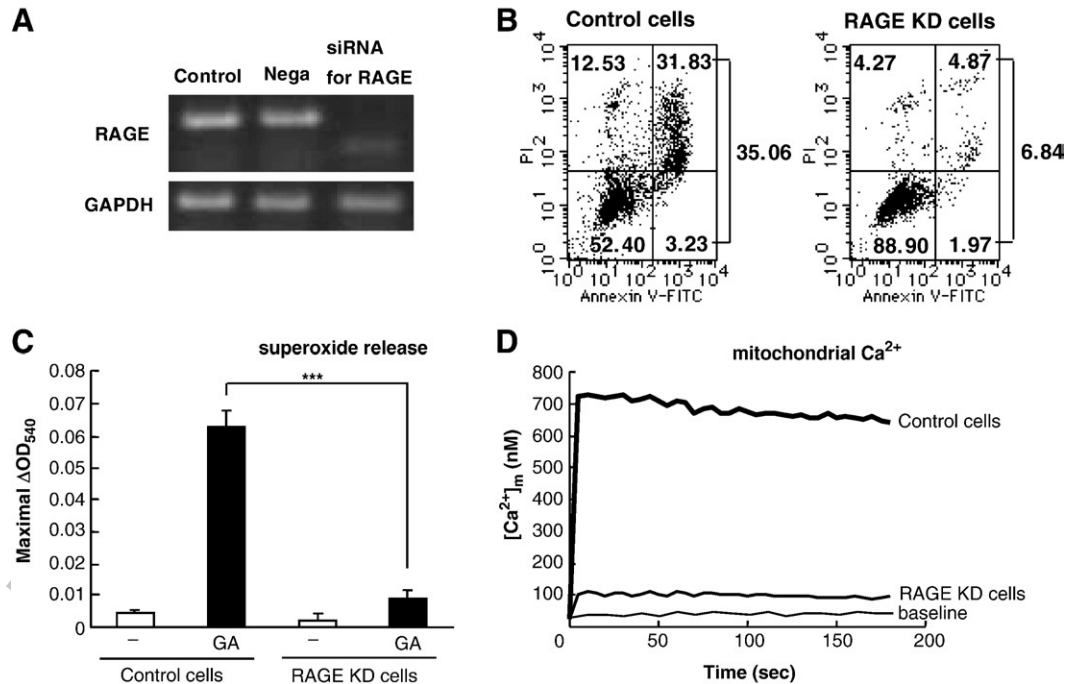


Fig. 11. RAGE mediates GA-induced mitochondrial death pathway. (A) RBL-2H3 cells in 6-well plates (2.5×10^5 cells/well) or in 24-well plates (5.0×10^4 cells/well) were transfected with siRNA for RAGE. As a negative control siRNA (Nega), a nontargeting scrambled siRNA with no sequence homology to any known gene sequences was used. The expression of RAGE mRNA was measured using RT-PCR at 48 h after the transfection of siRNA. (B) Adherent RAGE knocked down (RAGE-KD) cells (which had been cultured for 48 h after siRNA transfection in 24-well plates) or control cells were stimulated with 0.3 mg/ml GA for 18 h, and cell death was analyzed by annexin V/PI double-staining. (C) RAGE knocked down cells or control cells (1×10^6 cells/ml) were stimulated with 0.3 mg/ml GA, and O_2^- generation was measured by monitoring cytochrome c reduction (ΔOD_{540}) using a microplate reader. Statistical analysis data of the maximal ΔOD_{540} following exposure to GA were shown. The data represent the mean $\pm$ SE of three independent experiments. The statistical significance of differences was determined using an unpaired Student's *t*-test. ****p* < 0.001. (D) Rhod2-loaded RAGE knocked down cells or control cells (1×10^6 cells/ml) were stimulated with 0.3 mg/ml GA. Fluorescence was monitored for up to 3 min in a microplate fluorometer. These above data are representative of three independent experiments.

than mitochondrial $O_2^{\cdot -}$ generation, is important for induction of apoptosis. Since this $O_2^{\cdot -}$ release was completely blocked by bongrekic acid, $O_2^{\cdot -}$ seemed to be released via PTPs most likely voltage-dependent anion channel.

Although oxidative stress has been proposed to play a role in AGE-induced apoptosis, the mechanisms by which this stress mediates the apoptosis are unclear. We demonstrated that extracellular $O_2^{\cdot -}$ mediates mitochondrial Ca^{2+} dysregulation, another biochemical change associated with the induction of apoptosis in a number of cell types [47–49]. Accumulating evidence indicates that, under oxidative circumstances, mitochondrial Ca^{2+} accumulation can switch from a physiological beneficial process to a cell death signal. It has been thought that maintenance of the mitochondrial redox status and/or mitochondria Ca^{2+} homeostasis are essential for cell survival by preventing the release of pro-apoptotic substances, such as cytochrome c, from the mitochondria [47]. Opening of PTPs triggers the release of pro-apoptotic proteins, including cytochrome c, from the mitochondrial inter-membrane space into the cytoplasm, which activates the caspase-dependent death pathway. Our data clearly demonstrate that opening of PTPs is crucial for GA-induced mast cell apoptosis and the upstream biochemical events, including mitochondrial cytochrome c release, caspase-3/7 activation, and mitochondrial membrane potential reduction. Banki et al. [50] demonstrated that mitochondrial hyperpolarization precedes the collapse the mitochondrial membrane potential. Given that GA is much more effective than TNP-BSA in inducing apoptosis, GA is expected to evoke a higher mitochondrial hyperpolarization, thereby causing the collapse of the $\Delta\Psi_m$, resulting in the caspase-dependent execution of cell death. To date unlike TNP-BSA stimulation, although we observed no reproducible mitochondrial hyperpolarization after GA treatment for 18 h by flow cytometry, it is possible that the effect is observed earlier. We are currently investigating the effect of GA on the $\Delta\Psi_m$ under different conditions. Collectively, our data suggest that extracellular $O_2^{\cdot -}$ mediates GA-induced mitochondrial Ca^{2+} dysregulation, thereby provoking the mitochondrial death pathway. Further studies to clarify the mechanisms by which extracellular $O_2^{\cdot -}$ affects mitochondrial Ca^{2+} homeostasis are underway.

In conclusion, we have presented evidence that mast cells are cellular targets of AGE toxicity and that mitochondria-derived extracellular $O_2^{\cdot -}$ mediates the pro-apoptotic activity via mitochondrial Ca^{2+} dysregulation. These pieces of evidence support the view that AGE may modulate mast cell survival. Given that mast cells act as conductors of allergic and inflammatory reactions and play roles in innate and acquired immunity, AGE-induced mast cell death may be relevant to the altered immunological and inflammatory responses.

Acknowledgements

We thank the National Institute of Health Sciences (Japanese Collection of Research Bioresources) for providing the RBL-2H3 cells (Cell number, JCRB0023). This work was partially supported by a Grant-in-Aid “High-Tech Research Center” Project (2002–2006) for Private Universities matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

- [1] V. Monnier, A. Cerami, Nonenzymatic browning in vivo: possible process for aging of long-lived proteins, *Science* 211 (1981) 491–493.
- [2] R. Paul, A. Bailey, Glycation of collagen: the basis of its central role in the late complications of ageing and diabetes, *Int. J. Biochem. Cell Biol.* 28 (1996) 1297–1310.
- [3] J. Onorato, A. Jenkins, S. Thorpe, J. Baynes, Pyridoxamine an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine, *J. Biol. Chem.* 275 (2000) 21177–21184.
- [4] D. Sell, V. Monnier, Structure elucidation of a senescence cross-link from human extracellular matrix. Implication of pentoses in the aging process, *J. Biol. Chem.* 264 (1989) 21597–21602.
- [5] D. Dayer, J. Blackledge, S. Thorpe, J. Baynes, Formation of pentosidine during nonenzymatic browning of proteins by glucose. Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo, *J. Biol. Chem.* 266 (1991) 11654–11660.
- [6] H. Vlassara, M.R. Palace, Diabetes and advanced glycation endproducts, *J. Intern. Med.* 251 (2002) 87–101.
- [7] S. Schiekofer, M. Andrassy, J. Chen, G. Rudofsky, J. Schneider, T. Wendt, N. Stefan, P. Humpert, A. Fritsche, M. Stumvoll, E. Schleicher, H.U. Häring, P.P. Nawroth, A. Bierhaus, Acute hyperglycemia causes intracellular formation of CNL and activation of rat, p42/44 MAPK, and nuclear factor kappaB in PBMC, *Diabetes* 52 (2003) 621–633.
- [8] C. Min, E. Kang, S. Yu, S. Shinn, Y. Kim, Advanced glycation end products induce apoptosis and procoagulant activity in cultured human umbilical vein endothelial cells, *Diabetes Res. Clin. Pract.* 46 (1999) 197–202.
- [9] M. Lecomte, U. Denis, D. Ruggiero, M. Largarde, N. Wiernsperger, Involvement of caspase-10 in advanced glycation end-product-induced apoptosis of bovine retinal pericytes in culture, *Biochim. Biophys. Acta* 1689 (2004) 202–211.
- [10] H. Sekido, T. Suzuki, T. Jomori, M. Takeuchi, C. Yabe-Nishimura, S. Yagihishi, Reduced cell replication and induction of apoptosis by advanced glycation end products in rat Schwann cells, *Biochem. Biophys. Res. Commun.* 320 (2004) 241–248.
- [11] Z. Alikhani, M. Alikhani, C. Boyd, K. Nagao, P. Trackman, T. Graves, Advanced glycation endproducts enhance expression of pro-apoptotic genes and stimulate fibroblast apoptosis through cytoplasmic and mitochondrial pathways, *J. Biol. Chem.* 280 (2005) 12087–12095.
- [12] R.A. Kowluru, Effect of advanced glycation end products on accelerated apoptosis of retinal capillary cells under in vitro conditions, *Life Sci.* 76 (2005) 1051–1060.
- [13] C.S. Rosenberg, Wound healing in the patient with diabetes mellitus, *Nurs. Clin. North Am.* 25 (1990) 247–261.
- [14] H. Vlassara, The AGE-receptor in the pathogenesis of diabetic complications, *Diabetes Metab. Res. Rev.* 17 (2001) 436–443.
- [15] T. Wendt, N. Tanji, J. Guo, B.I. Hudson, A. Bierhaus, R. Ramasamy, B. Arnold, P.P. Nawroth, S.F. Yan, V.D. Agati, A.M. Schmidt, Glucose, glycation, and RAGE: implications for amplification and induction of cellular dysfunction in diabetic nephropathy, *J. Am. Soc. Nephrol.* 14 (2003) 1383–1395.
- [16] H. Vlassara, Protein glycation in the kidney: role in diabetes and aging, *Kidney Int.* 49 (1996) 1785–1804.
- [17] S. Horiuchi, T. Higashi, K. Ikeda, T. Saishoji, Y. Jinnouchi, H. Sano, R. Shibayama, T. Sakamoto, N. Araki, Advanced glycation end products and their recognition by macrophage and macrophage-derived cells, *Diabetes* 45 (1996) S73–76.
- [18] M. Nepper, A.M. Schmidt, J. Brett, S.D. Yan, F. Wang, Y.C. Pan, K. Elliston, D. Stern, A. Shaw, Cloning and expression of a cell surface receptor for advanced glycation end products of proteins, *J. Biol. Chem.* 267 (1992) 14998–15004.
- [19] A.M. Schmidt, S.D. Yan, S.F. Yan, D.M. Stern, The biology of the receptor for advanced glycation end products and its ligands, *Biochim. Biophys. Acta* 1498 (2000) 99–111.
- [20] H.M. Lander, J.M. Tauras, J.S. Ogiste, O. Hori, R.A. Moss, A.M. Schmidt, Activation of the receptor for advanced glycation end products triggers a p21 (ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress, *J. Biol. Chem.* 272 (1997) 17810–17814.
- [21] C. Lu, J.C. He, W. Cai, H. Liu, L. Zhu, H. Vlassara, Advanced glycation endproduct (AGE) receptor 1 is a negative regulator of the inflammatory response to AGE in mesangial cells, *Proc. Natl. Acad. Sci. USA* 101 (2004) 11767–11772.
- [22] G. Pugliese, F. Pricci, C. Iacobini, G. Leto, L. Amadio, P. Barsotti, L. Frigeri, D.K. Hsu, H. Vlassara, F.T. Liu, U.D. Mario, Accelerated diabetic glomerulopathy in galectin-3/AGE receptor 3 knockout mice, *FASEB J.* 15 (2001) 2471–2479.
- [23] A. Miyazaki, H. Nakyama, S. Horiuchi, Scavenger receptors that recognize advanced glycation end products, *Trends Cardiovasc. Med.* 12 (2002) 258–262.
- [24] J.G. Larkin, B.M. Frier, J.T. Ireland, Diabetes mellitus and infection, *Postgrad. Med. J.* 61 (1985) 233–237.
- [25] M. Delamare, D. Maudgandre, M. Moreno, M.C. Le Goff, H. Allannic, B. Genetet, Impaired leucocyte functions in diabetic patients, *Diabet. Med.* 14 (1997) 29–34.
- [26] Y. Ding, A. Kantarci, H. Hasturk, P.C. Trackman, A. Malabanan, T.E. Van Dyke, Activation of RAGE induces elevated $O_2^{\cdot -}$ generation by mononuclear phagocytes in diabetes, *J. Leukoc. Biol.* 81 (2007) 520–527.
- [27] K.S. Collison, R.S. Parhar, S.S. Saleh, B.F. Meyer, A.A. Kwaasi, M.M. Hammami, A.M. Schmidt, D.M. Stern, F.A. Al-Mohanna, RAGE-mediated neutrophil dysfunction is evoked by advanced glycation end products (AGEs), *J. Leukoc. Biol.* 71 (2002) 433–444.
- [28] S.J. Galli, M. Maurer, C.S. Lantz, Mast cells as sentinels of innate immunity, *Curr. Opin. Immunol.* 11 (1999) 53–59.
- [29] B.A. Sayed, A. Christy, M.R. Quirion, M.A. Brown, The master switch: the role of mast cells in autoimmunity and tolerance, *Annu. Rev. Immunol.* 26 (2008) 705–739.
- [30] Y. Suzuki, T. Inoue, T. Yoshimaru, C. Ra, Galectin-3 but not galectin-1 induces mast cell death by oxidative stress and mitochondrial permeability transition, *Biochim. Biophys. Acta* (2008) 924–934.
- [31] S. Nishida, M. Terashima, N. Tamiya, Amino acid sequences of phospholipases A_2 from the venom of *Pseudechis australis*, *Toxicon* 23 (1985) 87–104.
- [32] Y. Suzuki, T. Yoshimaru, T. Matsui, T. Inoue, O. Niide, S. Nunomura, C. Ra, FcεRI signaling of mast cells activates intracellular production of hydrogen peroxide: role in the regulation of calcium signals, *J. Immunol.* 171 (2002) 6119–6127.
- [33] J.H. Butterfield, D. Weiler, G. Deward, G.J. Gleich, Establishment of an immature mast cell line from a patient with mast cell leukemia, *Leuk. Res.* 12 (1998) 345–355.

- [34] Y. Suzuki, T. Yoshimaru, T. Inoue, C. Ra, Mitochondrial Ca^{2+} flux is a critical determinant of the Ca^{2+} dependence of mast cell degranulation, *J. Leukoc. Biol.* 79 (2006) 508–518.
- [35] G. Hajnoczky, L.D. Robb-Gaspers, M.B. Seitz, A.P. Thomas, Decoding of cytosolic calcium oscillations in the mitochondria, *Cell* 82 (1995) 415–424.
- [36] T. Inoue, Y. Suzuki, T. Yoshimaru, C. Ra, Reactive oxygen species produced up- or downstream of calcium influx regulate proinflammatory mediator release from mast cells: Role of NADPH oxidase and mitochondria, *Biochim. Biophys. Acta* (2008) 789–802.
- [37] J. Stolk, T.J. Hiltermann, J.H. Dijkman, A.J. Verhoeven, Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol, *Am. J. Respir. Cell Mol. Biol.* 11 (1994) 95–102.
- [38] H.C. Williams, K.K. Griendling, NADPH oxidase inhibitors: new antihypertensive agents? *J. Cardiovasc. Pharmacol.* 50 (2007) 9–16.
- [39] K.M. Robinson, M.S. Janes, M. Pehar, J.S. Monette, M.F. Ross, T.M. Hagen, M.P. Murphy, J.S. Beckman, Selective fluorescent imaging of superoxide in vivo using ethidium-based probes, *Proc. Natl. Acad. Sci. USA* 103 (2006) 15038–15043.
- [40] P. Mukhopadhyay, M. Rajesh, K. Yoshihiro, G. Haskó, P. Pacher, Simple quantitative detection of mitochondrial superoxide production in live cells, *Biochem. Biophys. Res. Commun.* 358 (2007) 203–208.
- [41] X. Du, K. Stotcklauser-Farber, P. Rosen, Generation of reactive oxygen intermediates, activation of NF- κ B, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? *Free Radic. Biol. Med.* 27 (1999) 752–763.
- [42] M. Brownlee, The pathobiology of diabetic complications: a unifying mechanism, *Diabetes* 54 (2005) 1615–1625.
- [43] T. Nishikawa, D. Edelstein, M. Brownlee, The missing link: a single unifying mechanism for diabetic complications, *Kidney Int.* 77 (2000) S26–S30.
- [44] T. Kislinger, C. Fu, B. Huber, W. Qu, A. Taguchi, S. Du Yan, M. Hofmann, S.F. Yan, M. Pischetsrieder, D. Stern, A.M. Schmidt, A. M. Ne-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression, *J. Biol. Chem.* 274 (1999) 31740–31749.
- [45] A. Schmitt, K. Bigl, I. Meiners, J. Schmitt, Induction of reactive oxygen species and cell survival in the presence of advanced glycation end products and similar structures, *Biochim. Biophys. Acta* 1763 (2006) 927–936.
- [46] A. San Martin, R. Foncea, F.R. Laurindo, R. Ebensperger, K.K. Griendling, F. Leighton, Nox1-based NADPH oxidase-derived superoxide is required for VSMC activation by advanced glycation end-products, *Free Radic. Biol. Med.* 42 (2007) 1671–1679.
- [47] D.R. Green, J.C. Reed, Mitochondria and apoptosis, *Science* 281 (1998) 1309–1312.
- [48] L.P. Zhu, X.D. Yu, S. Ling, R.A. Brown, T.H. Kuo, Mitochondrial Ca^{2+} homeostasis in the regulation of apoptotic and necrotic cell deaths, *Cell Calc.* 28 (2000) 107–117.
- [49] S. Orrenius, V. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, *Annu. Rev. Pharmacol. Toxicol.* 47 (2007) 143–183.
- [50] K. Banki, E. Hutter, N.J. Gonchoroff, A. Perl, Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling, *J. Immunol.* 162 (1999) 1466–1479.