Glycolaldehyde-derived advanced glycation end products promote macrophage proliferation via the JAK-STAT signaling pathway

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Abstract

Background: Advanced glycation end products (AGEs) are heterogeneous proinflammatory molecules produced by a non-enzymatic glycation reaction between reducing sugars (and their metabolites) and biomolecules with amino groups, such as proteins. Although increases in and the accumulation of AGEs have been implicated in the onset and exacerbation of lifestyle- or age-related diseases, including diabetes, their pathophysiological functions have not yet been elucidated in detail.

Methods and Results: The present study investigated the cellular responses of the macrophage cell line RAW264.7 stimulated by glycolaldehyde-derived AGEs (Glycol-AGEs) known as representative toxic AGEs. The results obtained showed that Glycol-AGEs significantly promoted the proliferation of RAW264.7 cells at a low concentration range (1-10 μg/mL) in a concentration-dependent manner. On the other hand, neither TNF-α production nor cytotoxicity were induced by the same concentrations of Glycol-AGEs. The increases observed in cell proliferation by low concentrations of Glycol-AGEs were also detected in receptor triple knockout (RAGE-TLR4-TLR2 KO) cells as well as in wild-type cells. Increases in cell proliferation were not affected by various kinase inhibitors, including MAP kinase inhibitors, but were significantly suppressed by JAK2 and STAT5 inhibitors. In addition, the expression of some cell cycle-related genes was up-regulated by the stimulation with Glycol-AGEs.

Conclusions: These results suggest a novel physiological role for AGEs in the promotion of cell proliferation via the JAK-STAT pathway.

1. Introduction

Diabetes mellitus is a chronic metabolic disorder that is characterized by persistent hyperglycemia due to poor insulin production or resistance. Persistent hyperglycemia causes systemic microangiopathy, resulting in serious diabetic complications, including diabetic nephropathy, neuropathy, and retinopathy, which markedly deteriorate the quality of life of patients [1]. Therefore, the control of blood glucose levels to the optimum with therapeutic drugs is very important in the treatment of diabetes [2]. However, the molecular mechanisms of pathogenesis by persistent hyperglycemia during the progression of diabetes have not yet been elucidated in detail.

Advanced glycation end products (AGEs) are heterogeneous proinflammatory molecules produced by the non-enzymatic glycation reaction of reducing sugars (or their metabolites) to various biomolecules, including proteins. During the progression of diabetes, persistent hyperglycemia promotes the increased production and accumulation of AGEs, which correlate with the severity of diabetes [3, 4]. Among the various subspecies of AGEs, glycolaldehyde-derived AGEs (Glycol-AGEs), which are produced by glycation with glycolaldehyde (a glucose metabolite), have been reported to exhibit more potent inflammatory activity, leading to oxidative stress in the kidneys or the accumulation of foam cells, which suggests their involvement in the pathogenesis of many lifestyle-related diseases, including diabetic nephropathy [5] and atherosclerosis [6]. However, the pathological consequences and physiological functions of the

accumulation of Glycol-AGEs remain unclear. On the other hand, an immunohistochemical analysis using specific antibodies against some subspecies of AGEs revealed that many AGEs-modified proteins were generally present in several normal peripheral tissues, suggesting the possible physiological roles for AGEs, such as post-translational modulators [7].

The present study investigated the effects of Glycol-AGEs on the cellular responses of the macrophage cell line, RAW264.7. Glycol-AGEs significantly promoted cell proliferation at low concentrations, which were incapable of inducing proinflammatory TNF-α production or cytotoxicity. The signaling pathways involved in this phenomenon were examined using kinase inhibitors and receptor knockout (KO) cells, and the physiological/pathophysiological significance of the results obtained was discussed.

2. Materials And Methods

2.1. Materials

The mouse macrophage cell line RAW264.7 is a product from ATCC (Rockville, MD, USA). The CellTiter 96® non-radioactive cell proliferation assay kit was purchased from Promega (Madison, WI, USA). The lactate dehydrogenase (LDH) cytotoxicity assay kit was from Nacalai Tesque (Kyoto, Japan). InSolution™ p38 MAP Kinase Inhibitor III (ML3403), ERK Inhibitor II (FR180204), and InSolution™ JNK Inhibitor II (SP600125) were from Merck Millipore (Burlington, MA, USA). Solcitinib, NSC42834, FM-381, deucravacitinib, fludarabine, cryptotanshinone, pimozole, and AS1517499 were from Selleck Biotech (Tokyo, Japan).

2.2. Preparation of Glycol-AGEs

Glycol-AGEs were prepared as previously described [8]. Bovine serum albumin (25 mg/mL) was incubated with 100 mM glycolaldehyde in 200 mM sodium phosphate buffer (pH 7.4) at 37ºC for 7 days under sterile conditions. The reaction mixture was dialyzed against phosphate-buffered saline at 4ºC for 2 days and used as Glycol-AGEs. The amount of endotoxin in the solution of Glycol-AGEs was confirmed to be less than 1 endotoxin unit (EU)/mL by ToxinSensor Gel Clot Endotoxin Assay single test kits from GenScript (Piscataway, NJ, USA)

2.3. Cell culture

RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Biosera, Nuaillé, France), 100 units/mL penicillin, and 100 µg/mL streptomycin (Nacalai Tesque, Kyoto, Japan), and maintained at 37ºC under 5% CO2. Cells were harvested with Accutase™ (Nacalai Tesque, Kyoto, Japan), and then cultured in 96-well microplates (Thermo Fischer Scientific, Waltham, MA, USA) for each assay.

2.4. Cell proliferation assay

After being stimulated with Glycol-AGEs (0, 0.1, 1, 10, and 100 µg/mL), cell proliferation activities were assessed using the MTT assay according to the manufacturer's protocol. Cells were seeded on 96-well

tissue culture plates at a density of 500 cells/well and incubated for 48, 72, 96, or 120 h with or without a stimulant and/or inhibitor at 37ºC under 5% CO2. Untreated cells served as the control group. After a stimulation for the indicated time, MTT solution was added to each well and mixed. Cells were incubated at 37ºC for 4 h under 5% CO2. Solubilization/Stop solution was added to each well after removal of the medium. After shaking the plate for 30 sec, the absorbance of the mixture was measured at 570 nm using the Varioskan LUX Multimode Microplate Reader (Thermo Scientific, Rockford, IL, USA), and optical density values were corrected by subtraction from those at 630 nm.

2.5. ELISA assay of TNF-α secretion

Cells were seeded on 96-well plates at 5×104 cells per well and cultured for 18 h with or without Glycol-AGEs (0, 1, 10, and 100 μg/mL) or lipopolysaccharide (LPS, 100 ng/mL) at 37ºC under 5% CO2. The concentration of TNF-α in the supernatant collected was measured by the BD OptEIA ELISA kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The optical density of each sample at 450 nm was measured using the Varioskan LUX Multimode Microplate Reader, and optical density values were corrected by subtraction from those at 570 nm.

2.6. Cytotoxicity assay

The cytotoxic activity of Glycol-AGEs was assessed by measuring the activity of LDH released from damaged cells according to the manufacturer's protocol. Cells were seeded at 500 cells per well on 96-well plates and cultured for 96 h with or without Glycol-AGEs at 37ºC under 5% CO2. After the stimulation, the supernatant from each well was transferred to new 96-well plates. Substrate solution was added to each well and mixed. The mixture was incubated for 20 min under darkness at room temperature and Stop solution was then added. Absorbance values at 490 nm were measured using the Varioskan LUX Multimode Microplate Reader.

2.7. Preparation of a triple receptor KO RAW264.7 clone (RAGE-TLR4-TLR2 KO clone) using the CRISPR/Cas9 approach

A triple receptor KO clone was established using the CRISPR/Cas9 method as previously described [9]. In brief, RAW264.7 cells were electroporated with the Cas9 protein (Dharmacon-Horizon Discovery, Cambridge, UK) and guide RNA using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA, USA). Guide RNAs were obtained by fusing predesigned CRISPR RNAs targeting RAGE, TLR4, or TLR2 and transactivating CRISPR RNA (Dharmacon-Horizon Discovery, Cambridge, UK) according to the manufacturer's protocol. Electroporated cells were cloned by limiting dilutions, and the genomic DNA sequences of the clones were verified by DNA sequencing (Eurofins Genomics, Tokyo, Japan). Clones with frameshift mutations in the genes coding RAGE, TLR4, and TLR2 were selected as KO clones. A triple receptor KO clone (RAGE-TLR4-TLR2 KO clone) was generated using a similar approach. A Western blot analysis confirmed that the clone obtained did not express any of the three receptors.

2.8. RT-PCR assay

Total RNA was isolated from treated cells using the total RNA isolation kit. The concentration and purity of extracted total RNA were assessed using the 260/280 nm absorbance ratio. cDNAs were prepared from 200 ng of total RNA using the PrimeScript™ RT reagent kit with a gDNA Eraser (TaKaRa Bio, Shiga, Japan). Real-time PCR was performed using the Thunderbird qPCR mix (TOYOBO, Osaka, Japan). Analyses were performed using Thermal Cycler Dice Real-Time System Lite (Takara Bio, Shiga, Japan).The forward and reverse primers used for this purpose were as follows:

Gapdh: Forward primer: TGTGTCCGTCGTGGATCTGA,

Gapdh: Reverse primer: TTGCTGTTGAAGTCGCAGGAG;

CycD1: Forward primer: CATGACCAGTGTGACTCAAAGCAA,

CycD1: Reverse primer: CTCAGACATGGCCCTAAACCTTC;

Cdk4: Forward primer: ACATGTGGAGCGTTGGCTGTA,

Cdk4: Reverse primer: CAACTGGTCGGCTTCAGAGTTTC;

Cdk6: Forward primer: CACGGTGCAGACATCAGGGTA,

Cdk6: Reverse primer: TGACCCGCTGAACCCAAAG;

Plk1: Forward primer: AGAGACCTACCTCCGGATCAAGAA,

Plk1: Reverse primer: CATTGAGCAACTCGTGAATGGTG;

Cdc25c: Forward primer: CCTTGGATTCATCTGGACCTCTG,

Cdc25c: Reverse primer: ATGTGCTACGCTTTGCAATCTTC;

CycB: Forward primer: CATGCTGGACTACGACATGGTG,

CycB: Reverse primer: ACATTCTTAGCCAGGTGCTGCATA.

PCR conditions were as follows: denaturation at 95ºC for 5 s and annealing and extension at 60ºC for 30 s (40 cycles). The transcript level of each gene was normalized to the expression level of GAPDH using the ddCt method.

2.9. Statistical analysis

Statistical analyses across multiple groups were performed using the Student's t-test, Tukey-Kramer method, or Dunnett's test. All data were analyzed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). P-values < 0.05 were considered to be significant. All data are presented as the mean ± SE of three independent experiments.

3. Results

3.1. Promotion of cell proliferation by Glycol-AGEs in macrophage-like RAW264.7 cells

The proliferation of RAW264.7 cells was significantly promoted by the stimulation with Glycol-AGEs in a concentration-dependent manner (Fig. 1A). The stimulation with 10 μg/mL for 96 h resulted in the greatest increase in proliferation, which was 3- to 5-fold greater than that in the non-stimulated control. Therefore, subsequent experiments were performed under the stimulation at 10 μg/mL for 96 h as the standard protocol. Figure 1B shows microscopic images of formazan deposited before the solubilization treatment in the MTT cell proliferation assay, with an increase being observed in the deposition of formazan in according to the stimulation with Glycol-AGEs.

RAW264.7 cells were stimulated with various concentrations of Glycol-AGEs for 96 hours, and the amounts of TNF-α produced in the culture supernatant were measured by ELISA. The results obtained showed that Glycol-AGEs at concentrations of 0–10 μg/mL did not induce the production of TNF-α, while 100 μg/mL only slightly induced its production. On the other hand, LPS as a positive control resulted in the stronger induction of TNF-α production at 0.1 μg/mL (Fig. 2A). In addition, when the cytotoxic activity of Glycol-AGEs was examined, cytotoxicity was not observed in the range of 0-100 μg/mL (Fig. 2B). These results confirmed that Glycol-AGEs significantly promoted cell proliferation at low concentrations, which were not capable of inducing TNF-α production or cytotoxicity.

The promotion of cell proliferation by Glycol-AGEs was then examined using RAW264.7 cells with the KO of three types of receptors (RAGE-TLR4-TLR2) using the CRISPR/Cas9 method. When cells were stimulated with Glycol-AGEs at 0-100 μg/mL for 96 h, no significant differences were observed in cell proliferation between the triple receptor KO clone and wild-type clone (Fig. 2C), indicating that the promotion of cell proliferation by Glycol-AGEs occurred through a mechanism that was independent of these three receptor pathways.

3.2. Involvement of the Janus kinase (JAK)/STAT pathway in the promotion of cell proliferation by Glycol-AGEs

In mammals, there are three MAP kinase families: p38/SAPK, ERK, and JNK, which are activated in the intracellular protein kinase cascade for the regulation of various cell functions, such as proliferation, differentiation, and cell death [10]. The involvement of intracellular MAP kinase in the promotion of cell proliferation by Glycol-AGEs was investigated using an inhibitor of each MAP kinase, and none of the MAP kinase inhibitors examined induced significant changes (Fig. 3).

JAK is a family of non-receptor tyrosine kinases that transduce cytokine-mediated signals via the JAK-STAT pathway. JAK kinase has been reported to function as a regulator of immune responses in macrophages and is also an essential protein phosphorylation enzyme for the intracellular signal transduction of proliferation, differentiation, and apoptosis [11]. Since four types of isoforms (JAK1,

JAK2, JAK3, and TYK2) have been identified as JAK kinases, a specific inhibitor was used to examine the contribution of each JAK kinase to the Glycol-AGE-induced promotion of cell proliferation (Fig. 4A). The promotion of cell proliferation by Glycol-AGEs was significantly inhibited by the selective JAK2 inhibitor NSC42834, with a 38% reduction being observed at 30 μM. Other selective inhibitors for JAK1, JAK3, and TYK2 had no effect.

The STAT family are intracellular transcription factors that mediate many aspects of cell-mediated immunity, proliferation, apoptosis, and differentiation [12]. STAT are mainly linked to and activated by JAK kinase. Dysregulation of the JAK-STAT system leads to tumorigenesis [13], resulting in secondary angiogenesis that enhances tumor survival and immunosuppression [14, 15]. Gene KO studies provided extensive evidence to show that the STAT protein is important for the development and function of the immune system and plays an essential role in maintaining immune tolerance and tumor surveillance [12].

Since JAK2 kinase selectively phosphorylates four types of STAT proteins, namely, STAT1, STAT3, STAT5, and STAT6 [16], the effects of selective STAT inhibitors were examined to elucidate the role of the STAT protein in the Glycol-AGE-induced promotion of cell proliferation. The results obtained showed that only pimozole, a selective inhibitor of STAT5, exerted significant inhibitory effects, inducing a reduction of 36% at 3 μM (Fig. 4B). Neither NSC42834 nor pimozole exerted any effect when added alone at the concentrations used in the present study. These results confirmed that the JAK2-STAT5 signaling pathway plays an essential role in the Glycol-AGE-induced promotion of cell proliferation.

3.3. Changes in cell cycle-related gene expression by the Glycol-AGE stimulation

Cell proliferation is closely associated with the expression of various factors that control the cell cycle [17]. It was expected that the expression levels of cell cycle-related genes, such as cyclins and cyclin-dependent kinases (CDKs), may be altered by a stimulation with Glycol-AGEs. Therefore, we investigated changes in the expression of cell cycle-related genes in response to a stimulation with Glycol-AGEs using the RT-PCR method. As shown in Fig. 5, the gene expression levels of the Cyclin D1-Cdk4-Cdk6 group [18] and Plk1-Cdc25c-Cyclin B group [19] were significantly higher following a stimulation with 10–100 μg/mL Glycol-AGEs than in the non-stimulated control. The rates of increases in gene expression levels were approximately 2-fold in the Cyclin D1-Cdk4-Cdk6 group or more than 3-fold in the Plk1-Cdc25c-Cyclin B group.

4. Discussion

Many research groups have reported that AGEs induce proinflammatory cytokine production through the stimulation of RAGE receptors, resulting in inflammatory diseases [3]. These signaling cascades are regarded as factors that exacerbate various lifestyle- and age-related diseases, particularly diabetic complications [20]. However, the pathological consequences and physiological functions of AGEs have not yet been characterized in detail due to their structural heterogeneity and the existence of various receptors other than RAGE.

Among AGEs, Glycol-AGEs have been reported to suppress mitochondrial functions in Achilles tendon-derived fibroblasts [21] and induce apoptosis in osteoblasts by endoplasmic reticulum stress [22]. Furthermore, the intraperitoneal administration of Glycol-AGEs to rats induced the functional and structural remodeling of cardiomyocytes, resulting in cardiac dysfunction [23].

In the present study, Glycol-AGEs, which are representative toxic AGEs, promoted the proliferation of RAW264.7 cells via the JAK-STAT pathway. This effect was biphasic, showing a concentration-dependent increase at a low concentration range of 0–10 μg/mL and attenuation at 100 μg/mL. TNF-α was produced in cells exposed to 100 μg/mL Glycol-AGEs, which induced an inflammatory state via the autocrine effect. Although TNF-α induces cell death, including apoptosis [24], the LDH release assay revealed no cytotoxicity by Glycol-AGEs at 100 μg/mL. These results suggest that no relationship exists between TNF-α production and the attenuation of cell proliferation by a stimulation with Glycol-AGEs at 100 μg/mL. A previous study reported that Tob1, a modulator of the expression and activities of cell cycle proteins, such as CDKs, inhibited the proliferation of hepatocytes under normal conditions; however, its expression was down-regulated by hepatectomy and returned to its original level after cell regeneration, resulting in the biphasic regulation of cell proliferation by a change in Tob1 expression [25]. Therefore, although the biphasic effects of the promotion of macrophage proliferation in this study may be regulated by an unknown modulator, such as Tob1, further studies are warranted to clarify this issue. Since AGEs are constantly present in healthy tissues that do not show a significant inflammatory response, the promotion of macrophage proliferation by Glycol-AGEs may play a physiological role in maintaining or boosting innate immune responses or may function as a presymptomatic risk factor for inflammatory diseases, such as hyperplasia and fibrosis.

We previously established triple receptor KO RAW264.7 cells lacking RAGE, TLR4, and TLR2 to examine AGEs-induced inflammatory responses. These triple KO cells did not produce TNF-α, even when stimulated with glyceraldehyde-derived AGEs at 500 μg/mL [9]. To elucidate the involvement of inflammatory-related receptors (RAGE-TLR4-TLR2) in the Glycol-AGEs-induced promotion of cell proliferation, the proliferation of KO cells stimulated with Glycol-AGEs was compared with that of wild-type cells. The obtained results showed that Glycol-AGEs promoted the proliferation of KO and wild-type cells to the same extent, and indicated that cell proliferation was induced by Glycol-AGEs mediated by a receptor pathway other than RAGE, TLR4, and TLR2. A number of non-inflammatory AGEs receptors, such as CD36, FEEL, LOX-1, SR-A, and SR-BI, have been identified; however, they were shown to function as scavenger receptors for AGEs but not as the cell proliferative receptors [26, 27, 28, 29]. The receptors responsible for Glycol-AGEs-induced cell proliferation remain unknown, and the significance of the present results has yet to be established.

We found that the JAK2/STAT5 signaling pathway was important in the Glycol-AGEs-induced promotion of cell proliferation. Additionally, its proliferative effects were accompanied by the up-regulated expression of some cell-cycle genes, which plateaued at 10 μg/mL. The JAK2/STAT5 signaling pathway was previously shown to contribute to the production of GM-CSF in microglia [30] and MCP-1 in the monocytic cell line U937 [31] in their activated states. Furthermore, it has been associated with

erythropoietin receptors, prolactin receptors [32, 33], and some cytokine receptors for IL-2, 3, and 5 [34, 35, 36], suggesting its involvement in the proliferation of some cells, including fibroblasts and immune cells. Therefore, JAK/STAT appears to play an important role in the Glycol-AGEs-induced promotion of cell proliferation.

The present results revealed that Glycol-AGEs promoted the proliferation of macrophages via the JAK2/STAT5 pathway at concentrations that were incapable of inducing the production of TNF-α. These results suggest that Glycol-AGEs in vivo contribute not only to the exacerbation of pathological conditions by inducing excessive inflammatory responses, but also to the maintenance and activation of innate immunity, including macrophage proliferation. Furthermore, these findings may present a novel physiological potential of glycation as the post-transcriptional modifications. The significance of the Glycol-AGEs-induced promotion of cell proliferation remains unclear and, thus, further studies are warranted.

Declarations

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Figures

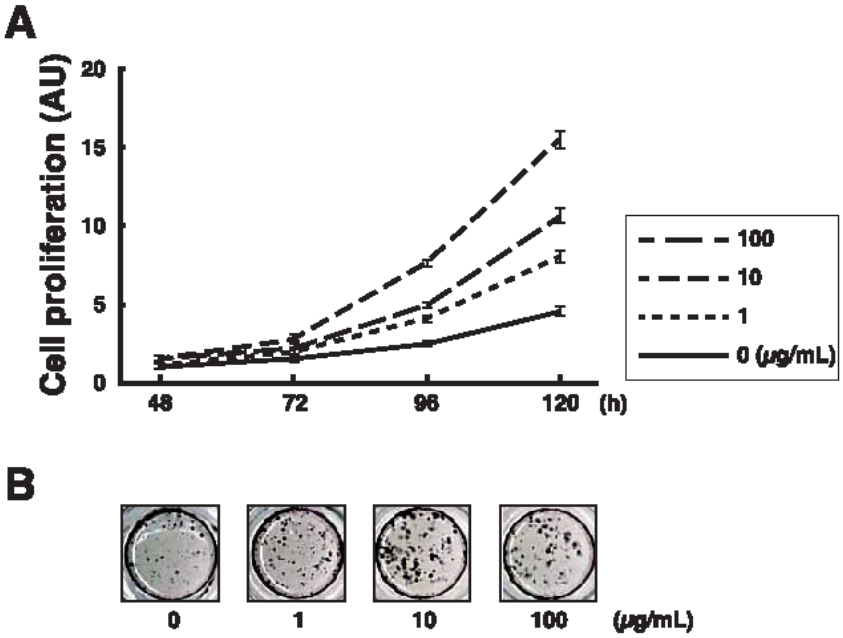

Figure 1

Glycol-AGEs promote the proliferation of RAW 264.7 cells. (A) RAW264.7 cells were cultured for 48, 72, 96, and 120 h with Glycol-AGEs (0, 1, 10, and 100 μg/mL) in 96-well multiplates. After an incubation for the indicated time, cell growth was measured by the MTT colorimetric assay. The ratio of cell proliferation was presented as arbitrary units (AU) relative to the value of unstimulated cells at 48 h. Data represent the mean ± SE of three independent experiments. (B) After being stimulated by Glycol-AGEs at the

indicated concentration for 96 h, cells were incubated in MTT assay solution for 4 h. Representative microscopic images of formazan crystals in each well were photographed.

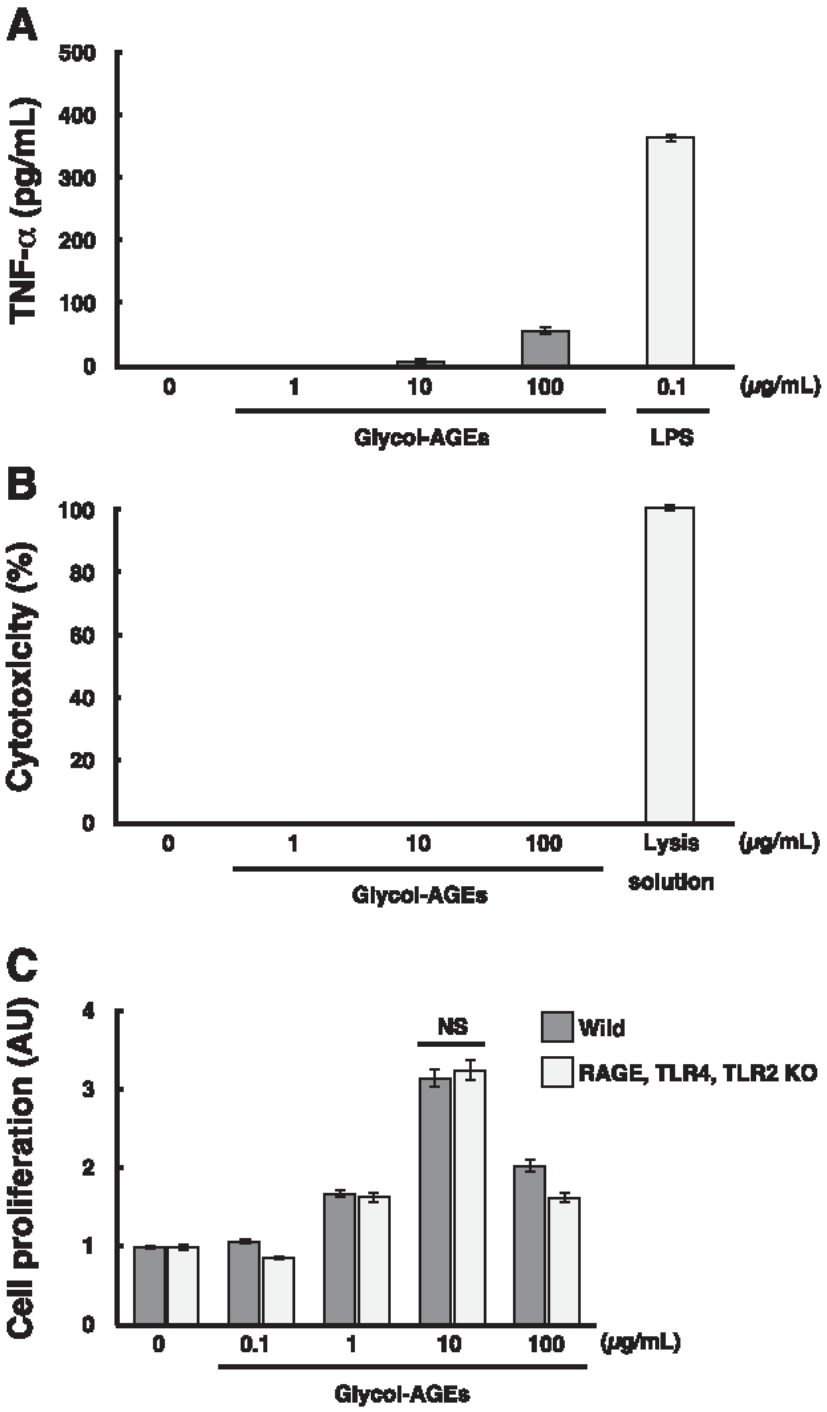

Figure 2

The inflammatory response induced by Glycol-AGEs is independent of cell proliferation. (A) RAW264.7 cells were cultured for 96 h with Glycol-AGEs (0, 1, 10, and 100 μg/mL) in 96-well multiplates. The

amount of TNF-α produced in the culture medium was assayed by ELISA according to manufacturer's protocol. LPS (0.1 μg/mL) was used as a positive control. (B) Supernatants were transferred to new 96-well multiplates, and the substrate solution was added to measure the activity of LDH released from damaged cells. Absorbance at OD490 was measured by a photometer, following by mixing with stop solution. Cytotoxicity was calculated according to the manufacturer's protocol. Data represent the mean ± SE of three independent experiments. (C) Triple receptor knockout RAW264.7 cells lacking three types of receptors (RAGE-TLR4-TLR2) were cultured for 96 h with Glycol-AGEs (0, 0.1, 1, 10, and 100 μg/mL) in 96-well multiplates. After the incubation, cell proliferation was measured using the MTT colorimetric assay. The ratio of cell proliferation was presented as arbitrary units (AU), relative to the value of unstimulated cells. A similar experiment was performed with wild-type RAW264.7 cells. Data represent the mean ± SE of three independent experiments. NS indicates no significant difference by the Student's *t*-test.

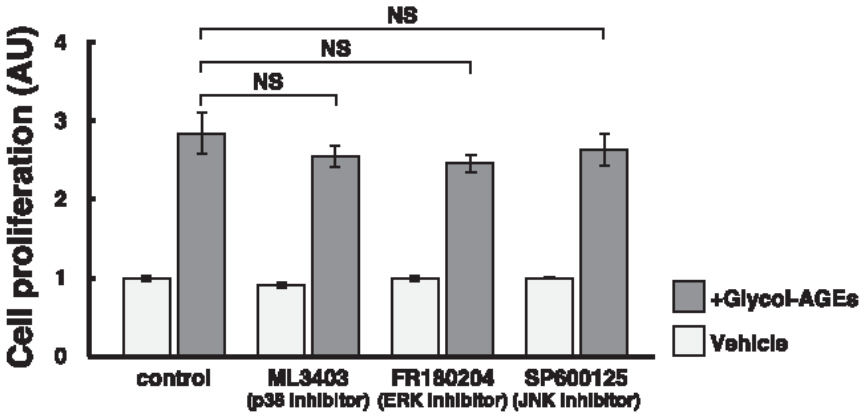

Figure 3

Effects of MAP kinase inhibitors on Glycol-AGEs-induced cell proliferation. RAW264.7 cells were cultured with or without MAP kinase inhibitors for 96 h in the presence of 10 μg/mL Glycol-AGEs in 96-well multiplates. The concentrations used were 400 nM ML3403 (IC50=380 nM for p38), 500 nM FR180204 (IC50=510 nM for ERK1, 330 nM for ERK2), and 100 nM SP600125 (IC50=40 nM for JNK1 and JNK2, 90 nM for JNK3). After the incubation, cell proliferation was measured using the MTT colorimetric assay.

The ratio of cell proliferation was presented as arbitrary units (AU) relative to the values of unstimulated cells without MAP kinase inhibitors. Data represent the mean ± SE of three independent experiments. NS indicates no significant difference by the Tukey-Kramer method.

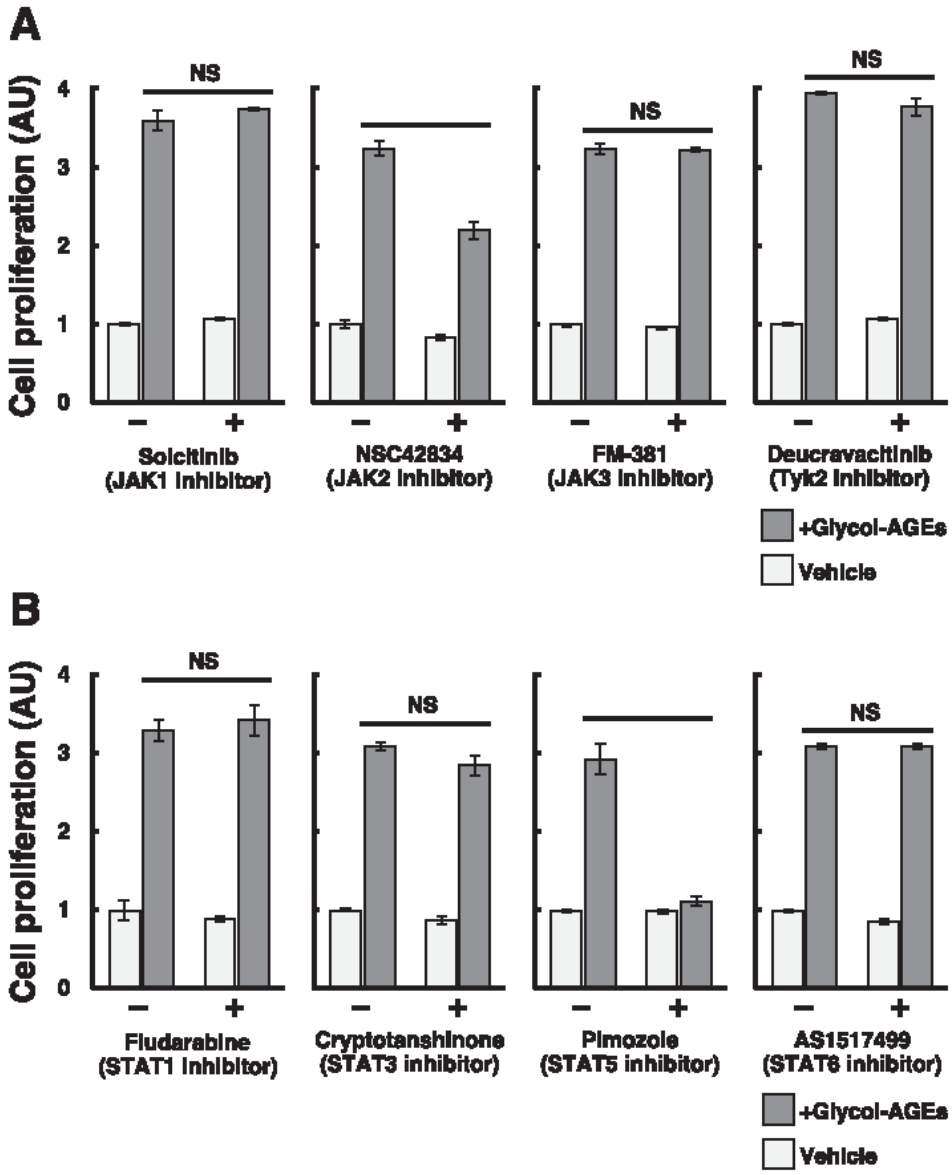

Figure 4

Involvement of the JAK2-STAT5 pathway in the promotion of cell proliferation by Glycol-AGEs. RAW264.7 cells were cultured with or without JAK/STAT inhibitors for 96 h in the presence of 10 μg/mL Glycol-AGEs in 96-well multiplates. A) Cells were treated with the indicated inhibitors for JAK1, JAK2, JAK3, and Tyk2, the concentrations of which were 30 nM solcitinib (IC50=10 nM for JAK1), 30 μM NSC42834 (IC50=15 μM for JAK2), 300 pM FM-381 (IC50=128 pM for JAK3), and 3 nM deucravacitinib (IC50=1 nM for Tyk2), respectively. B) Cells were treated with the indicated inhibitors for STAT1, STAT3, STAT5, and STAT6, the concentrations of which were 30 μM fludarabine (IC50=30 μM for STAT1), 5 μM cryptotanshinone (IC50=4.6 μM for STAT3), 3 μM pimozole (IC50=5 μM for STAT5), and 30 nM AS1517499 (IC50=21 nM for STAT6), respectively. After the incubation, cell proliferation was measured using the MTT colorimetric assay. The ratio of cell proliferation was presented as arbitrary units (AU) relative to the value of unstimulated cells without each inhibitor. Data represent the mean ± SE of three independent experiments. **, $p<0.001$ by the Student's t-test. NS: not significant

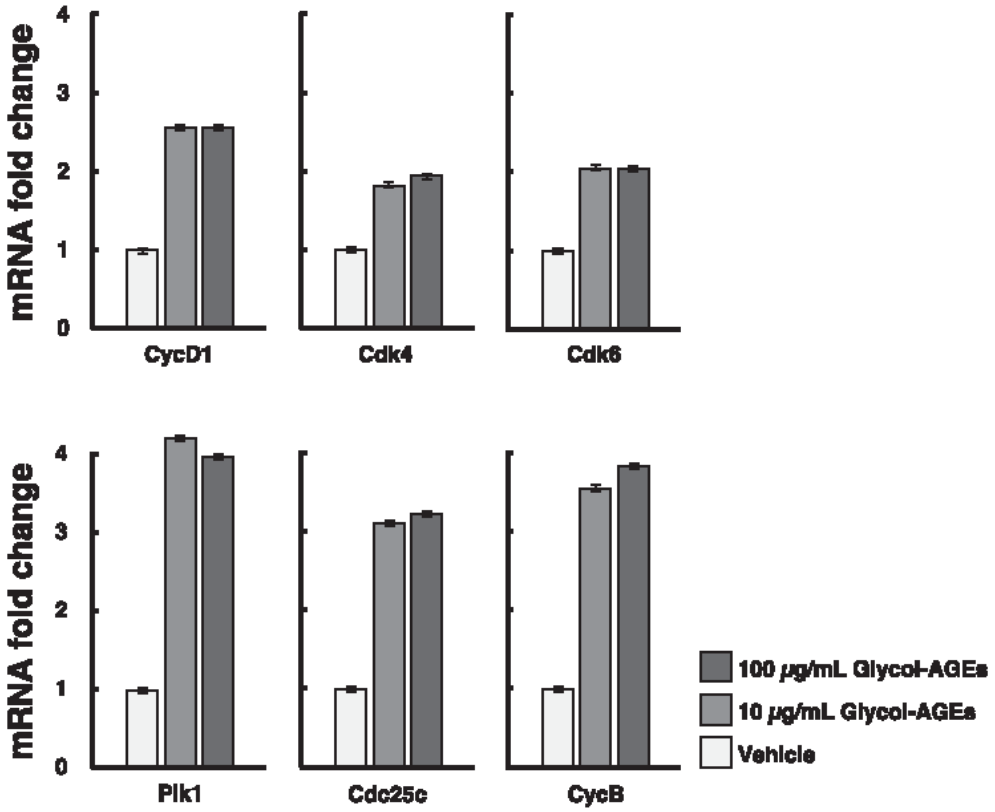

Figure 5

mRNA expression of cell cycle-related genes in RAW264.7 cells stimulated with Glycol-AGEs. After RAW264.7 cells were stimulated for 96 h with Glycol-AGEs (0, 10, and 100 μg/mL), total mRNA was isolated. The expression of cell cycle-related genes was analyzed by real-time PCR. Changes in the expression of each mRNA were normalized against GAPDH mRNA. Data represent the mean ± SE of three independent experiments. **, $p<0.001$ versus the vehicle control by Dunnett's test.