

Advanced glycation end products regulate interleukin-1 β production in human placenta

Kotomi SENO¹⁾, Saoko SASE¹⁾, Ayae OZEKI¹⁾, Hironori TAKAHASHI²⁾, Akihide OHKUCHI²⁾, Hirotada SUZUKI²⁾, Shigeki MATSUBARA²⁾, Hisataka IWATA¹⁾, Takehito KUWAYAMA¹⁾ and Koumei SHIRASUNA¹⁾

¹⁾Laboratory of Animal Reproduction, Department of Animal Science, Tokyo University of Agriculture, Kanagawa 243-0034, Japan

²⁾Department of Obstetrics and Gynecology, Jichi Medical University, Tochigi 329-0498, Japan

Abstract. Maternal obesity is a major risk factor for pregnancy complications, causing inflammatory cytokine release in the placenta, including interleukin-1 β (IL-1 β), IL-6, and IL-8. Pregnant women with obesity develop accelerated systemic and placental inflammation with elevated circulating advanced glycation end products (AGEs). IL-1 β is a pivotal inflammatory cytokine associated with obesity and pregnancy complications, and its production is regulated by NLR family pyrin domain-containing 3 (NLRP3) inflammasomes. Here, we investigated whether AGEs are involved in the activation of NLRP3 inflammasomes using human placental tissues and placental cell line. In human placental tissue cultures, AGEs significantly increased IL-1 β secretion, as well as *IL-1 β* and *NLRP3* mRNA expression. In human placental cell culture, although AGE treatment did not stimulate IL-1 β secretion, AGEs significantly increased *IL-1 β* mRNA expression and intracellular IL-1 β production. After pre-incubation with AGEs, nano-silica treatment (well known as an inflammasome activator) increased IL-1 β secretion in placental cells. However, after pre-incubation with lipopolysaccharide to produce pro-IL-1 β , AGE treatment did not affect IL-1 β secretion in placental cells. These findings suggest that AGEs stimulate pro-IL-1 β production within placental cells, but do not activate inflammasomes to stimulate IL-1 β secretion. Furthermore, using pharmacological inhibitors, we demonstrated that AGE-induced inflammatory cytokines are dependent on MAPK/NF- κ B/AP-1 signaling and reactive oxygen species production in placental cells. In conclusion, AGEs regulate pro-IL-1 β production and inflammatory responses, resulting in the activation of NLRP3 inflammasomes in human placenta. These results suggest that AGEs, as an endogenous and sterile danger signal, may contribute to chronic placental cytokine production.

Key words: Advanced glycation end products, Inflammation, NLRP3 inflammasome, Placenta

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Maternal obesity has recently been increasing in developed countries [1]. Obesity is a major risk factor in pregnancy complications, such as gestational diabetes, spontaneous miscarriage, intrauterine growth restriction, and preeclampsia [1, 2]. Obesity represents a low-grade chronic systemic inflammation [3], and maternal obesity increases the risk of the offspring developing obesity and insulin resistance in the later stages of life [4–8].

The placenta is a vital organ for pregnancy and produces cytokines to regulate placental function [9]. Levels of pro-inflammatory cytokines, such as interleukin (IL)-1 β , IL-6, IL-8 and tumor necrosis factor- α (TNF α), are increased in the serum and placental tissue of pregnant women with obesity when compared with those in pregnant women of normal weight [4, 8, 10, 11]. In addition, pregnant women with obesity also develop accelerated systemic and placental inflam-

ation with elevated circulating advanced glycation end products (AGEs) [10, 12]. AGEs consist of heterogeneous, reactive, and irreversibly crosslinked molecules formed from the non-enzymatic glycation of proteins, lipids, and nucleic acids [13, 14]. In general, AGEs interact with the receptor for advanced glycation end product (RAGE) and/or toll-like receptor 4 (TLR4) to induce inflammatory responses (the production of cytokines) [15, 16]. However, the mechanism underlying these inflammatory responses via AGEs during pregnancy remains unclear.

IL-1 β is one of the pivotal inflammatory cytokines associated with obesity and pregnancy complications, and its production is regulated by nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasomes [17–20]. NLRP3 inflammasomes comprise three different proteins—NLRP3, an apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 (CASP1, known as IL-1 β -converting enzyme). In most cells, optimal IL-1 β production is dependent on two separate signals [17]. Signal 1 is a stimulus involving lipopolysaccharide (LPS) and is needed to produce the IL-1 β precursor (pro-IL-1 β) in cells before inflammasome activation. Signal 2 consists of IL-1 β secretion and is regulated by CASP1 activation, which is induced by NLRP3 inflammasome activators, such as ATP,

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Correspondence: K Shirasuna (e-mail: ks205312@nodai.ac.jp)

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nano-silica, nigericin, or cholesterol crystal [17–20]. NLRP3 inflammasomes are involved in the pathogenesis of inflammatory diseases, including metabolic syndrome, type 2 diabetes, cardiovascular diseases, gout, and silicosis [17–20]. It has been reported that uric acid and nano-silica, a known NLRP3 activator, stimulates IL-1 β secretion via the NLRP3 inflammasome in human trophoblast cells [21]. In addition, we and other groups have recently reported that pregnancy complications are improved in NLRP3-deficient mice because placental inflammation is suppressed [22–24]. However, the role of NLRP3 inflammasomes in placental function is poorly understood.

In the present study, we hypothesized that obesity-related AGEs stimulate IL-1 β production and secretion via the NLRP3 inflammasome. To test this hypothesis, we investigated the effect of AGEs on mRNA expression of NLRP3 inflammasome constituted molecules and IL-1 β secretion using human placental tissues and human trophoblast cell line *in vitro*.

Materials and Methods

Tissue collection

Human placentae were obtained from a total of three women, who delivered healthy, singleton infants at term (patients who gave informed consent were obtained using protocols approved by the Jichi Medical University Ethics Committee). Tissues were obtained within 10 min of delivery and dissected fragments were placed in ice-cold phosphate buffered saline (PBS). Placental tissue was bluntly dissected to remove visible connective tissue and cut into small pieces (40–60 mg wet weight). These placental tissue pieces were placed on a cell culture insert membrane (0.4 μ m membrane, 24-well plate, Thermo Fisher Scientific, Waltham, MA, USA) with 1 ml Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12; Life Technologies, Carlsbad, CA, USA) supplemented with antibiotics including amphotericin B and gentamicin (Sigma-Aldrich, St. Louis, MO, USA), and 5% fetal calf serum (FCS, ICN Pharmaceuticals, Costa Mesa, CA, USA).

Cell culture of human trophoblast cell line

Human first-trimester trophoblast cells (Sw.71, trophoblast cell line) were kindly provided by Professor Gil Mor [25]. The Sw.71 cells produced various types of cytokines, including IL-1 β , IL-6, IL-8, and TNFA, were used as *in vitro* model of uterine implantation [26]. It has also been reported that Sw.71 cells express key inflammasome components, including NLRP3 and ASC [27]. Cells were cultured in DMEM/F-12 supplemented with antibiotics, sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan), non-essential amino acids (Wako Pure Chemical Industries), and 5% FCS. After reaching confluence, the cells were harvested and plated at a concentration of 1×10^5 cells/well in a 48-well culture plate (Thermo Fisher Scientific).

Experimental conditions

Placental tissue culture experiment; human placental tissues were incubated with glycoaldehyde-AGEs-BSA (200 μ g/ml; BioVision, Milpitas, CA, USA), BSA as a control for AGEs (BioVision), or LPS (100 ng/ml; Sigma-Aldrich) for 6 h at 37°C. Supernatant and tissues were collected for enzyme-linked immunosorbent assay

(ELISA), western blotting, and real-time RT-PCR, and stored at -20°C or -80°C before use.

Placental cell culture experiment; Sw.71 placental cells were washed twice in PBS and treated with AGEs-BSA (200 μ g/ml), BSA, or LPS (100 ng/ml) for 24 h at 37°C. After serum starvations and priming with AGEs or LPS for 24 h, cells were treated with nano-silica (100 μ g/ml, Micromod Partikeltechnologie GmbH, Rostock, Germany) for 24 h at 37°C, to investigate the role of AGEs on inflammasome activation. To further explore the relationships between AGEs and inflammatory cytokine production, various pharmacological inhibitors, including SB203580 [a mitogen-activated protein kinase (MAPK) p38 inhibitor, 20 μ M, Merck Millipore, Belize, MA, USA], SP600125 [a Jun N-terminal kinase (JNK) inhibitor, 20 μ M, Merck Millipore], Bay 11-7082 (a NF- κ B activation inhibitor, 2 μ M, Merck Millipore), SR11302 (an AP-1 inhibitor, 10 μ M, Merck Millipore), *N*-acetyl-L-cysteine (NAC, an antioxidant, 5 mM, Wako Pure Chemical Industries), or diphenyleneiodonium (DPI, a NADPH inhibitor, 2 μ M, Sigma-Aldrich) were pre-incubated in the medium for 1 h. Supernatant and cell lysate were collected for ELISA, real-time RT-PCR, or western blot analysis, and stored at -20°C or -80°C before use.

Determination of cytokines

Levels of IL-1 β , IL-6, or IL-8 were determined using a human ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Real-time RT-PCR

Total RNA was prepared using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. RNA extraction and cDNA production were performed using a commercial kit (ReverTra Ace; Toyobo, Osaka, Japan). Real-time RT-PCR was performed using the CFX Connect™ Real Time PCR (Bio-Rad, Hercules, CA, USA) and a commercial kit (Thunderbird SYBR qPCR Mix; Toyobo) to detect mRNA expressions of *IL-1 β* , *NLRP3*, *ASC*, *CASP1*, *IL-6*, or *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. The following antisense and sense primers were used: *IL-1 β* (5'-TGATGGCTTATTACAGTGGCAATG-3' and 5'-GTAGTGGTGGTGGGAGATTTCG-3', NM000576), *NLRP3* (5'-GAGAGACCTTTATGATGAGAAAGCA-3' and 5'-GCATATCACAGTGGGATTTCGAA-3', BC143363), *ASC* (5'-AACCCAAGCAAGATGCGGAAG-3' and 5'-TTAGGGCCTGGAGGAGCAAG-3', BC013569), *CASP1* (5'-GAAGCTCAAAGGATATGGAAACAAA-3' and 5'-AAGACGTGTGCGGCTTGACT-3', X65019), *IL-6* (5'-AAATTCGGTACATCCTCGACGG-3' and 5'-GGAAGGTTTCAGGTTGTTTTCTGC-3', M54894) and *GAPDH* (5'-AAATGAGCCCCAGCCTTCT-3' and 5'-AGGATGTCAGCGGGAGCCGG-3', M33197). RT-qPCR was performed in duplicate with a final reaction volume of 20 μ l containing 10 μ l SYBR Green, 7.8 μ l distilled water, 0.1 μ l 100 μ M forward and reverse primers, and 2 μ l of cDNA template. The amplification program consisted of a 5 min denaturation at 95°C followed by 40 cycles of amplification (95°C for 15 sec, 60°C for 30 sec, and 72°C for 20 sec). Expression levels of each target gene were normalized to corresponding GAPDH threshold cycle (CT) values using the $\Delta\Delta$

CT comparative method [28].

Western blot analysis

Lysates from the cell culture were prepared using RIPA buffer (Wako Pure Chemical Industries). Cells were washed with cold PBS and incubated with RIPA buffer for 15 min on ice. Cell lysates were subsequently transferred into 1.5 ml tubes and centrifuged at $12,000 \times g$ for 20 min at 4°C. Supernatants were transferred to a fresh tube and stored at -80°C before analysis. A total of 10 μ g protein was loaded per lane and separated by 10% SDS-PAGE. The expression of pro-IL-1 β and β -actin (ACTB) was analyzed by western blot. After transfer onto polyvinylidene fluoride membranes, nonspecific antibody binding was blocked for 1 h at room temperature using Immunoblock (DS Pharma Biomedical, Osaka, Japan). Then, membranes were incubated for 24 h at 4°C with anti-IL-1 β antibody (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-ACTB antibody (1:10000, Sigma-Aldrich), followed by an incubation for 1 h with secondary antibody conjugated horseradish peroxidase (HRP; 1:1000, GE Healthcare, UK, Buckinghamshire, UK). Immunoreactive bands were visualized by Western BLoT Quant HRP Substrate (GE Healthcare) using ImageQuant LAS 4000 (GE Healthcare). The results represent at least 3 independent experiments. Quantitative analysis of bands was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). Differences between treatment groups were identified using unpaired *t*-tests. Multiple comparisons were made using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. A P-value of < 0.05 was considered statistically significant.

Results

Effects of AGEs on inflammatory cytokine production in human placental tissues

First, we tested whether AGEs could induce inflammatory responses using healthy human placental tissue cultures. LPS was used as a positive control for induction of inflammatory responses. Both AGE and LPS treatments significantly increased *IL-1 β* and *NLRP3* mRNA expression (Fig. 1A and B), but did not affect *ASC* and *CASP1* mRNA expression in human placental tissues (Fig. 1C and D). On the other hand, both AGEs and LPS significantly increased pro-IL-1 β production (Fig. 1E) and IL-1 β secretion (Fig. 1F) in human placental tissues. In the present study, proinflammatory cytokines, including IL-6 and IL-8, were used as positive markers for inflammatory responses, because we previously reported that AGEs and LPS stimulated IL-6 and IL-8 secretion from human placental cells [16]. As expected, both AGEs and LPS significantly increased IL-6, and IL-8 secretion from human placental tissues (Fig. 1G and H). These findings suggest that AGEs up-regulate inflammatory cytokine production and secretion in human placenta.

Effects of AGEs on IL-1 β production in the Sw.71 human placental cell line

Next, we investigated the effects of AGEs and LPS on IL-1 β

production systems in Sw.71 cells (a human placental cell line). Both AGEs and LPS significantly increased *IL-1 β* mRNA expression (Fig. 2A) and pro-IL-1 β protein production (Fig. 2B) in Sw.71 cells. Contrary to the human placental tissue data, AGE and LPS treatment did not affect *NLRP3*, *ASC* and *CASP1* mRNA expression (Fig. 2C–E). Previously, we reported that AGEs and LPS stimulate inflammatory cytokine secretion, including IL-6 and IL-8, from human Sw.71 placental cells [16]. To support the previous results, both AGEs and LPS significantly increased *IL-6* mRNA expression in Sw.71 cells (Fig. 2F).

Effects of AGEs on IL-1 β secretion in the Sw.71 human placental cell line

Two signals are required for mature IL-1 β secretion from the NLRP3 inflammasome [17]. The first signal is the transcription and translation of pro-IL-1 β by priming stimuli, including LPS. The second signal activates autocatalytic cleavage of pro-CASP1 to CASP1, to secrete IL-1 β from pro-IL-1 β . We used nano-silica for the second signal to activate the NLRP3 inflammasome. As shown in Fig. 3A (left side), both AGEs and LPS alone (without nano-silica) did not affect IL-1 β secretion in Sw.71 placental cells. However, after AGE or LPS priming for 24 h to induce pro-IL-1 β production within the cells, nano-silica treatment clearly stimulated IL-1 β secretion in Sw.71 placental cells (Fig. 3A, right side), indicating the role of AGEs and LPS as “Signal 1” for pro-IL-1 β production. We checked the potential role of AGEs as “Signal 2” to activate the inflammasome system in Sw.71 placental cells. After LPS priming, AGE treatment did not affect IL-1 β secretion, whereas nano-silica treatment significantly stimulated IL-1 β secretion in Sw.71 cells (Fig. 3B). These findings suggest that AGEs stimulate pro-IL-1 β production within the cells, but do not activate the inflammasome to stimulate IL-1 β secretion.

Intracellular AGE signaling pathways on inflammatory cytokine production in the Sw.71 human placental cell line

It is well known that MAPK and transcription factors, including NF- κ B and AP-1 are important in AGE and LPS signal transduction [29]. To elucidate the signaling mechanisms, we aimed to clarify which of these pathways is involved in AGE- and LPS-amplified inflammatory cytokine production using specific pharmacological inhibitors to block each individual signaling pathway. Blockage of each individual pathway (SB203580 as a p38 MAPK inhibitor, SP600125 as a JNK inhibitor, Bay11-7082 as a NF- κ B inhibitor, and SR11302 as an AP-1 inhibitor) significantly attenuated AGEs-induced intracellular IL-1 β production (Fig. 4A), extracellular IL-1 β secretion stimulated by nano-silica (Fig. 4B), and extracellular IL-6 secretion (Fig. 4C) in human Sw.71 placental cells. These findings indicate that the activation of all of these four pathways is necessary for effective induction of cytokines by AGEs in human Sw.71 cells.

Role of ROS for AGE-induced cytokine production in the Sw.71 human placental cell line

The increase in inflammatory cytokine secretion after AGE treatment led us to hypothesize that reactive oxygen species (ROS) production involves AGE-induced inflammation in Sw.71 cells. Indeed, we already reported that AGEs stimulated ROS production and that AGE-induced ROS production is important for stimulating

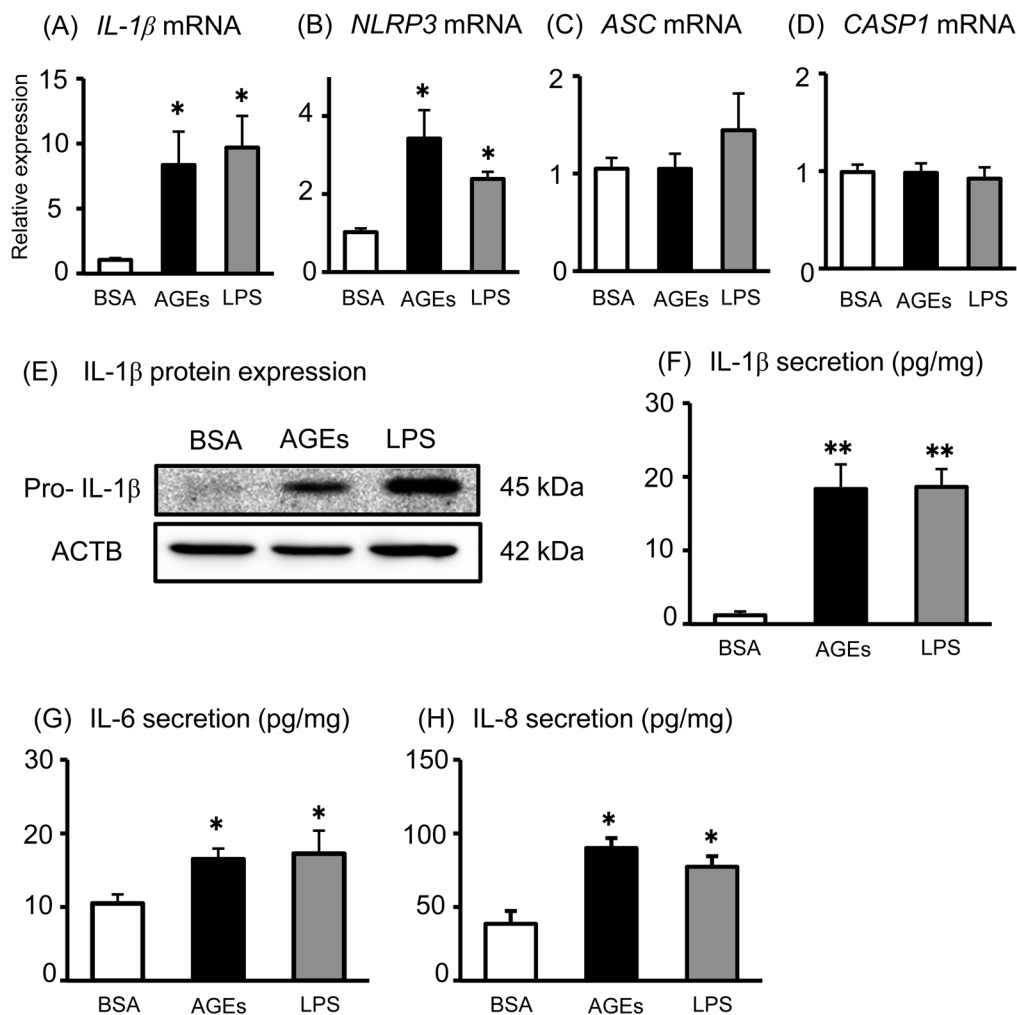


Fig. 1. Effects of AGEs on inflammatory cytokine production in human placental tissues. Human placental tissues were incubated for 6 h with BSA, AGEs (200 μg/ml), or LPS (100 ng/ml). (A–D) After incubation, the *IL-1β*, *NLRP3*, *ASC* and *CASP1* mRNA levels were measured using qRT-PCR. Pro-IL-1β and ACTB protein levels were subsequently quantified in the tissue lysates by western blot (E). IL-1β (F), IL-6 (G), and IL-8 (H) levels in the supernatant were determined using ELISA. Data are expressed as means ± SEM (n = 4). Significant differences were identified using ANOVA followed by Bonferroni's multiple comparison test; * P < 0.05 and ** P < 0.01.

cytokine production, including IL-6 and IL-8 [16]. To investigate the importance of ROS for AGE-induced IL-1β production, we tested the effect of the antioxidants NAC and DPI on Sw.71 cells. Treatment with NAC or DPI significantly decreased the extracellular IL-1β secretion induced by AGE priming with nano-silica (Fig. 5A). In addition, we showed that pre-treatment of NAC attenuated AGE-induced *IL-1β* mRNA expression (Fig. 5B). Thus, our findings indicated that ROS production is an upstream factor in AGE-induced inflammatory cytokine production, including IL-1β and IL-6, in human placental cells.

Discussion

Increasing evidence indicates that AGEs and IL-1β are associated with preeclampsia and obesity in pregnant women [1, 2, 30–32].

In the present study, we investigated whether AGEs are involved in IL-1β production and secretion via NLRP3 inflammasomes in human placenta. Our novel results demonstrated that (1) in human placental tissues, AGEs directly increase both the transcription and secretion of IL-1β; (2) In human placental cell line, AGEs stimulate pro-IL-1β production, resulting in the acceleration of mature IL-1β secretion by NLRP3 inflammasome activation; (3) AGE-induced inflammatory cytokines are dependent on MAPK/NF-κB/AP-1 signaling and ROS production.

In human placental tissues, AGE treatment directly stimulated mature IL-1β secretion, together with an increase in *IL-1β* and *NLRP3* mRNA transcription. Similar to our present results, it has been reported that AGEs increased the *in vitro* release of IL-1β from human placenta in a tissue culture system [12]. However, we showed that AGEs markedly induced pro-IL-1β production, regulating transcription of

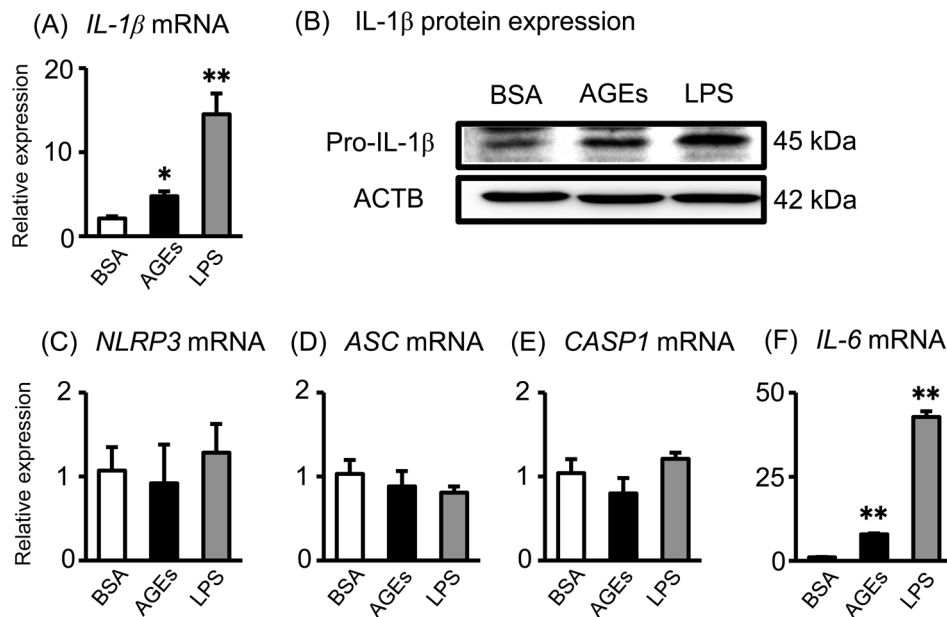


Fig. 2. Effects of AGEs on IL-1 β production in the Sw.71 human placental cell line. (A and B) Sw.71 cells were incubated for 24 h with BSA or AGEs (200 μ g/ml), and LPS (100 ng/ml); The *IL-1 β* mRNA levels were measured using qRT-PCR. Pro-IL-1 β and ACTB protein levels were subsequently quantified in the cell lysates by western blot. (C–F) After incubation, the *NLRP3*, *ASC* and *CASP1* mRNA levels were measured using qRT-PCR. Significant differences were identified using ANOVA, followed by Bonferroni's multiple comparison test; * $P < 0.05$ and ** $P < 0.01$.

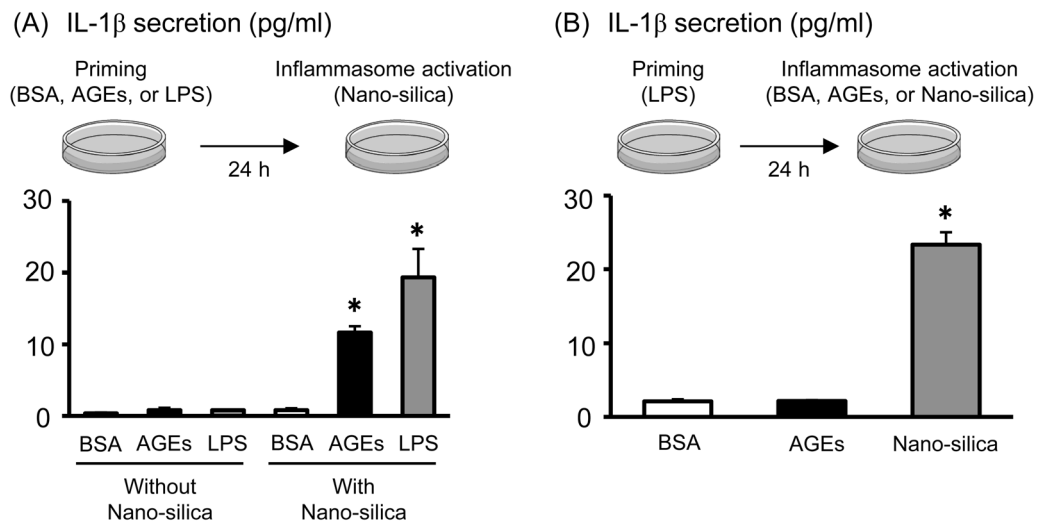


Fig. 3. Effects of AGEs on inflammasome activation in the Sw.71 human placental cell line. (A) Sw.71 cells were pre-incubated for 24 h with BSA, AGEs (200 μ g/ml), or LPS (100 ng/ml). After priming, cells were treated with nano-silica (100 μ g/ml) for 24 h to activate NLRP3 inflammasomes. (B) Sw.71 cells were pre-incubated for 24 h with LPS (100 ng/ml). After priming, cells were treated with BSA, AGE (200 μ g/ml) or nano-silica (100 μ g/ml) for 24 h. IL-1 β levels in the supernatant were determined using ELISA. Data are expressed as mean \pm SEM ($n = 4$). Significant differences were identified using ANOVA, followed by Bonferroni's multiple comparison test; * $P < 0.05$ and ** $P < 0.01$.

IL-1 β mRNA, whereas AGEs did not stimulate IL-1 β secretion in human placental cells. We believed that these differences regarding IL-1 β secretion by AGEs were due to the conditional differences between placental cells (cell lines) and placental tissues (composed of many types of cells, including placental cells, endothelial cells, and

immune cells). Indeed, numerous immune and inflammatory cells, including macrophages, NK cells, lymphocytes and neutrophils, reside in the placenta and decidua [9]. In addition, activated macrophages are the major secretors of IL-1 β via NLRP3 inflammasomes. In fact, AGEs, as well as LPS, independently increased IL-1 β secretion

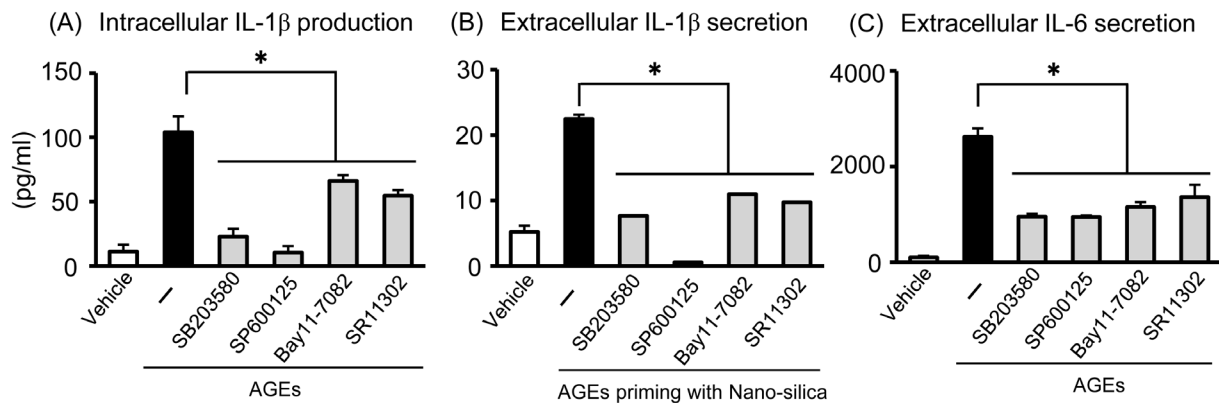


Fig. 4. Intracellular AGE signaling pathways on inflammatory cytokine production in the Sw.71 human placental cell line. Sw.71 cells were pre-incubated for 1 h with specific pharmacological inhibitors (SB203580 as a p38 MAPK inhibitor, SP600125 as a JNK inhibitor, Bay11-7082 as a NF- κ B inhibitor, and SR11302 as an AP-1 inhibitor). (A) After inhibitor treatments, cells were incubated for 24 h with AGEs (200 μ g/ml). (B) After inhibitor treatments, cells were incubated for 24 h with AGEs (200 μ g/ml), followed by nano-silica (100 μ g/ml) treatment for 24 h. (C) After inhibitor treatments, cells were incubated for 24 h with AGEs (200 μ g/ml). Intracellular IL-1 β , and IL-1 β and IL-6 levels in the supernatant were measured using ELISA. Data are expressed as mean \pm SEM (n = 4). Significant differences were identified using ANOVA, followed by Bonferroni's multiple comparison test; * P < 0.05.

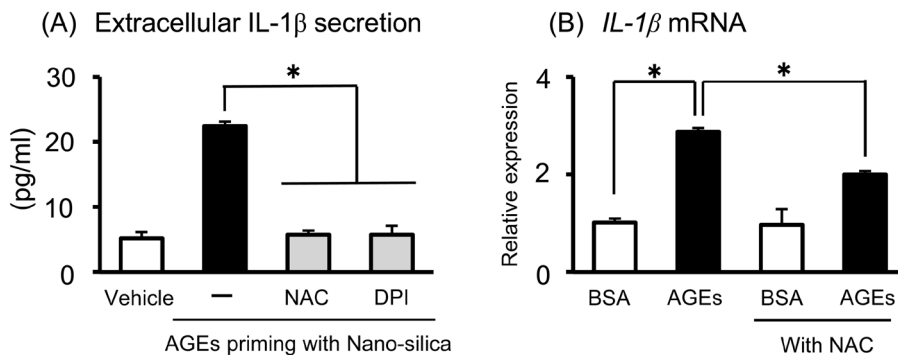


Fig. 5. ROS pathways of AGEs and their effect on inflammatory cytokine production in the Sw.71 human placental cell line. Sw.71 cells were pre-incubated for 1 h with specific pharmacological inhibitors (NAC as an antioxidant, DPI as a NADPH inhibitor). (A) After inhibitor treatments, cells were incubated for 24 h with AGEs (200 μ g/ml), and then treated with nano-silica (100 μ g/ml) for 24 h. IL-1 β levels in the supernatant were determined using ELISA. (B) After NAC treatments, cells were incubated for 24 h with AGEs (200 μ g/ml). The IL-1 β mRNA levels were measured using qRT-PCR. Data are expressed as mean \pm SEM (n = 4). Significant differences were identified using ANOVA, followed by Bonferroni's multiple comparison test; * P < 0.05.

by human peritoneal macrophages in a dose-dependent manner [33]. In addition, inflammatory immune cells accumulate in the adipose tissues of obese patients, and AGEs directly increased IL-1 β secretion without LPS priming in the adipose tissue of women with gestational diabetes [12]. These findings led us to hypothesize that (1) immune cells within human placental tissues respond to AGE treatment to induce IL-1 β secretion, and (2) cell-to-cell interactions (communication between placenta cells and immune cells) within the placenta are important for AGEs to directly induce mature IL-1 β secretion. Further investigation is needed to clarify the mechanisms of AGE-induced IL-1 β secretion, its related signaling pathways, and the key cell types involved in IL-1 β production and secretion in human placental tissues.

Next, we investigated the possible role of AGEs on IL-1 β production

and secretion using the human Sw.71 placental cell line. We showed that AGEs did not regulate mature IL-1 β secretion, but regulated pro-IL-1 β production, and nano-silica treatment stimulated IL-1 β secretion after AGE-priming in placental cells. Therefore, these results indicate that AGEs act as "Signal 1" to produce pro-IL-1 β , not as "Signal 2" to activate NLRP3 inflammasomes within human placental cells, suggesting that AGEs have a key role in pro-IL-1 β production following NLRP3 inflammasome activation. Contrary to our present study, Song *et al.* [34] recently demonstrated that using human primary nucleus pulposus cells, AGEs alone can activate not only pro-IL-1 β production as "Signal 1" within the cells, but also the NLRP3 inflammasome assembly on mitochondria as "Signal 2", resulting in elicitation of mature IL-1 β secretion. We considered cell type or cell condition (primary cells isolated from tissues or established

cell lines) as possible reasons for differences in experimental outcome.

We further investigated the possible signaling pathways of AGE-induced inflammatory cytokine production in human Sw.71 placental cells. Recently, we reported that exposure to AGEs resulted in the activation of inflammatory responses, inducing IL-6 and IL-8 secretion, and AGE-induced IL-6 secretion was dependent on NF- κ B activation through RAGE and/or TLR4 in human Sw.71 placental cells [16]. Accumulating evidence has indicated that activation of both MAPK (p38, JNK, extracellular signal-regulated kinase 1/2) and transcription factors, including NF- κ B and AP-1, are required for AGE-amplified inflammatory cytokine production [29, 35, 36]. In the present study, we confirmed that both MAPK (p38 and JNK) and NF- κ B/AP-1 are essential for AGE-induced IL-1 β production and secretion, as well as IL-6 secretion, in human placental cells.

ROS are harmful mediators of inflammation, and ROS inhibition suppresses NLRP3 inflammasome activation and inflammatory responses [22]. We previously reported that AGEs significantly stimulated ROS production, ROS inhibitor experiments confirmed that ROS are essential for IL-6 secretion by AGEs in human Sw.71 cells [16]. In the present study, we used the ROS inhibitors NAC (an antioxidant) and DPI (an NADPH oxidase inhibitor) to examine the importance of ROS, and found that these significantly blocked AGE-induced IL-1 β production, due to the inhibition of *IL-1 β* mRNA transcription, resulting in the suppression of IL-1 β secretion via NLRP3 inflammasome activation. Indeed, ROS generation is essential as an upstream factor for NLRP3 inflammasome-dependent IL-1 β secretion [37]. Therefore, it appears that ROS production is important for both inflammatory cytokine production and inflammasome activation in placental cells.

In conclusion, we demonstrated that exposure to AGEs resulted in the activation of NLRP3 inflammasome via pro-IL-1 β production and inflammatory responses, inducing IL-6 and IL-8 secretion in human placenta. MAPK/NF- κ B/AP-1 signaling and ROS production play pivotal roles in AGE-induced inflammatory responses. These results suggest that AGEs, as an endogenous and sterile danger signal, may contribute to chronic placental cytokine production, resulting in pregnancy related complications such as preeclampsia. Further insight into the relationships between placental inflammation, obesity, and pregnancy complications will be useful for developing therapeutic strategies to treat these complications.

Conflict of interest: The authors declare no conflict of interest.

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