The effect of high glucose concentrations on HMGB1 production in MG-63 osteoblast-like cells

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This thesis is based on the following article and additional results in term of the effect of the high glucose on the α-SMA mRNA expression (Fig. 8):
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Abstract

Diabetes is a metabolic disorder that causes a long-term hyperglycemic state with complications that affect multiple tissues, including bone. HMGB1, a nonhistone chromosomal binding protein, is released from the nucleus of damaged cells and secreted extracellularly, where it mediates inflammatory responses. Hyperglycemia induces HMGB1 expression in cell types associated with diabetic complications. Therefore, this study evaluated the effect of a high glucose concentration on HMGB1 production in MG-63 osteoblast-like cells. MG-63 cells were cultured in the presence of glucose at 5.5 mM (control) or 25.0 mM (high glucose). The mRNA levels of HMGB1, HMGB1 receptors (RAGE, TLR2, and TLR4), HSP90AA1, inflammatory cytokines (IL-6 and TNF-α), and α-SMA were analyzed by quantitative PCR. The protein levels of HMGB1 and TNF-α were evaluated by ELISA, immunofluorescence staining, and Western blotting. The mRNA levels of HMGB1, HSP90AA1, RAGE, TLR2, TLR4, TNF-α, IL-6 and α-SMA were higher in the high glucose group than in the control group. Also, high glucose upregulated the HMGB1 protein level. Anti-HMGB1 antibodies partially blocked the high glucose-induced increase in the TNF-α, but not IL-6 and α-SMA, mRNA levels. The TNF-α protein level in the presence of high glucose was also decreased by anti-HMGB1 antibodies. In conclusion, high glucose induced the expression and secretion of HMGB1 in MG-63 cells, suggesting that the extracellular HMGB1 induces TNF-α expression in osteoblasts under high glucose conditions.

Introduction

Diabetes is a metabolic disorder that causes a long-term hyperglycemic state with complications in multiple tissues, including the heart, brain, kidney, retina, and nerves[1]. Blood vessels are injured, and the functions of cells involved in immune and tissue repair are suppressed, under chronic hyperglycemic conditions[2,3]. In bone tissue, remodeling is continuously performed by osteoblasts and osteoclasts[4], and the risks of osteoporosis and bone loss resulting from periodontitis are increased in diabetic patients[5,6]. The healing process is also prolonged[7], which restricts the choices of orthopedic (if surgical wounds span bone tissue) and dental treatments available to diabetic patients.

High mobility group box 1 (HMGB1) is a nonhistone chromosomal binding protein involved in several nuclear events, such as DNA replication and translation[8]. HMGB1 released from the nucleus into the cytoplasm and secreted extracellularly mediates inflammatory responses[9]. HMGB1 is passively secreted when cells undergo necrosis or damage. HMGB1 is also actively secreted by monocytes, macrophages, mature dendritic cells, natural killer cells, and endothelial cells upon stimulation by oxidative stress, bacterial pathogens, and inflammatory cytokines[10,11]. Extracellular HMGB1 is a ligand for the receptor for advanced-glycation end products (RAGE), Toll-like receptor (TLR) 2, and TLR4, which are upstream of signals associated with the inflammatory response[12]. Therefore, HMGB1 is an alarmin that responds to disturbances in homeostasis.

HMGB1 is associated with the onset and progression of diabetes and its complications. The HMGB1 level was higher in the circulating blood of diabetic patients than that of healthy controls[13]. In an animal model, hyperglycemia caused by glucose infusion increased the serum HMGB1 level[14]. HMGB1 expression was higher in the ganglion cell layer of retina tissue and tubular epithelial cells in rats with streptozotocin-induced hyperglycemia compared with non-hyperglycemic rats[15,16]. Therefore, hyperglycemia induces HMGB1 expression in immune cells, vascular endothelial cells, and cells that reside in tissues with a high risk of diabetic complications, and the elevation of HMGB1 expression is linked to dysfunction of the pancreas, insulin resistance, and the onset of diabetic complications.

HMGB1 and its receptors are expressed in most organs, including bone[17]. In bone tissue, osteoblasts interact with osteoclasts and osteocytes via a cytokine network[18]. Osteoblasts have features associated with osteogenesis, such as high alkaline phosphatase activity and production of bone matrix proteins. Osteoblasts also produce cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, in a manner involving HMGB1 and the RAGE/TLR axis[19,20]. Thus, osteoblasts are essential components of the cytokine network in bone tissue. In diabetes, osteoblasts are continuously exposed to high glucose concentrations. The effect of hyperglycemia on HMGB1 expression and secretion in osteoblasts remains unexplored. Therefore, the effect of a high glucose concentration on HMGB1 production in MG-63 osteoblast-like cells was evaluated in this study.

Materials and Methods

Cell culture and stimulation with a high glucose concentration

The human osteosarcoma cell line, MG-63 cells (Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) were seeded in 100 mm culture dishes and cultured in Dulbecco's modified Eagle's medium (DMEM; Fujifilm Wako Pure Chemical, Osaka, Japan) at 37°C in a 5% CO_2 humidified chamber[21]. Semi-confluent cells were harvested by digestion with trypsin EDTA (Gibco BRL, Rockville, MD, USA) and seeded in a 6- or 24-well culture plate at $5 \times 10^4/cm^2$. To examine the effects of a high glucose concentration, cells were cultured in DMEM containing D-glucose at 5.5 mM (control) or 25.0 mM (high glucose). Heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and penicillin–streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) were added at 10% and 1%, respectively, to DMEM-containing glucose. To examine the effect of extracellular HMGB1 on the expression of inflammatory cytokines, 1.0 μg/ml anti-HMGB1 antibodies (Shino-Test, Sagamihara, Japan) was added. The medium was replaced every 2 days.

RNA isolation and qPCR

Total RNA was isolated from cells using the RNA Purification Kit (NucleoSpin, TaKaRa Bio, Otsu, Japan). cDNA was synthesized from 1 μg RNA using the RNA PCR Kit (PrimeScript, TaKaRa Bio). The cDNA mixture was diluted 1:2 in sterile water and subjected to PCR analysis. The reactions were performed in 25 μl of a mixture containing TB Green premixed Ex Taq™ solution (TaKaRa Bio), diluted cDNA, and 8 μM sense and antisense primers. The PCR assays were performed on a thermal cycler (CFX Connect Real-Time PCR system, Bio-Rad, Hercules, CA, USA) and analyzed using the instrument's software. The PCR protocol was 35 cycles at 95°C for 5 s and 60°C for 20 s. The specificity of the PCR products was confirmed by melting curve analysis. Calculated gene expression levels were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sets used for PCR are listed in Table 1.

ELISA

After 5 or 7 days of culture, cells were starved of FBS for 24 h, and the culture supernatants were collected. The HMGB1 level in the culture supernatant was measured using an ELISA kit (Shino-Test) according to the manufacturer's instructions. The values were converted to ng/ml using a standard curve.

Immunofluorescence analysis

Cells were seeded on glass coverslips and cultured as described above. Cells were fixed and permeabilized with methanol (−20°C) and 0.5% Triton X-100, respectively. The permeabilized cells were blocked with 1% bovine serum albumin, and incubated with a mouse monoclonal antibody against human HMGB1 (1:500 dilutions, Abcam, Cambridge, UK) followed by an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:200 dilutions, Thermo Fisher Scientific, Waltham, MA, USA). Immunoreactivity was detected, and images were acquired using the BZ-810 microscope in fluorescence mode (Keyence Co., Osaka, Japan).

Western blotting

After 7 days of culture, cells were collected in cell lysis buffer (0.5% Triton X-100, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM ethylenediaminetetraacetic acid, and 25 mM Tris-HCl, pH 7.4). The cell lysates were centrifuged (10,000×g for 10 min), and the protein concentrations of supernatants were determined using the Bradford Assay Kit (Bio-Rad). Proteins (10 μg) were subjected to sodium dodecyl sulfate–polyacrylamide (4-20%) gel electrophoresis and transferred to PVDF membranes using the Trans-Blot Turbo Transfer System® (Bio-Rad Laboratories). Non-specific reactions were prevented by blocking the membranes with 5% skim milk for 1 h, and the membranes were washed with Tris-buffered saline containing 0.5% Tween-20 (TBST). The membranes were incubated with a 1:500 dilution of mouse monoclonal antibodies against human TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a 1:5,000 dilution of rabbit polyclonal antibodies against human GAPDH (Merck Millipore, Temecula, CA, USA) for 24 h at 4°C. Membranes were washed with TBST and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated rat anti-mouse

secondary antibodies (Rockland Immunochemicals, Limerick, PA, USA) or mouse anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 60 min at room temperature (15-25°C). The target proteins were detected using the Western ECL Substrate Kit (Bio-Rad Laboratories). Before reprobing with different antibodies, the membrane was incubated with Restore PLUS Western blot stripping buffer (Thermo Scientific) at room temperature for 15 min.

Statistical analysis

All data were presented as mean ± SEM, and subjected to Student's *t*-test (Figs. 1, 3, 4, 5) or one-way analysis of variance followed by Tukey's multiple comparison test (Figs. 6, 8). Differences with $P < 0.05$ were considered significant. Prism 6.0 (GraphPad Software, San Diego, CA) was used for the statistical analyses.

Results

Effect of high glucose on the HMGB1 mRNA level

MG-63 cells were cultured in 5.5 mM (control) and 25.0 mM (high) glucose, and the HMGB1 mRNA level was examined by qPCR on days 3, 5 and 7 (Fig. 1). HMGB1 expression in the high glucose group increased over time, and it was significantly higher by 1.2-, 1.9-, and 2.7-fold on days 3, 5, and 7, respectively, compared with the control.

Effect of high glucose on the HMGB1 protein level

The HMGB1 protein level in MG-63 cells was evaluated by immunofluorescence analysis on days 3 and 5 (Fig. 2). Intranuclear and extranuclear HMGB1 was detected in both groups; intranuclear HMGB1 was detected more frequently, and extranuclear HMGB1 less frequently, in the control group than the high glucose group.

Effect of high glucose on the HSP90AA1 mRNA level

The HSP90AA1 mRNA levels in MG-63 cells in the control and high glucose groups are shown in Fig. 3. The HSP90AA1 level was significantly increased in the high glucose group compared with the control group by 1.4-, 1.6-, and 1.9-fold on days 3, 5, and 7, respectively.

Effect of high glucose on HMGB1 protein secretion

The HMGB1 protein concentrations in the culture supernatant from cells in the control group were 43.4 ± 0.7 and 53.5 ± 1.3 ng/ml on days 5 and 7 of culture, respectively. The HMGB1 protein concentrations in the culture supernatant from cells in the high glucose group were 107.3 ± 1.3 and 138.7 ± 3.5 ng/ml on days 5 and 7, respectively. The between-group differences were significant on days 5 and 7 (Fig. 4).

Effect of high glucose on the mRNA levels of HMGB1 receptors

The mRNA levels of HMGB1 receptors (RAGE, TLR2, and TLR4) in cells from the control and high

glucose groups on day 7 are shown in Fig. 5. The mRNA levels of RAGE, TLR2, and TLR4 were significantly increased in the high glucose compared with the control group by 2.3-, 1.8-, and 1.7-fold, respectively.

Effect of anti-HMGB1 antibodies on the high glucose-induced expression of TNF-α and IL-6

The mRNA levels of TNF-α and IL-6 in cells were evaluated on day 7 of culture (Fig. 6). The mRNA levels of TNF-α and IL-6 were significantly increased in the high glucose group compared with the control group by 30.9- and 3.0-fold, respectively. The high glucose-induced expression of TNF-α was inhibited by approximately 68% by anti-HMGB1 antibodies. However, anti-HMGB1 antibodies did not influence the high glucose-induced expression of IL-6. In Western blots, the intensity of the band corresponding to TNF-α was reduced by anti-HMGB1 antibodies in the high glucose group (Fig. 7).

Effect of anti-HMGB1 antibodies on the high glucose-induced expression of the myoblast differentiation maker

The mRNA levels of alpha-smooth muscle actin (α-SMA), the marker of myoblast differentiation, in cells were evaluated on day 7 of culture (Fig. 8). The mRNA levels of α-SMA were significantly increased in the high glucose group compared with the control group by 2.3-fold. Anti-HMGB1 antibodies did not block the high glucose-induced expression of α-SMA.

Discussion

Chronic hyperglycemia damages cells, and HMGB1, a damage-associated molecular pattern[10], is associated with diabetic complications[11,12]. High glucose concentrations induce HMGB1 expression in vascular endothelial cells[22], mesangial cells[23], cardiomyocytes[24], retinal pigment epithelial cells[25], and gingival epithelial cells[26]. In diabetes, osteoblasts are influenced by hyperglycemia, and their response to high glucose concentrations has been evaluated. It was reported that high glucose concentrations upregulate HMGB1 expression in bone marrow stromal cells[27]. In this study, the effect of a high glucose concentration on HMGB1 expression and secretion by osteoblasts was examined using MG-63 osteoblastic cells.

Charoonpatrapong *et al*. reported that osteoblast-like cell lines, including MG-63 cells, express HMGB1, and that extracellular secretion of HMGB1 is induced by recombinant parathyroid hormone in rat osteosarcoma cells[17]. Qiu *et al*. showed that hypoxia induced HMGB1 secretion from MC3T3E1 murine osteoblast-like cells and MG-63 cells[28]. In this study, HMGB1 protein was detected extra- rather than intranuclearly in cells cultured under high glucose concentrations. Moreover, the culture supernatant from cells cultured under the high glucose condition had a higher HMGB1 protein level compared with the control cells. Therefore, the high glucose concentration facilitated HMGB1 translocation from the nucleus to the cytosol and its extracellular secretion. The high glucose concentration increased the mRNA level of HMGB1 and promoted its secretion from MG-63 cells. Similar effects of high glucose concentrations on HMGB1 expression were reported in mesangial cells[23] and gingival epithelial cells[26]. In brief, not only the release but also the synthesis of HMGB1 was induced by the high glucose concentration in MG-63 cells.

HMGB1 is released during the process of lysosome and autophagy[29]. Kim *et al*. reported that overexpression of HSP90AA1, a chaperone protein induced under stress conditions, promoted HMGB1translocation and secretion via autophagy machinery[30]. In this study, the HSP90AA1 mRNA level was increased in MG-63 cells cultured under a high glucose concentration. Therefore, high glucose concentrations might induce the release of HMGB1 from MG-63 cells via a mechanism involving autophagy.

In bone tissue, osteoblasts, osteocytes, and osteoclasts interact via cytokines. High glucose concentrations

induce the expression of inflammatory cytokines in osteoblasts[31-33]. The previous study that investigated the effects of high glucose on mesangial cells reported that high glucose-induced HMGB1 has been implicated in the expression of TNF-α and IL-6[23]. In this study, RAGE, TLR2, and TLR4 (HMGB1 receptors)[12,13] were upregulated in MG-63 cells cultured under high glucose conditions. Based on these findings, the effect of anti-HMGB1 antibodies on TNF-α expression under high glucose conditions was evaluated. The high glucose concentration increased the TNF-α mRNA and protein level, an effect inhibited by anti-HMGB1 antibodies. The IL-6 mRNA level was also elevated by the high glucose concentration, an effect that was not inhibited by anti-HMGB1 antibodies. Although the reason for the different effects of antibodies on the high glucose-induced expression of these two cytokines is unclear, ligands other than HMGB1 may be involved in the high glucose-induced expression of cytokines in osteoblasts. Some S100 proteins can bind to RAGE[34]. Intracellular S100 proteins are involved in transmitting calcium signaling, whereas secreted S100 proteins function as cytokines via RAGE[34]. Takagi *et al*. reported that S100A9, a secreted S100 protein, increased IL-6 and RANKL expression in osteocyte-like cells[35]. Also, inhibition of RAGE and TLR4 expression by siRNA transfection diminished S100A-induced IL-6 expression[35]. Moreover, S100B induced the secretion of IL-6 and TNF-α from lung alveolar epithelial cells[36]. In this study, anti-HMGB1 antibodies inhibited the high glucose-induced TNF-α expression, but its effect was partial. Therefore, S100 proteins might be involved in the upregulation of IL-6 and TNF-α in MG-63 cells under high glucose conditions.

Wang *et al*. previously reported that the expression of osteogenic markers including Runx2, and osteocalcin and mineralized nodule formation was suppressed in MG-63 cells cultured in the 25.5 mM glucose[37]. Moreover, Qui *et al*. revealed that the expression of not only HMGB1 but also α-SMA was induced by hypoxia in MC3T3-E1 cells and MG-63 cells[28]. They also suggested that hypoxia induced the differentiation of MC3T3-E1 and MG-63 cells into fibroblast via HMGB1, since knockdown of RAGE by siRNA attenuated this effect of HMGB1[28]. In the present study, the mRNA level of α-SMA was increased in MG-63 cells cultured under high glucose conditions. Thus, high glucose might also induce the differentiation of MG-63 cells into fibroblast. In addition, this effect of high glucose concentration might be independent of HMGB1, because anti-HMGB1

antibodies did not inhibited the upregulation of α-SMA expression under high glucose concentration.

In the high glucose group, HMGB1 was retained in the nucleus in some cells. Intranuclear HMGB1 regulates gene transcription[8]; therefore, it is important to investigate the function of intranuclear HMGB1 under high glucose conditions. Moreover, HMGB1 released from osteoblasts can influence osteoclasts and osteocytes. Further research on osteoclasts and osteocytes is needed to analyze the functions of HMGB1 produced by osteoblasts under high glucose conditions.

In conclusion, a high glucose concentration increased the expression and secretion of HMGB1 in MG-63 osteoblasts, and HMGB1-specific antibodies partially inhibited the high glucose-induced TNF-α expression. Therefore, extracellular HMGB1 may be involved in TNF-α expression in osteoblasts under high glucose conditions.

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Table and Figures

Talbe 1. Primer sequences

Gene target		Sequence 5'-3'	GenBank accession number
HMGB1	F:	AAGGGTCTGATAGCTTTCAGTAGCA	NM_001370341
	R:	AGACTTAAGCTGTGTACGGT	
HSP90AA1	F:	CAGTACGCTTGGGAGTCCTC	NM_005348
	R:	TTTGTTCCACGACCCATAGG	
RAGE	F:	AGCTGTGTGGCCACCCATT	NM_001206936
	R:	GGGCTATCTTCTGCTTCCCT	
TLR2	F:	GAAAGCTCCCAGCAGGAACATC	NM_001318796.2
	R:	GAATGAAGTCCCGCTTATGAAGACA	
TLR4	F:	TCATTGGTGTGTCGGTCCTCA	NM_003266
	R:	CCATACTTTATGCAGCCAGCAAGA	
TNF-α	F:	GAAATGGAGGCAATAGGTT	MH180383.1
	R:	AGCCGTGGGTCAGTATGT	
IL-6	F:	ACTCACCTCTTCAGAACGAATTG	NM000600.5
	R:	CCATCTTTGGAAGGTTCAGGTTG	
α-SMA	F:	GTTCCGCTCCTCTCTCCAAC	NM_001406462.1
	R:	SCGCTGGAGGACTTGCTTTT	

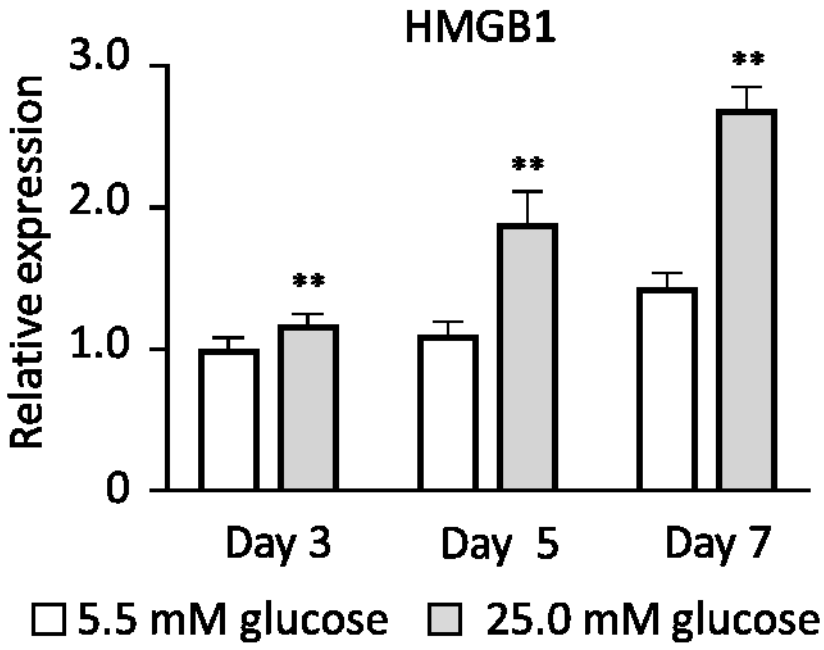

Figure 1. Effect of a high glucose concentration on the HMGB1 mRNA level.

The HMGB1 mRNA levels in MG-63 cells cultured in 5.5 and 25.0 mM glucose for 3, 5, and 7 days were determined by qPCR. Error bars indicate the SEM (n = 6). $^{**}P < 0.01$ (*vs*. the control).

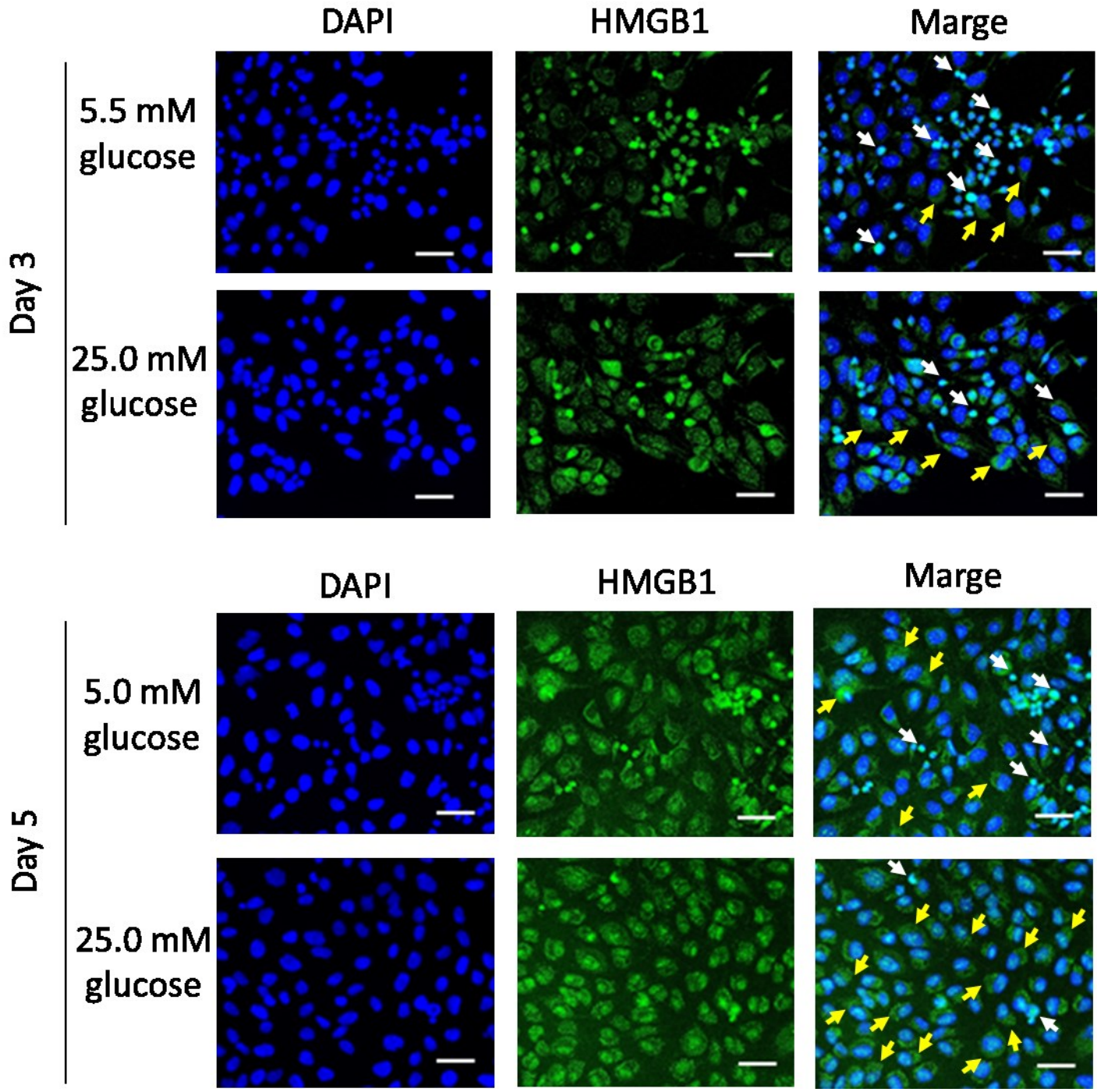

Figure 2. Effect of a high glucose concentration on the HMGB1 protein level.

The HMGB1 protein levels in MG-63 cells cultured in 5.5 and 25.0 mM glucose for 3 and 5 days were determined by immunofluorescence staining. White and yellow arrows represent typical intranuclear and extranuclear HMGB1, respectively. Scale bar, 100 μm.

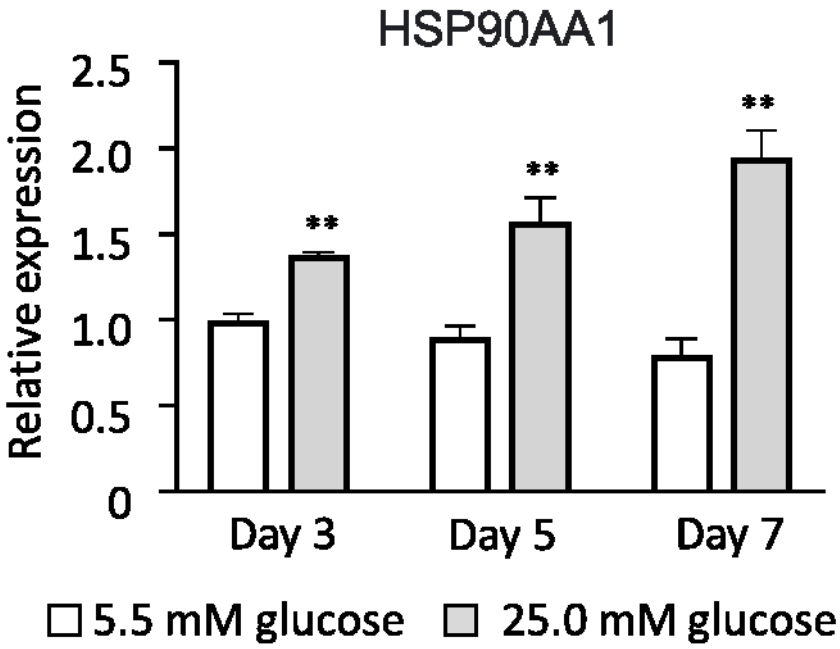

Figure 3. Effect of a high glucose concentration on the HSP90AA1 mRNA level.

The HSP90AA1 mRNA levels in MG-63 cells cultured in 5.5 and 25.0 mM glucose for 3, 5, and 7 days were determined by qPCR. Error bars indicate the SEM (n = 6). $^{**}P < 0.01$ (vs. control).

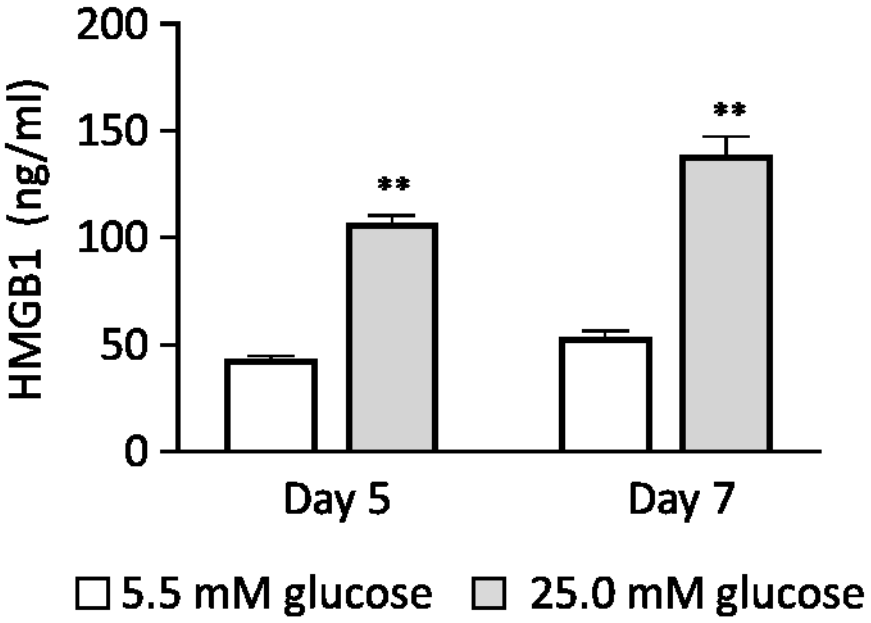

Figure 4. Effect of a high glucose concentration on HMGB1 protein secretion.

The HMGB1 protein levels in the culture supernatant of MG-63 cells cultured in 5.5 and 25.0 mM glucose for 5 and 7 days were determined by ELISA. Error bars indicate the SEM (n = 6). $^{**}P < 0.01$ (vs. control).

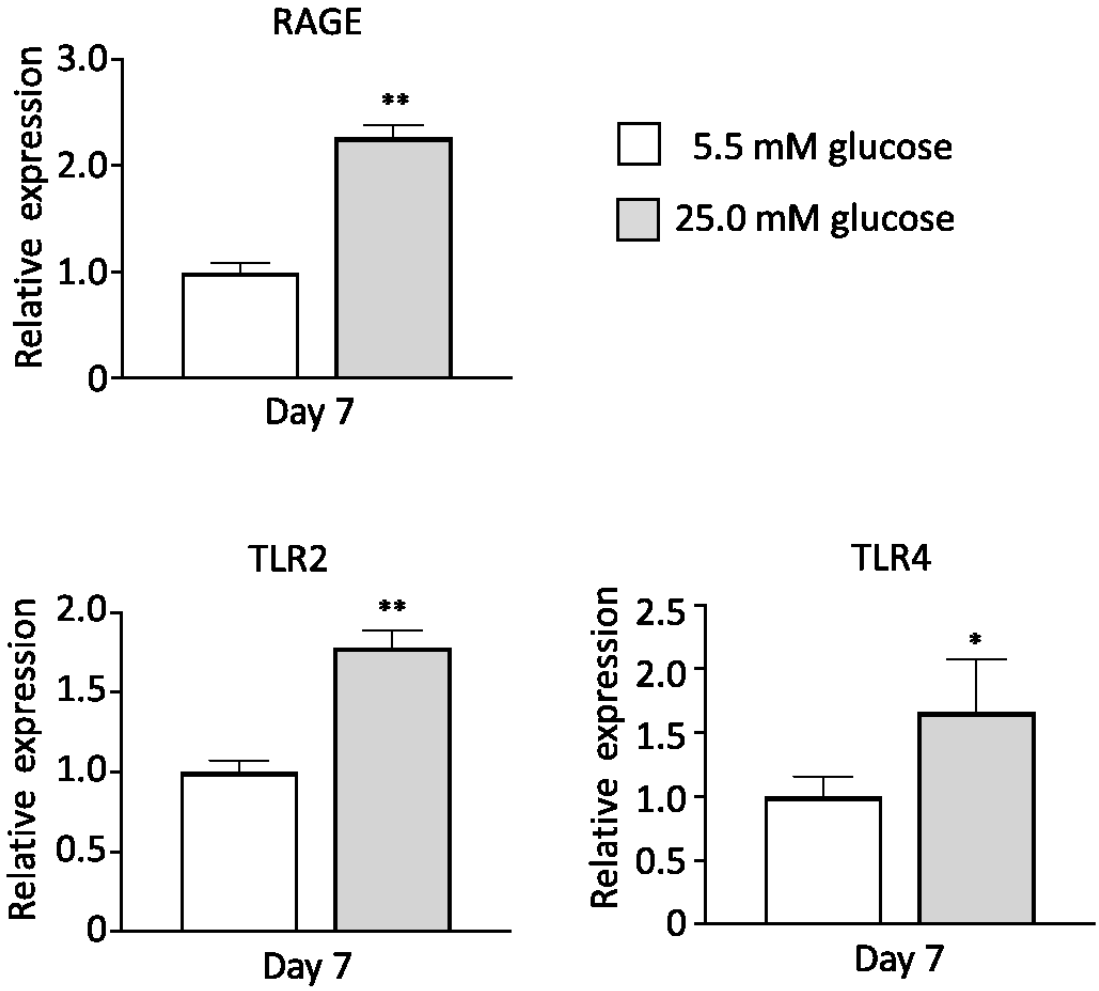

Figure 5. Effect of a high glucose concentration on the mRNA levels of HMGB1 receptors.

The mRNA levels of RAGE, TLR2, and TLR4 in MG-63 cells cultured in 5.5 and 25 mM glucose for 7 days were determined by qPCR. Error bars indicate the SEM (n = 6). *$P < 0.05$, **$P < 0.01$ (vs. control).

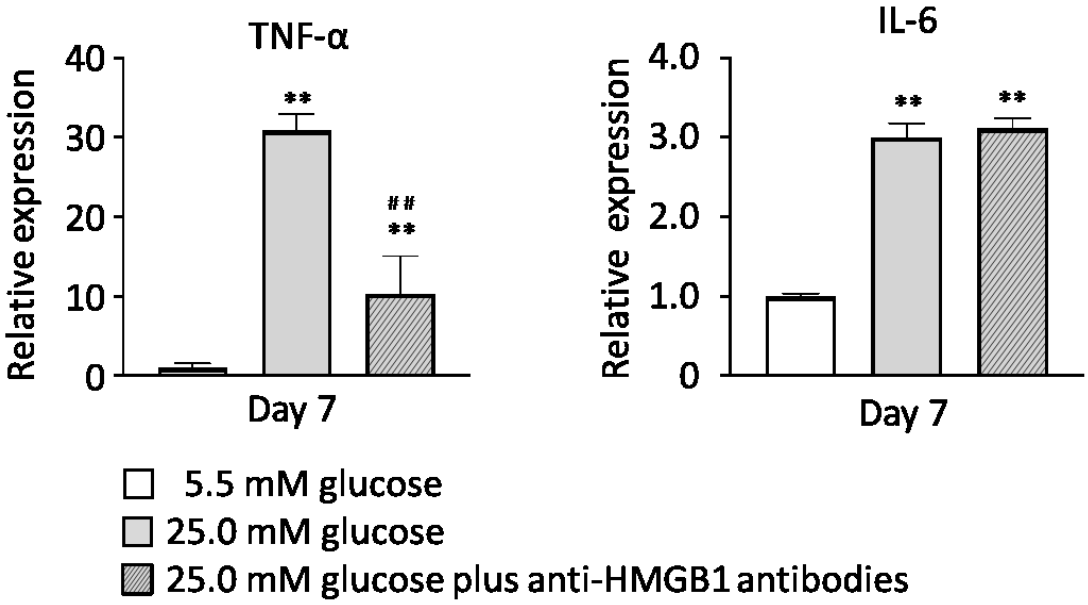

Figure 6. Effect of HMGB1 neutralizing antibodies on the high glucose-induced increase in the mRNA levels of TNF-α and IL-6.

The mRNA levels of TNF-α and IL-6 in MG-63 cells cultured in 5.5 mM glucose (control), 25.0 mM glucose (high glucose), and high glucose plus 1 μg anti-HMGB1 antibodies for 7 days were determined by qPCR. Error bars indicate the SEM (n = 6). $^{**}P < 0.01$ (vs. control), $^{\#\#}P<0.01$ (vs. 25.0 mM glucose).

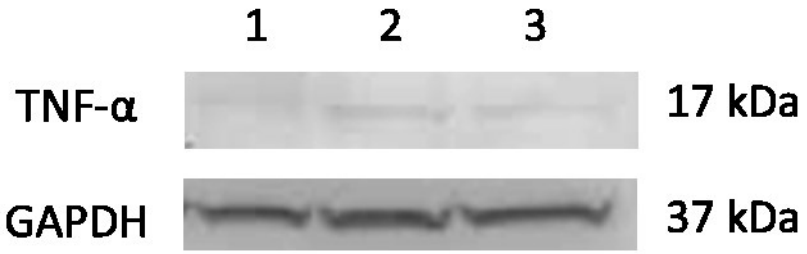

Figure 7. Effect of anti-HMGB1 antibodies on the high glucose-induced increase in the TNF-α protein level. The TNF-α protein level was analyzed by Western blotting. Lane 1: 5.5 mM glucose (control); lane 2: 25.0 mM glucose (high glucose); lane 3: high glucose plus anti-HMGB1 neutralizing antibodies.

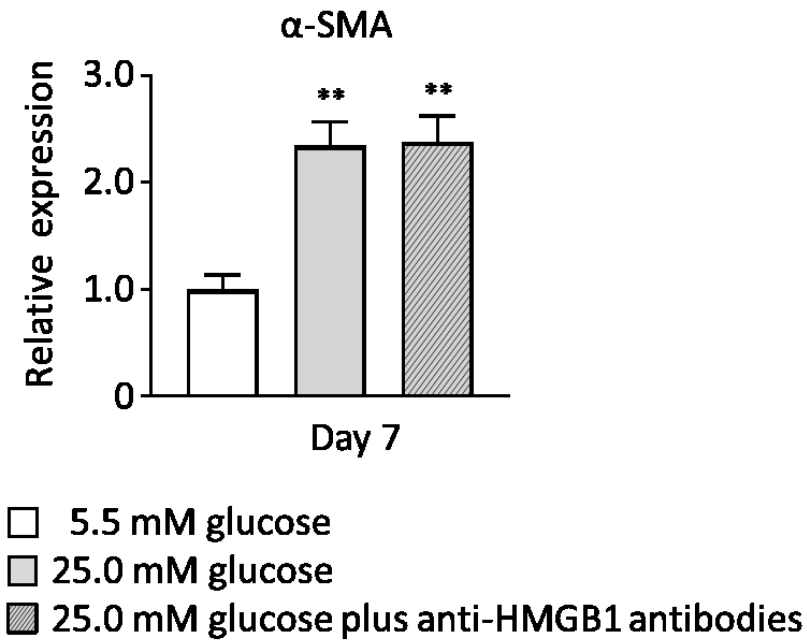

Figure 8. Effect of a high glucose concentration on α-SMA mRNA level.

The α-SMA mRNA levels in MG-63 cells cultured in 5.5 and 25.0 mM glucose for 7 days were determined by qPCR. Error bars indicate the SEM (n = 6). $^{**}P < 0.01$ (vs. control).