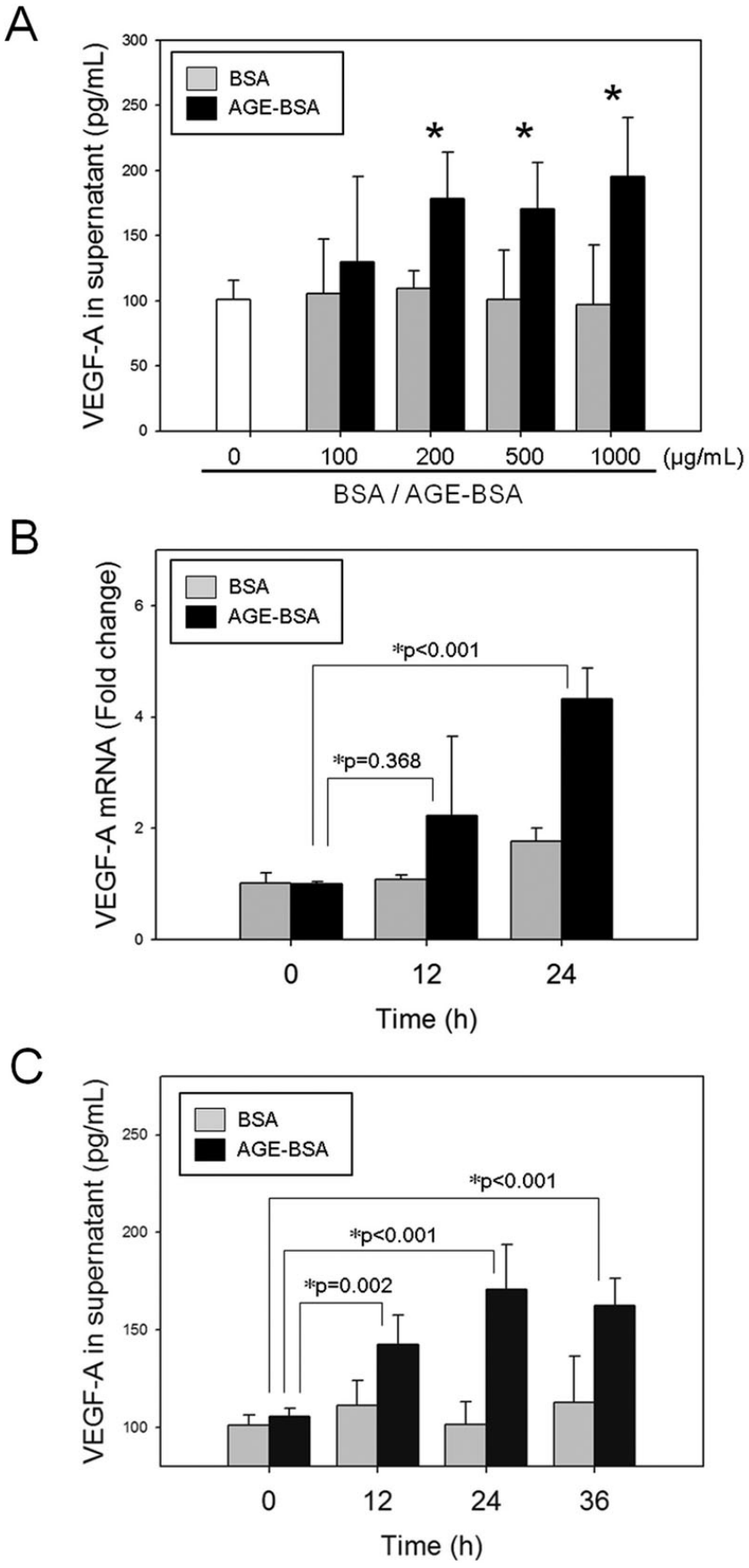
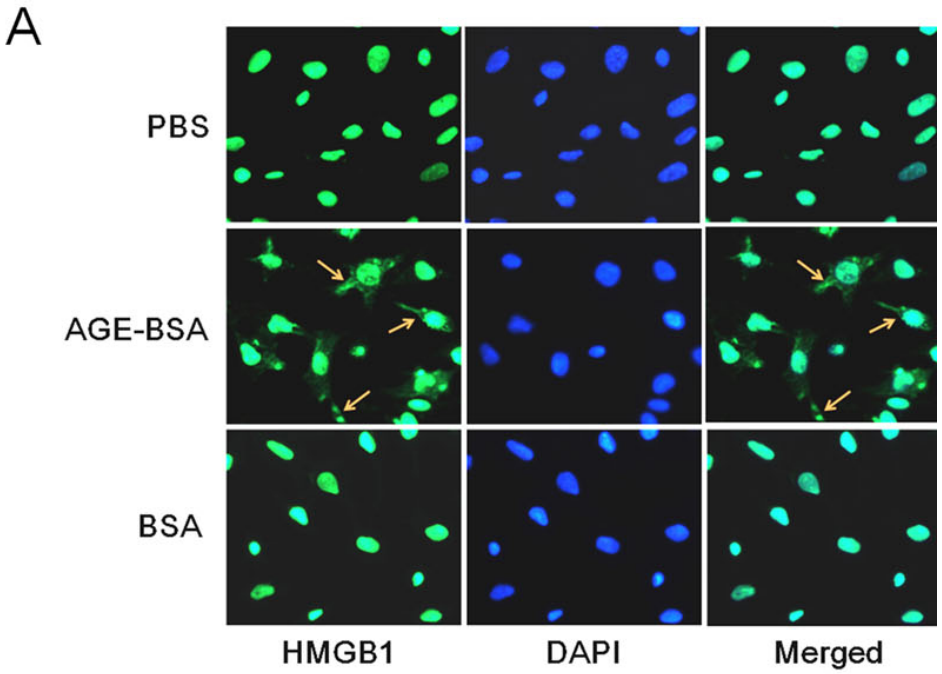


Figure 1. Advanced glycation end products–BSA (AGE-BSA) treatment results in vascular endothelial growth factor (VEGF)-A upregulation in retinal ganglion cell (RGC)-5 cells. **A**: The VEGF-A concentration in the media of RGC-5 cells treated with 100 to 1,000 μg/ml of AGE-BSA for 24 h was detected with enzyme-linked immunosorbent assay (ELISA). (n=8, * $p<0.05$, between AGE-BSA and BSA control). **B**: The change in the VEGF-A mRNA levels of RGC-5 cell was detected with real-time qPCR after incubation with AGE-BSA (200 μg/ml) for 12 to 24 h (n=3). **C**: VEGF-A concentration in conditioned media increased over time after incubation of RGC-5 cells with 200 μg/ml of AGE-BSA (n=8).



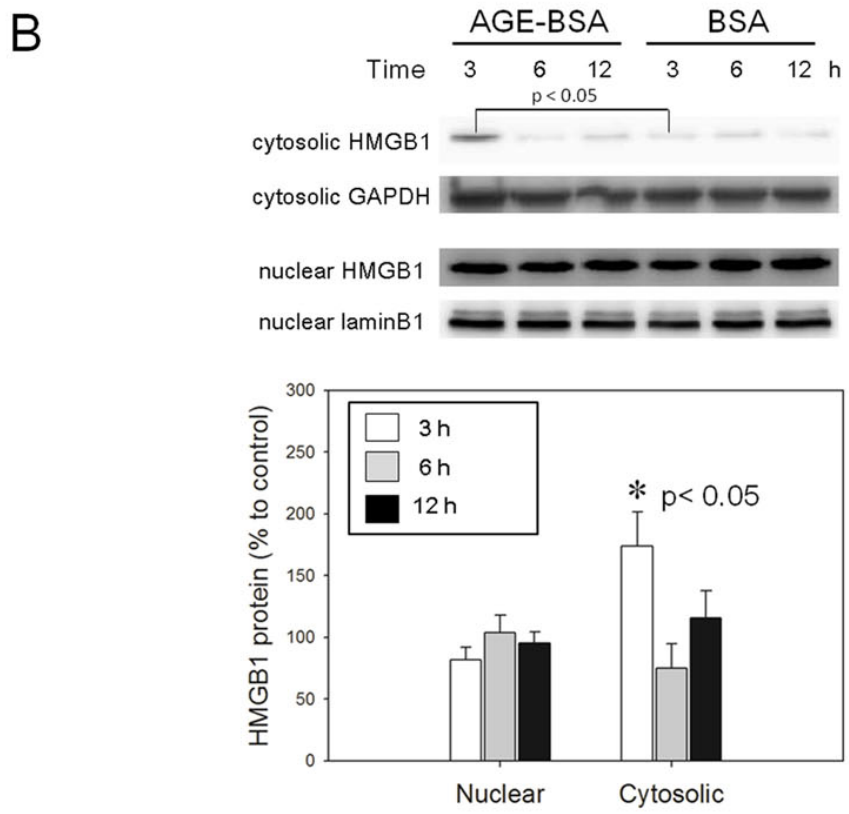


Figure 2. The distribution of high-mobility group box protein 1 (HMGB1) in retinal ganglion cell (RGC)-5 cells changed following treatment with advanced glycation end products–BSA (AGE-BSA). **A**: Immunofluorescence photographs (400× magnification) show that HMGB1 appears in the cytoplasm (arrow) of RGC-5 cells at 3 h after treatment with 200 µg/ml of AGE-BSA, while HMGB1 remains in the nucleus of RGC-5 cells treated with either BSA (200 µg/ml) or PBS. The left column shows the distribution of HMGB1 protein with green fluorescence. The central column shows the nucleus stained with 4',6-diamidino-2-phenylindole (DAPI; blue), and the right column shows merged pictures. **B**: Subcellular fractionation of proteins shows a significant increase of HMGB1 levels in the cytosol of RGC-5 cells after incubation with AGE-BSA for 3 h (n=4, p<0.05, BSA-treated cells were used as control). The level of HMGB1 in the nucleus was not different between groups over time.

h (p<0.001, Figure 1B). A time-dependent increase in VEGF-A concentration was detected in the medium after treatment of RGC-5 cells with AGE-BSA for 12, 24, and 36 h (p=0.002, p<0.001, and p<0.001, respectively, Figure 1C).

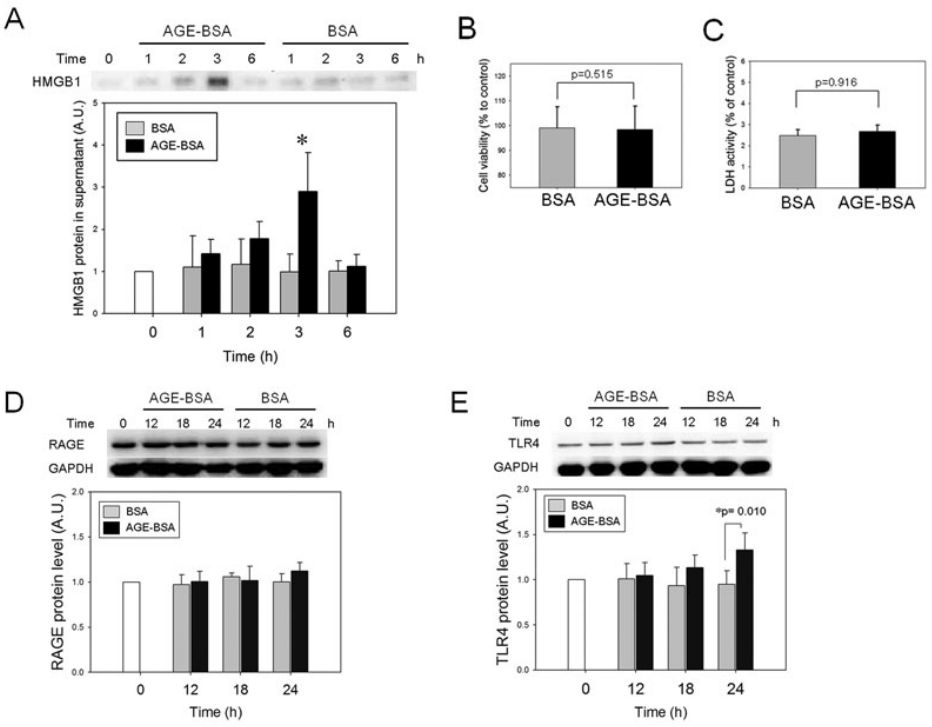


Figure 3. Advanced glycation end products–BSA (AGE-BSA) treatment results in the release of high-mobility group box protein 1 (HMGB1) from retinal ganglion cell (RGC)-5 cells. **A**: HMGB1 in the culture media after treatment of RGC-5 cells with 200 µg/ml of AGE-BSA or BSA alone was detected by western blot analysis. The release of HMGB1 into the media reached significance at 3 h after treatment with AGE-BSA, compared to the BSA control (n=4, * p<0.05; A.U.represents arbitrary unit). **B**: XTT test and (**C**) lactate dehydrogenase (LDH) assay conducted after incubation of RGC-5 cells with 200 µg/ml of AGE-BSA or BSA alone for 24 h. No significant difference was found between groups (n=6, p=0.515 for XTT and p=0.916 for the LDH test). **D**: RAGE and (**E**) TLR4 protein levels were assayed with western blot analysis. The levels of RAGE in the RGC-5 were not different between groups over time. However, a mild but significant increase in the TLR4 protein was detected after AGE-BSA treatment at 24 h (n=5, p=0.010).

Advanced glycation end products induce translocation and release of high-mobility box 1 protein from RGC-5 cells: Immunofluorescence studies demonstrated that HMGB1 appeared in the cytoplasm after 3 h of incubation with AGE-BSA. Meanwhile, HMGB1 remained localized in the nucleus in control RGC-5 cells treated with BSA or DPBS only (Figure 2A). Significant increases in cytosolic HMGB1 in RGC-5 cells were detected with 3 h of AGE-BSA (200 µg/ml) when compared with the BSA treatment. Nuclear HMGB1 levels remained relatively the same during 3 to 12 h of AGE-BSA treatment. Western blot analysis of intracellular protein of RGC-5 showed that AGE-BSA induced a significant change in the cytosolic HMGB1 by 173.7%±27.6% at 3 h (p=0.029), which then decreased to 81.0%±14.6% at 6 h (p=0.686) and 105.3%±20.7% at 12 h (p=0.343) when compared with the BSA-treated cells. AGE-BSA caused insignificant changes in nuclear HMGB1 by 83.3%±15.1%, 97.1±24.0%, and 96.8%±14.1% (p=0.275, p=0.827, and p=0.827, respectively) at 3, 6, and 12 h, comparing to BSA-treated cells (Figure 2B). Western blot analysis showed a significant increase in HMGB1 in the culture medium of RGC-5 cells incubated with AGE-BSA for 3 h (p<0.005) but not at any other time points (Figure 3A). The viability of the RGC-5 cells assayed with the XTT test and the cytotoxicity assessed with LDH showed no significant difference between the AGE-BSA treated and control groups (Figure 3B,C).

The expressions of RAGE and TLR4 were also assessed with western blot analysis. The RAGE protein levels at 12, 18, and 24 h compare to the baseline in AGE-BSA (200 µg/ml) treated RGC-5 cells were 98.6%±12.5%, 107.4%±2.7%, and 112.1%±9.4%, respectively, and 96.8%±12.6%, 101.9% ±15.6%, and 101.3%±6.3%, respectively, in the BSA-treated cells (Figure 3D). The difference between treatments was not significant (p=0.699, p=0.413, and p=0.114 for 12, 18, and 24 h, respectively). The TLR4 protein levels at 12, 18, and 24 h compared to the baseline in the RGC-5 cells treated with AGE-BSA (200 µg/ml) were 104.5%±14.4%, 113.6% ±14.1%, and 132.6%±19.0%, respectively, and 101.1% ±17.0%, 93.4%±20.4%, and 94.6%±15.4%, respectively, in the BSA-treated cells (Figure 3E). A significant increase in the TLR4 protein was detected at 24 h after the AGE-BSA treatment (p=0.931, p=0.111, and p=0.010 for 12, 18, and 24 h, respectively).

Reactive oxidative species generation in RGC-5 cells treated with advanced glycation end products and the reverse of high-mobility box 1 protein secretion with antioxidant N-acetyl-L-cysteine: ROS production in the RGC-5 cells treated with AGE-BSA was evaluated using a peroxide-dependent oxidation of DCFH-DA. A significant increase in ROS from baseline was observed in the RGC-5 cells at 3 h after incubation with AGE-BSA, compared with the BSA control (249.1%±38.4% and 153.9%±7.0%, respectively, p=0.002,

Figure 4 A,B). Pretreatment of RGC-5 cells with 1, 5, and 10 mM NAC, a known antioxidant [24,25], significantly suppressed the elevation of HMGB1 in a dose-dependent manner (p=0.215, p=0.004, and p<0.001, respectively, Figure 4C).

c-Jun N-terminal kinase phosphorylation is induced in RGC-5 cells treated with advanced glycation end products and is blocked by a high-mobility box 1 protein inhibitor: HMGB1 and RAGE activate MAPK signaling pathways [26-28]. In the present study, we focused on activation of MAPKs by HMGB1 in RGC-5 cells incubated with AGE-BSA or BSA for 1.5 to 4.5 h. The results showed that only phosphorylated JNK2/3, but not phosphorylated p38, ERK, or JNK1, increased significantly at 3 h after treatment with AGE-BSA (Figure 5A). The timing of the JNK2/3 activation was consistent with the release of HMGB1 from AGE-BSA–treated RGC-5 cells in the supernatant. GZ (100 μM), which binds and inhibits the cytokine activities of HMGB1 [29,30], SP600125 (10 μM), and NAC (10 mM), successfully blocked the increase of phosphorylated JNK2/3 in RGC-5 cells treated with AGE-BSA for 3 h (Figure 5B). Treatment with GZ (100 μM), NAC (5 mM), and NAC (10 mM) for 24 h showed insignificant changes in RGC-5 cell viability on the XTT, which were 94.6%±5.4%, 97.9%±1.1%, and 98.8%±0.7% relative to control (sterile PBS; p=0.134, p=1.000, and p=1.000, respectively, Figure 5C).

Advanced glycation end products induced vascular endothelial growth factor-A upregulation is high-mobility box 1 protein dependent and works through the c-Jun N-terminal kinase pathway: We used the HMGB1 inhibitor GZ to block HMGB1 and found a dose-dependent decrease in VEGF-A concentration in the culture media at 24 h after the RGC-5 cells were treated with AGE-BSA. GZ doses of 50, 100, and 200 μM suppressed VEGF-A concentration elevation by 38.0%±77.6%, 67.2%±28.1%, and 79.8%±30.0%, respectively (p=0.831, p=0.021, and p=0.006, respectively, Figure 6A). However, NAC at doses of 5 and 10 mM also inhibited VEGF-A concentration elevation by 79.0%±18.6%, and 94.1%±11.2%, respectively (p<0.001 and p<0.001, respectively, Figure 6B). Furthermore, specific inhibitors of MAPK signaling pathways, namely, SP600125 (10 μM) for JNK, SB230580 (5 μM) for p38, PD98059 (10 μM), and U0126 (10 μM) for ERK, were studied. Pretreatment of RGC-5 cells with these inhibitors before AGE-BSA treatment resulted in a decrease in VEGF-A concentration in the supernatant by 15.8%±72.6%, 95.2%±26.9%, −0.2%±89.1%, and −9.8%±74.8%, respectively (p=1.000, p=0.015, p=1.000, and p=1.000, respectively). The results showed that only SP600125, the specific inhibitor of the JNK pathway, could inhibit the increase of VEGF-A at 24 h after incubation with AGE-BSA (Figure 6C).

DISCUSSION

Previous studies have confirmed the effects of AGEs on the pathogenesis of a variety of diseases, including retinal microangiopathy [31-35]. In this study, we confirmed a unique role played by HMGB1 in AGE-BSA-induced VEGF-A secretion from RGC-5 cells. Our results are consistent with recent studies that showed that HMGB1 provides a strong proangiogenic stimulus both in vitro and in vivo [12,13]. It has been demonstrated that

HMGB1 stimulates membrane ruffling and repair of a mechanically wounded endothelial cell monolayer, causes endothelial cell sprouting, and stimulates neovascularization of chicken embryo chorioallantoic membrane via RAGE [12]. The crucial role of HMGB1 has also been demonstrated in diabetic mice for ischemia-induced angiogenesis through a VEGF-dependent mechanism [13].

VEGF-A has been recognized as a critical mediator of diabetic retinopathy in experimental and clinical studies [34-36]. However, the modulation of VEGF-A in the retina of diabetic patients before the stage of proliferative retinopathy with neovascularization is not completely clear. We have demonstrated that HMGB1 might play a role in the upregulation of VEGF-A in retinal ganglion cells after exposure to AGEs. Binding of glycyrrhizin to HMGB1inhibits the phosphorylation and physiologic activities of HMGB1 in vitro [29]. Glycyrrhizin has also been reported to inhibit the chemoattractant and mitogenic activities of HMGB1, and only has a weak inhibitory effect on its intranuclear DNA-binding function [30]. In our study, blocking HMGB1 with glycyrrhizin successfully inhibits AGE-BSA-induced upregulation of VEGF-A.

Blocking HMGB1 suppressed the AGEs-induced upregulation of VEGF-A in our study. We deduce that the extracellular HMGB1 protein works as a cytokine or a cofactor that amplifies the effect of the AGE-RAGE axis, in an autocrine/paracrine manner, and mediates the secretion of survival factors including VEGF-A for counteracting the oxidative stress. Glycyrrhizin used in our study suppressed 38.0% to 79.8% of VEGF-A upregulation in response to AGEs, instead of totally suppressing it; therefore, a reduced level of VEGF-A upregulation and secretion from RGC-5 cells was induced by AGEs without augmentation of HMGB1. Another possible explanation for the effect of glycyrrhizin on suppression of VEGF-A upregulation is the other signaling pathway triggered by HMGB1 outside the AGE-RAGE axis. In our study, an increase in the TLR4 protein level was detected in RGC-5 cells treated by AGEs for 24 h. HMGB1 can form highly inflammatory complexes with single-stranded DNA, lipopolysaccharide, interleukin-1beta, and nucleosomes, which interact with TLR9, TLR4, interleukin 1 receptor, and TLR2 receptors, respectively [37]. HMGB1 isolated from cells cultured in the presence of IL-1β, IFN-γ, and TNF-α had enhanced proinflammatory activities through binding to mediators such as IL-1β [38]. One recent study showed that Toll-interleukin 1 receptor domain-containing adaptor protein and MyD88, which are known to be adaptor

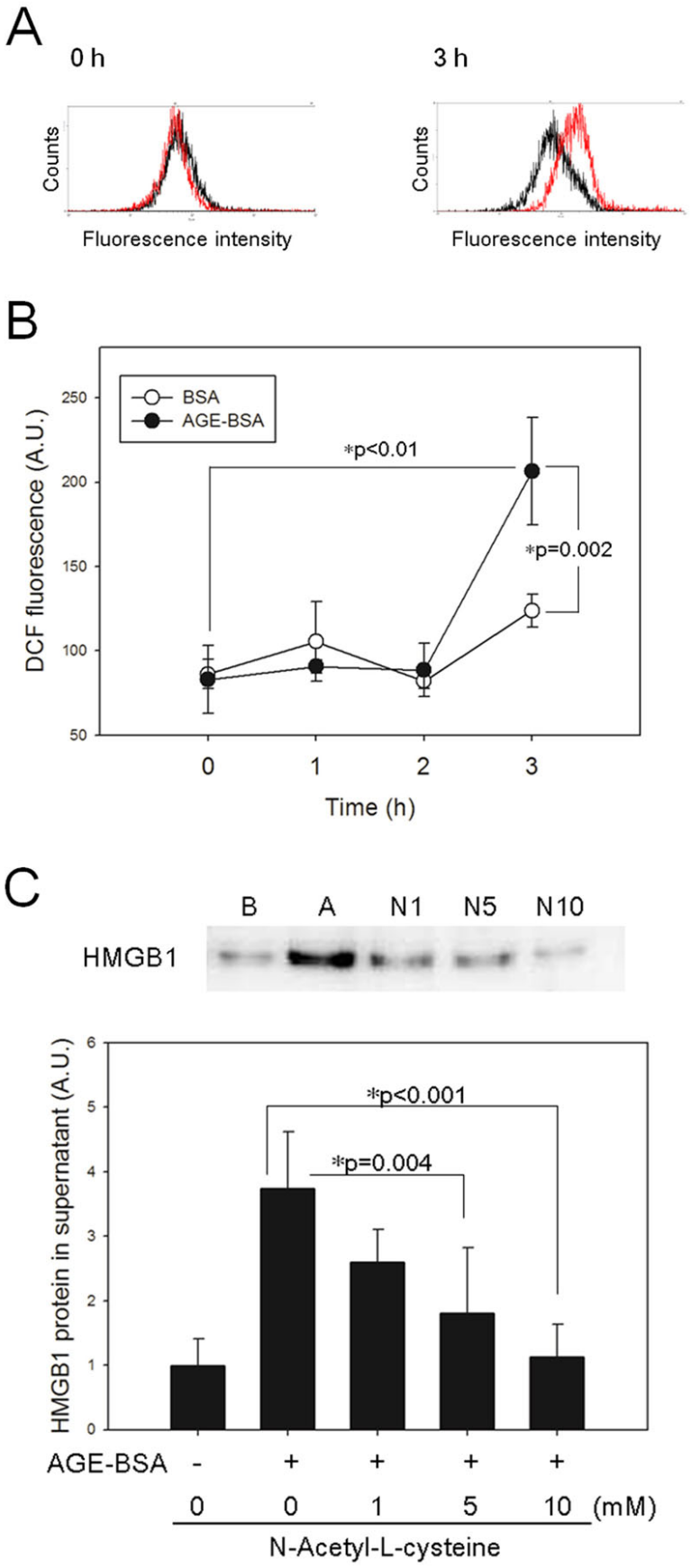


Figure 4. Reactive oxidative species (ROS) could be the trigger for release of high-mobility group box protein 1 (HMGB1) in retinal ganglion cell (RGC)-5 cells. **A**: Flow cytometric histogram shows an increase in ROS at 3 h in RGC-5 cells treated with advanced glycation end products–BSA (AGE-BSA; red) or BSA alone (black). **B**: Histogram shows that DCF fluorescence changes with time (n=3). **C**: Pretreatment of RGC-5 cells with the antioxidant N-acetyl-L-cysteine (NAC) reduces the AGE-BSA-induced HMGB1 protein levels in the supernatant at 3 h in a dose-dependent manner. Abbreviations: **A**, AGEs; **B**, BSA; N1, N5, and N10: RGC-5 cells pretreated with NAC at concentrations of 1, 5, and 10 mM respectively (n=4).

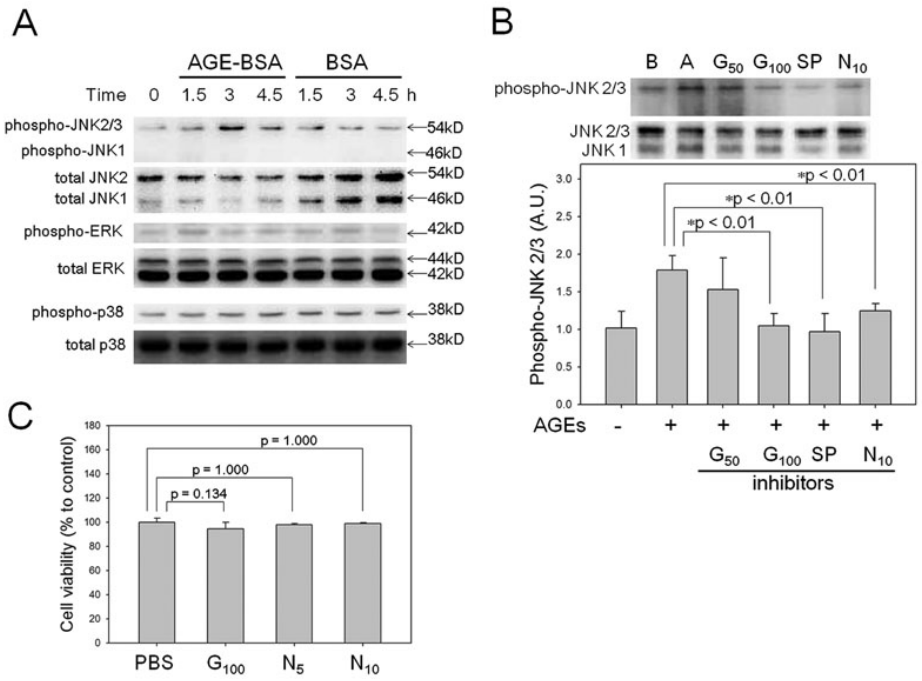


Figure 5. Advanced glycation end products–BSA (AGE-BSA) treatment induces c-Jun N-terminal kinase (JNK)2/3, and JNK2/3 induction is reversed by a high-mobility group box protein 1 (HMGB1) blocker. **A**: The phosphorylations of JNK, extracellular-signal-regulated kinase (ERK), and p38 in mitogen-activated protein kinase (MAPK) signaling in retinal ganglion cell (RGC)-5 cells treated with 200 μg/ml of AGE-BSA for more than 3 h were detected by western blot analysis. Only phospho-JNK2/3, but not phospho-ERK or phospho-p38, significantly increased at 3 h after treatment with AGE-BSA compared with the BSA control (n=4). **B**: Glycyrrhizin (100 μM), SP600125 (10 μM), and NAC (10 mM) decreased AGEs-induced upregulation of phospho-JNK2/3 (n=4). **C**: Treatment with GZ and NAC for 24 h showed insignificant changes relative to sterile PBS in cell viabilities of RGC-5 assayed with XTT (n=6). Abbreviations: **A**, AGE-BSA; **B**, BSA; G_{50} and G_{100}, glycyrrhizin at 50 and 100 μM; SP, SP600125; N_5 and N_{10}, NAC at 5 and 10 mM.

proteins for TLR2 and TLR4, bound to the phosphorylated RAGE after ligand binding and transduced a signal to downstream molecules [39]. TLR signaling pathways have been shown to mediate VEGF production following acute myocardial ischemia-reperfusion [40], and lipopolysaccharide-induced prostaglandin (PG)I2/prostacyclin receptor interaction in macrophage [41]. The role of TLRs in RGC-5 cells and VEGF-A upregulation is the direction of our future study.

HMGB1 is released from neural cells in response to stresses such as chemical ischemia, oxidative stress by hydrogen peroxide, and excitotoxicity by glutamate [42]. In this study, HMGB1 was detected in the cytosol of RGC-5 cells and was released into culture media within 3 h of treatment with AGE-BSA. The cytosolic HMGB1 in RGC-5 cells could be the result of translocation from nuclei. The immunofluorescence studies in our study showed a pattern of cytosolic translocation, which has been reported in murine macrophage-like RAW 264.7 cells treated with hydrogen peroxide [43]. The release of HMGB1 is an active process rather than a passive phenomenon from damaged nuclei or necrotic cells, as there are no significant changes in cell viability and cytotoxicity in RGC-5 cells treated with AGE-BSA. Active secretion of HMGB1 from the RGC-5 cell line has not been demonstrated before the present study. However, the insignificant changes in HMGB1 from nuclear fraction in our study suggest that secretion of newly synthesized HMGB1 protein by RGC-5 may also be possible, which needs to be clarified with further studies.

The elevation of levels of intracellular ROS following AGE-BSA treatment could be responsible for triggering active secretion of HMGB1 from RGC-5 cells. BSA is commonly used as a control for evaluating the effect of AGEs in experimental researches such as in vitro study for diabetic nephropathy with cultured mesangial cells [5], and proximal tubular cell injury via peroxisome proliferator-activated receptor-gamma activation [33]. However, the generation of ROS is a potential drawback of using BSA for in vitro studies. BSA in a concentration of 10 mg/ml induced endoplasmic reticulum stress, and stimulated the production of cellular ROS in renal proximal tubule cells [44]. Although the concentration of BSA used in our study is only 1/50 of that used for renal proximal tubule cells, an increase of ROS was still observed in the BSA-treated RGC-5 cells. In our study, the AGE-BSA-induced ROS levels were significantly higher than those of BSA alone at 3 h of treatment, indicating the effect of advanced glycation overrode BSA itself.

Various intracellular signaling pathways, including ERK1/2 and stress-activated protein kinase/JNK, have been shown to be activated downstream of HMGB1 in the study for scratch wound closure of HaCaT keratinocyte [27], and hippocampal dysfunction in single-Ig-interleukin-1 related

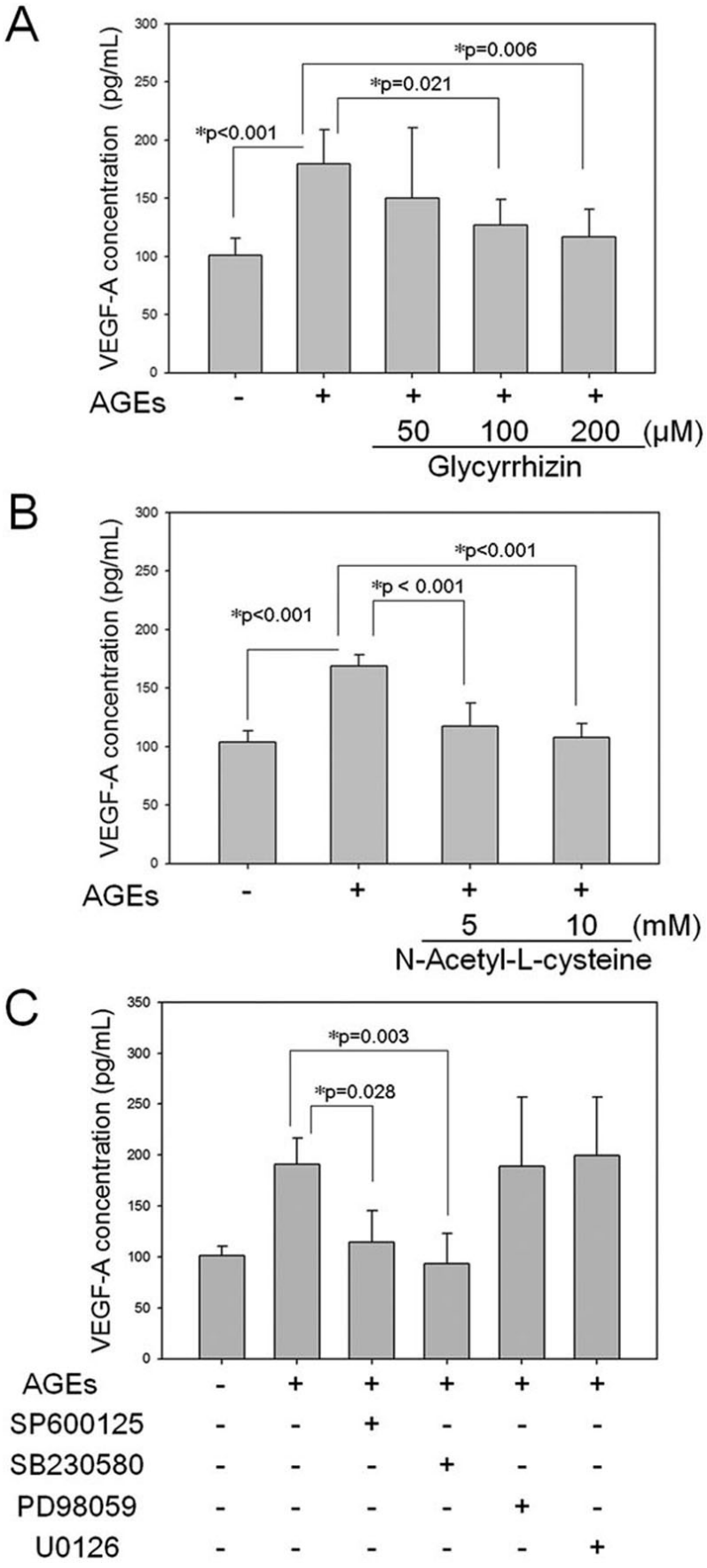


Figure 6. Advanced glycation end products–BSA (AGE-BSA) induced vascular endothelial growth factor (VEGF)-A expression is modulated by glycyrrhizin, antioxidant N-acetyl-L-cysteine (NAC), or specific c-Jun N-terminal kinase (JNK) inhibitor in retinal ganglion cell (RGC)-5 cells. Both glycyrrhizin (**A**) and NAC (**B**) significantly decreased the AGE-BSA-induced increase of VEGF-A concentration in the culture media of RGC-5 cells (n=8). (**C**) AGE-BSA-induced increase of VEGF-A concentration in the culture media of RGC-5 (n=8), was significantly decreased by SP600125 (a specific JNK inhibitor), but not SB230850 (5 μM; p38 inhibitor), as well as PD98059 and U0126 (10 μM each; extracellular-signal-regulated kinase [ERK] inhibitors).

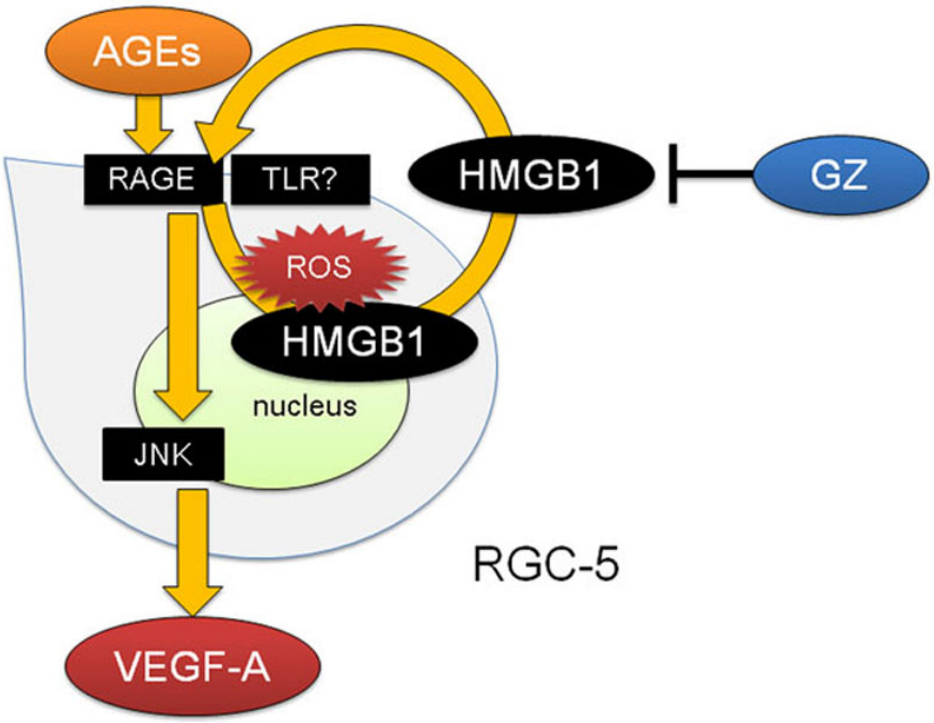


Figure 7. Hypothetic diagram of high-mobility group box protein 1 (HMGB1)-mediated upregulation of vascular endothelial growth factor (VEGF)-A in retinal ganglion cell (RGC)-5 cells in response to advanced glycation end products (AGEs) stimulation. The AGEs cause a rise in intracellular reactive oxidative species (ROS), which results in the release of HMGB1 into the extracellular space. Extracellular HMGB1 augments the signal via RAGE or TLR and mediates secretion of VEGF-A through the JNK signaling pathway, which was blocked by the HMGB1 inhibitor glycyrrhizin (GZ).

receptor (SIGIRR)-deficient mice [28]. Interaction between AGEs and RAGE on the cell surface may also signal via p38 and other MAPKs. In this study, we found unique upregulation of phospho-JNK2/3, but not phospho-p38 or phospho-ERK1/2, at the time of HMGB1 secretion in RGC-5 cells treated with AGE-BSA. Blocking the JNK pathway with a specific inhibitor effectively reduces AGE-BSA-induced VEGF-A production by RGC-5 cells; this effect was not observed in blocking p38 or ERK1/2. These results suggest preferential involvement of the JNK2/3 pathway in AGEs-induced VEGF-A production; however, the molecular mechanisms underlying this phenomenon await clarification.

In conclusion, based on the results of this study, we propose that the HMGB1 protein plays a critical role in AGE-BSA-induced upregulation of VEGF-A in RGC-5 cells, which involves ROS production and the JNK signaling pathway (Figure 7). The findings from a single cell line study may not be sufficient to explain the pathophysiology of diabetic retinopathy involving the entire retina; however, we believe our results provide the framework for future studies investigating the role of HMGB1 in diabetic retinopathy.

ACKNOWLEDGMENTS

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