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HMGB1 enhances AGE-mediated VSMC proliferation via an increase in 5-LO-linked RAGE expression

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ABSTRACT

Receptors for advanced glycation end-product (RAGE) play a pivotal role in the progression of proliferative vascular diseases. However, the precise mechanisms regulating RAGE expression in vascular smooth muscle cells (VSMCs) of the injured vasculatures is unclear. Given the potential importance of 5-lipoxygenase (5-LO) derived mediators in cellular responses mediated by RAGE, this study aimed to evaluate in VSMCs treated with high mobility group box 1 (HMGB1): 1) the RAGE expression; 2) the AGE-induced VSMC proliferation; 3) the role of 5-LO signaling in HMGB1-induced RAGE expression. In cultured human VSMCs stimulated with HMGB1 (100 ng/ml), RAGE mRNA and protein expression were markedly increased along with an increase in AGE-mediated VSMC proliferation. Both of these effects were markedly attenuated in cells pretreated with zileuton (1–10 μM), a 5-LO inhibitor, as well as in cells transfected with 5-LO siRNA, suggesting a potential involvement of 5-LO signaling in HMGB1-mediated RAGE expression in VSMCs. Moreover, 5-LO expression, accompanied by production of leukotrienes was markedly increased in HMGB1-stimulated VSMCs, which was attenuated in cells deficient of TLR2 or RAGE. Taken together, our results suggest that HMGB1-induced increase in 5-LO expression enhances RAGE expression in VSMCs, which stimulates AGE-mediated VSMC proliferation. Thus, the 5-LO-RAGE signaling axis in VSMCs might serve as a potential therapeutic target for vascular remodeling in the injured vasculature.

1. Introduction

Endoluminal vascular interventional procedures cause vascular injury and subsequent release of endogenous damage-associated molecular patterns, including high-mobility group box 1 (HMGB1) [1]. The extracellular HMGB1 may bind directly to cell surface receptors on somatic and immune cells mediating intracellular signaling cascades [2,3]. The functions of HMGB1 are known to be dependent on molecular binding partners and redox status [2,4,5]. In vascular smooth muscle cells (VSMCs), HMGB1 has been shown to increase inflammatory cytokine production via activation of NLRP3 inflammasome [6], and play a pivotal role in vascular remodeling of the injured vasculatures.

VSMCs de-differentiate to a proliferative/synthetic phenotype in response to vascular injury and various pathological states including neointima hyperplasia [7,8]. In proliferative vascular lesions, several key proteins within the leukotriene cascades, including 5-lipoxygenase (5-LO) and 5-LO-activating protein (FLAP) are highly expressed [9,10]. Moreover, in FLAP-deficient mice, neointima hyperplasia in the injured arteries was significantly attenuated by reduction of inflammatory cytokine release from FLAP-deficient macrophages [11]. In line with the results of our previous studies in which 5-LO was shown to play a pivotal role in the development of vascular remodeling diseases [12–14], the role of 5-LO pathways in neointima formation has also been described [11].

The importance of 5-LO-derived mediators in cellular responses mediated by pattern recognition receptors, including receptor for advanced glycation end-product (RAGE) has been previously described [15,16]. RAGE expression is markedly upregulated in VSMCs at the site of vascular injury [17], and cell surface expression of RAGE is reported to be partly regulated by NF-κB [18,19]. Further, 5-LO metabolites have been suggested to participate in in vivo and in vitro responses to ligands for pattern recognition receptors [20,21]. Although the importance of 5-LO signaling in RAGE expression has been suggested in the previous

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studies [16], there is a strong requirement for detailed studies on mechanisms regulating RAGE expression in VSMCs, in order to develop effective therapeutic strategies for proliferative vascular diseases.

The increased RAGE expression in VSMCs has been suggested to be a key player in the development of proliferative vascular diseases [22]. Given the importance of 5-LO in cellular responses mediated by RAGE, we hypothesized that the HMGB1-5-LO signaling axis might directly influence VSMC proliferation in the injured vasculature via the RAGE signaling pathway. Thus, the role of HMGB1 in regulating RAGE expression in VSMCs was evaluated in the present study. In addition, we also evaluated the importance of 5-LO signaling in HMGB1-induced RAGE expression and subsequent AGE-induced VSMC proliferation.

2. Materials and methods

2.1. Chemicals and antibodies

Recombinant human HMGB1 was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Zileuton ((±)-N-hydroxy-N-(1-benzo[*b*] thien-2-ylethyl)urea) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Advanced glycation end product–BSA (AGE-BSA) was purchased from BioVision (Milpitas, CA, USA). 5-LO (sc136195, 50 μg/0.5 ml) and TLR4 (sc293072, 200 μg/ml) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Beverly, MA, USA). TLR2 (ab9100, 0.1 mg/ml) and RAGE (ab89911, 0.5 mg/ml) antibodies were purchased from Abcam (Cambridge, MA, USA). β-Actin (sc47778, 200 μg/ml) antibody was purchased from Santa Cruz Biotechnology Inc.

2.2. Cell culture

Human aortic VSMCs (hVSMCs) were purchased from the ATCC (Manassas, VA, USA). hVSMCs were grown in culture dishes using smooth muscle cell growth medium (Gibco BRL, Grand Island, NY, USA), smooth muscle growth supplement (Gibco BRL), 10% fetal bovine serum, antibiotic-antimycotic solution (Gibco BRL), and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. hVSMCs between passage 4 and 8 were used for experiments.

2.3. Cell proliferation assay

hVSMC proliferation rates were determined by MTT and cell count assays. In brief, cells (a total of 1×10^5 cells) were treated with MTT working solution (EZ-Cytox, Daeil Laboratories, Seoul, ROK) and then incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. The absorbance value of the solution was obtained at a wavelength of 450 nm by ELISA. Equal number of cells were seeded in 6 well. And then cells were starved in 1% FBS for 24 h and were treated with anti-RAGE antibody (10 μg/ml), HMGB1 (100 ng/ml) and AGE (30 μg/ml). Cells were counted using a hemocytometer. Relative proliferation rates were determined by comparing cells with control.

2.4. RNA isolation and RT-PCR

5-LO and RAGE mRNA expression in hVSMCs was quantified by RT-PCR using GAPDH as an internal control. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, NY, USA), in accordance with the manufacturer's instructions. RNA (1 μg) was reverse transcribed into cDNA using the ImProm-II reverse transcription system (Promega, Madison, WI, USA). PCR amplification was carried out using 5-LO-specific primers (forward, 5′-AGT ACC TGA CCG TGG TGA TCT TCA-3′ reverse, 5′-TCA GAT GGC CAC ACT GTT CGG AAT-3′) and RAGE-specific primers (forward, 5′-GC TGT CAG CAT CAG CAT CAT-3′ reverse, 5′-AT TCA GTT CTG CAC GCT CCT-3′). Equal amounts of RT-PCR products obtained were separated on 1–1.5% agarose gel stained with ethidium bromide. Signals from bands were quantified using the UN-SCAN-IT GEL 7.1 program and data were expressed as relative GAPDH densities.

2.5. Western blot analysis

hVSMC lysates were prepared in ice-cold lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), and equal amounts of protein were separated on 8–10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham-Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked with 5% skim milk in Tris buffered saline Tween-20 (TBST) for 2 h at room temperature and then incubated overnight with primary antibody at 4 °C. Following this, membranes were washed with TBST, incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature and then chemiluminescence intensities were measured using the LAS-3000 System (Fuji Photo Film, Minato-ku, Tokyo, JP). Membranes were re-blotted using anti-β-actin antibody as an internal control. Signals from bands were quantified using the UN-SCAN-IT GEL 7.1 program and data were expressed as relative β-actin densities.

2.6. Measurement of leukotriene B4 (LTB4) production

As an indicator for 5-LO activity, LTB4 production was measured in the culture media using an ELISA kit from R&D Systems, Inc. The culture supernatant of HMGB1-stimulated hVSMCs was collected, and then ELISA was performed in accordance with the manufacturer's instructions.

2.7. Small interfering RNA (siRNA) preparation and transfection

5-LO, TLR2, TLR4 and RAGE siRNA oligonucleotides were synthesized by Bioneer (Daejeon, ROK). The siRNA negative control duplex was used as the control oligonucleotide. siRNA molecules were transfected into the cells using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions.

2.8. Statistical analysis

Results were expressed as means ± standard error of the means (SEMs). One-way analysis of variance (ANOVA) followed by Dunnett multiple comparison test was performed. Student's *t*-test was used to determine significant differences between two groups. Statistical significance was accepted for p values < 0.05.

3. Results

3.1. Effects of HMGB1 on AGE-induced VSMC proliferation

To determine the role of HMGB1 on VSMC proliferation, hVSMCs were treated with HMGB1 (30 and 100 ng/ml) for 24 h or with HMGB1 (100 ng/ml) for 24 and 48 h, and then cell proliferation was measured by MTT and cell count assays. As shown in Fig. 1, although exposure to HMGB1 (30 and 100 ng/ml) had no direct effects on VSMC proliferation, AGE (30 μg/ml)-induced VSMC proliferation was markedly increased in cells stimulated with HMGB1 (100 ng/ml), which was attenuated in cells pretreated with anti-RAGE antibody. These results suggested that HMGB1 enhanced AGE-induced VSMC proliferation via modulation of AGE receptor signaling.

3.2. Effects of HMGB1 on RAGE expression in VSMCs

To determine the effects of HMGB1 on RAGE expression, the mRNA and protein levels of RAGE in cells stimulated with HMGB1 were measured by RT-PCR and Western blot, respectively. When cultured hVSMCs were stimulated with HMGB1 (100 ng/ml) for 0 to 48 h, the levels of RAGE mRNA started to increase at 12 h, and continuously

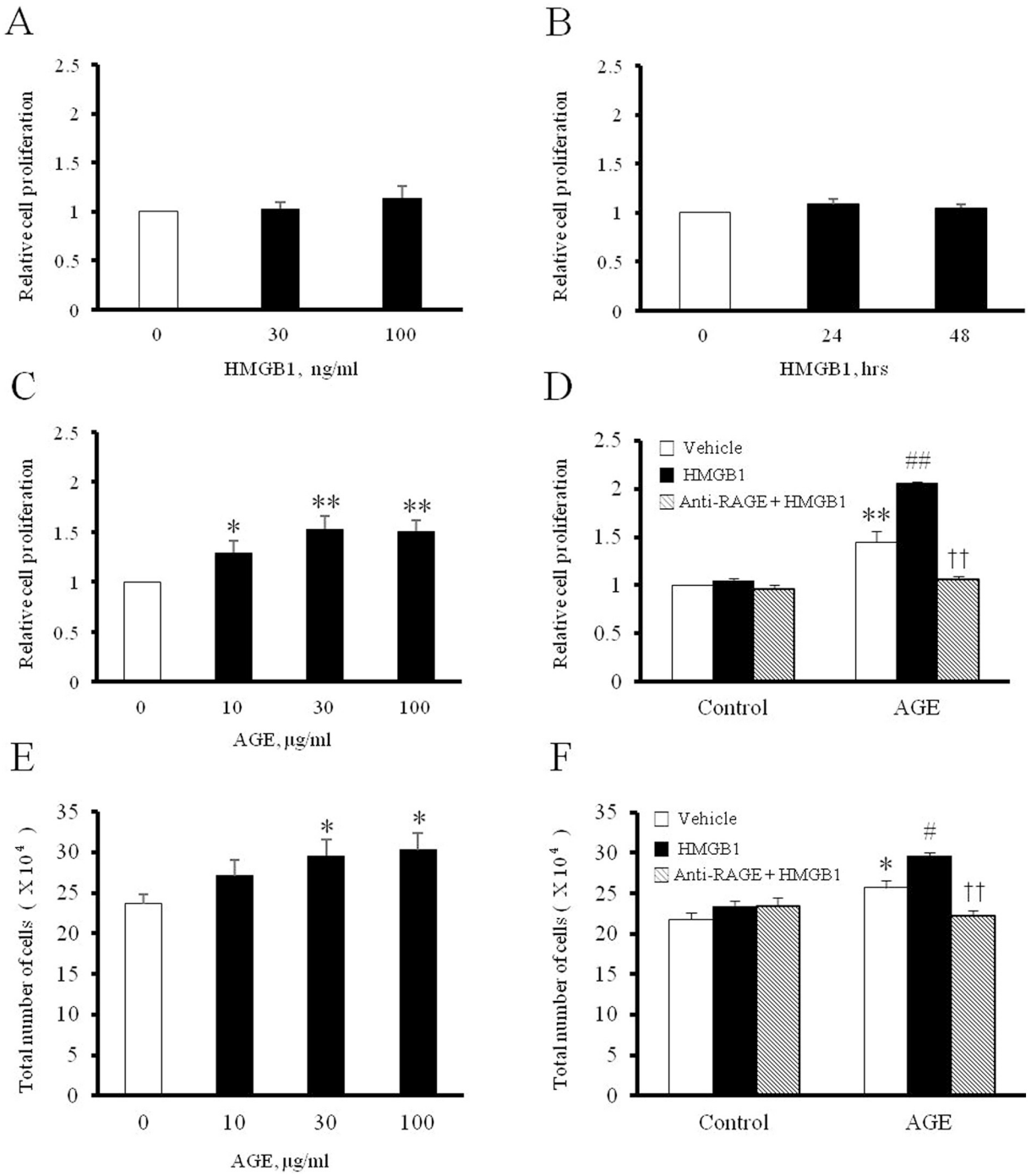

Fig. 1. Effects of HMGB1 on AGE-induced proliferation of hVSMCs.
(A and B) hVSMCs were treated with HMGB1 (30 and 100 ng/ml) for 24 h or with HMGB1 (100 ng/ml) for 24 and 48 h. Cell proliferation was determined by MTT assay, and relative cell proliferation was expressed as the means ± SEMs of 4 independent experiments. (C and E) hVSMCs were treated with AGE (0 to 100 μg/ml) for 24 h. Relative cell proliferation determined by MTT and cell count assays was expressed as the means ± SEMs of 3–4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding value at 0. (D and F) hVSMCs were pre-treated with HMGB1 (100 ng/ml) for 24 h, and then stimulated with AGE (30 μg/ml) for 24 h in the presence or absence of anti-RAGE (10 μg/ml). Relative cell proliferation determined by MTT and cell count assays was expressed as the means ± SEMs of 3–4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. corresponding control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. corresponding vehicle, and $^{\dagger\dagger}P < 0.01$ vs. corresponding value in HMGB1 group.

increased until 48 h. Likewise, the RAGE protein level in cells started to increase at 12 h post HMGB1 treatment, and continuously increased thereafter in a time-dependent manner (Fig. 2).

3.3. The role of 5-LO in HMGB1-induced RAGE expression in VSMCs

Since zileuton has little effects on several related enzymes, including 12-LO, 15-LO and cyclooxygenase even at concentrations up to 100 μM [23], 1–10 μM concentration of zileuton was used to inhibit 5-LO in this study. In cultured hVSMCs, HMGB1-induced increase in RAGE expression was markedly attenuated in cells pretreated with zileuton (1–10 μM) as well as in cells depleted of 5-LO using siRNA, suggesting a potential involvement of 5-LO in HMGB1-induced RAGE expression (Fig. 3A and B). In cells stimulated with HMGB1, AGE-induced VSMC

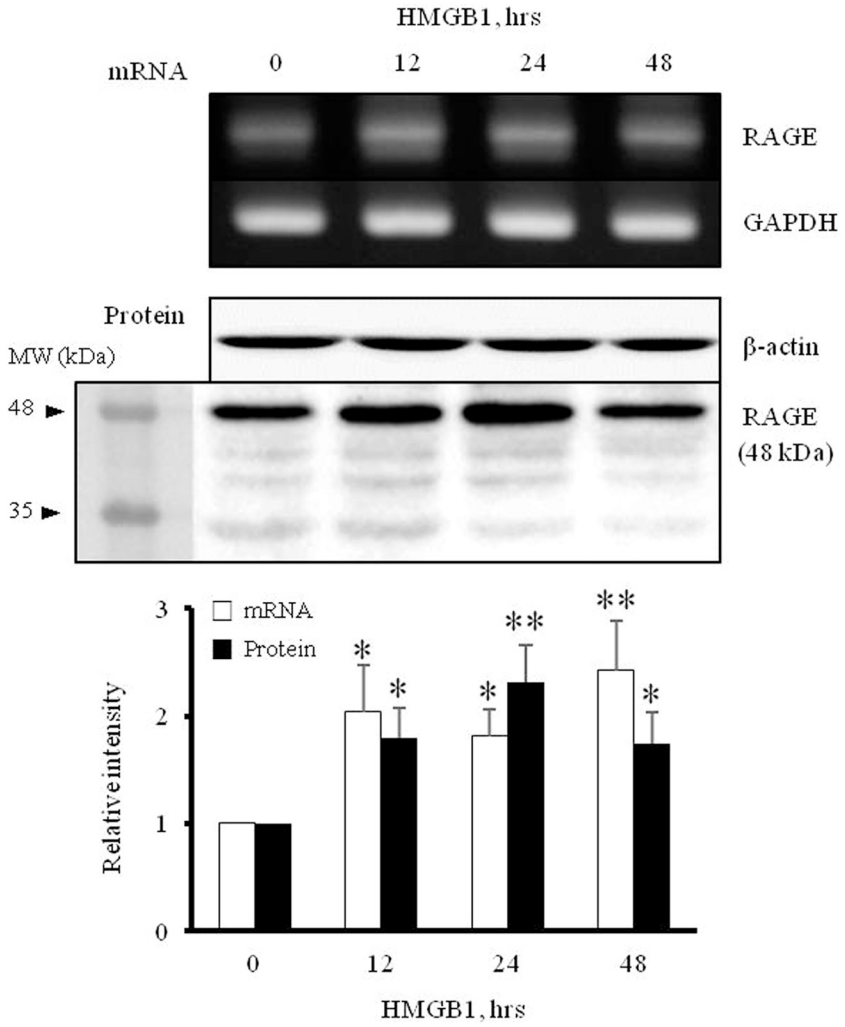

Fig. 2. Effects of HMGB1 on RAGE expression in hVSMCs.
hVSMCs were treated with HMGB1 (100 ng/ml) for 0 to 48 h, and then the mRNA and protein levels of RAGE were determined by RT-PCR and Western blot, respectively. GAPDH and β-actin were used as internal controls. Blots are representative of 3–4 independent experiments. Bottom: Relative intensities were expressed as the means ± SEMs of 3–4 independent experiments. *P < 0.05, **P < 0.01 vs. corresponding value at 0. MW, molecular weight.

proliferation was also markedly increased, which was attenuated in cells pretreated with zileuton (10 μM) as well as in 5-LO-depleted cells (Fig. 3C and D). These results suggested a pivotal role of 5-LO in regulating RAGE expression and subsequent AGE-induced VSMC proliferation.

3.4. Effects of HMGB1 on 5-LO expression in VSMCs

To determine the effects of HMGB1 on 5-LO expression in hVSMCs, cells were stimulated with 100 ng/ml HMGB1 for 12 h or 24 h, and then 5-LO mRNA and protein levels were determined by RT-PCR and Western blotting, respectively. As shown in Fig. 4A and B, 5-LO mRNA expression in HMGB1-treated cells was increased at 12 h, following which its expression increased in a dose-dependent manner. Likewise, 5-LO protein in HMGB1-treated cells was markedly expressed at 24 h, following which its expression increased in a dose-dependent manner. In a separate experiment, LTB4 production was measured using ELISA to determine the functional role of 5-LO expressed in HMGB1-stimulated cells. As shown in Fig. 4C and D, LTB4 production in HMGB1 (100 ng/ml)-treated cells was gradually increased for up to 48 h, and dose-dependency was observed up to 100 ng/ml. These results suggested that 5-LO-derived metabolites might be involved in RAGE expression in HMGB1-stimulated VSMCs.

As shown in Fig. 5, HMGB1 receptors including TLR2, TLR4 and RAGE were found to be consistently expressed in cultured hVSMCs. Thus, we determined the individual role of these receptors in 5-LO expression in HMGB1-stimulated VSMCs using VSMCs transfected with siRNAs for TLR2, TLR4, or RAGE. In cells transfected with scrambled siRNA duplex (negative controls), HMGB1 increased the protein levels of 5-LO, which was significantly attenuated in cells deficient of TLR2 or RAGE.

4. Discussion

In the present study, the surface expression of RAGE was markedly increased in HMGB1-stimulated hVSMCs and was accompanied by an increase in AGE-mediated cellular proliferation. Both HMGB1-induced RAGE expression and AGE-induced VSMC proliferation were markedly attenuated by inhibition of 5-LO signaling, suggesting a potential involvement of 5-LO signaling in HMGB1-induced RAGE expression in hVSMCs.

Although VSMCs are the major cell type in blood vessel walls and play pivotal roles in the process of the proliferative vascular diseases, but little is known of the role played by VSMCs in vascular remodeling in the damaged vasculatures. In addition to macrophages and endothelial cells, VSMCs have been identified as a major source of HMGB1

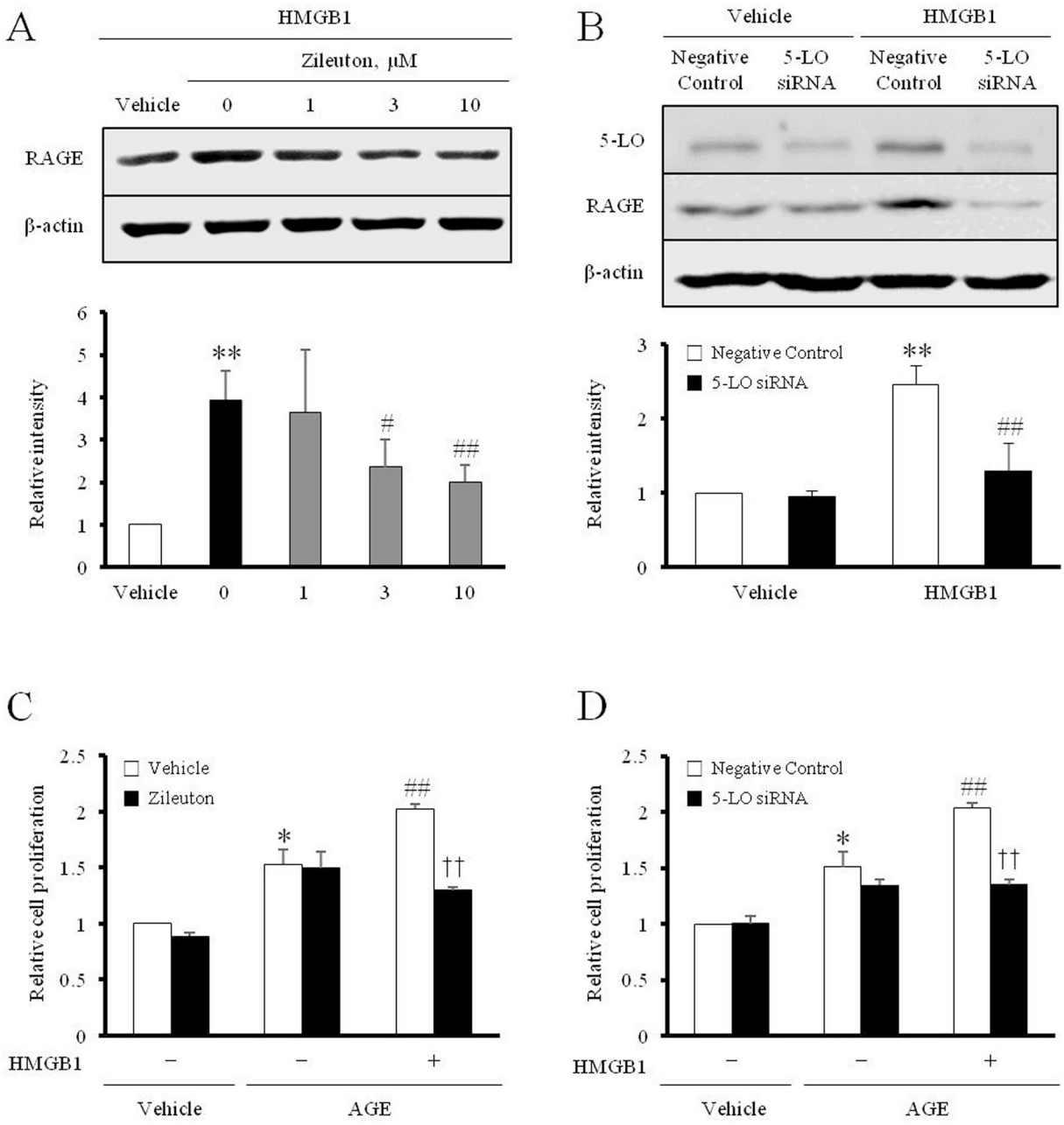

Fig. 3. Role of 5-LO in HMGB1-induced RAGE expression with subsequent proliferation of hVSMCs.
(A and B) hVSMCs were pre-treated with zileuton (0 to 10 μM) for 1 h or transfected with 5-LO siRNA (200 nM) for 48 h, and then stimulated with HMGB1 (100 ng/ml) for 24 h. The protein levels of RAGE were determined by Western blotting using β-actin as an internal control. Blots are representative of 5 independent experiments. Bottom: Relative intensities were expressed as the means ± SEMs of 5 independent experiments. $^{**}P < 0.01$ vs. corresponding vehicle or negative control and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. corresponding control or negative control. (C and D) hVSMCs were pre-treated with zileuton (10 μM) for 1 h or transfected with 5-LO siRNA (200 nM) for 48 h, and then stimulated with HMGB1 (100 ng/ml) for 24 h. These cells were treated with AGE (30 μg/ml) for 24 h, and then cell proliferation was determined by MTT assay. Relative cell proliferation was expressed as the means ± SEMs of 3 independent experiments. $^{*}P < 0.05$ vs. corresponding vehicle, $^{\#\#}P < 0.01$ vs. corresponding value in HMGB1-untreated AGE group and $^{\dagger\dagger}P < 0.01$ vs. corresponding value in HMGB1-treated group.

production in atherosclerotic lesions [1,24,25]. When cells are exposed to stress, nuclear HMGB1 can be translocated outside the cells, and extracellular HMGB1 may bind directly to the cell surface receptors [2,3], where it elicits the production of proinflammatory mediators [26,27]. Among various cell membrane pattern recognition receptors including toll-like receptors (TLR2 and TLR4) and RAGE, the binding of RAGE to its ligands has been importantly implicated in the progression of vascular remodeling [28,29].

Vascular injury initiates inflammatory cell infiltration into damaged tissues, followed by increased production of endogenous DAMPs including HMGB1, which is one of the best characterized DAMPs [1]. HMGB1 primarily resides in the nuclei of quiescent cells [30], but its release is increased in the injured vasculature [1,24]. Reportedly, HMGB1 levels in atherosclerotic plaque were increased, and it has been suggested to be involved in vascular remodeling via the potentiation of inflammatory processes [31,32]. Recent evidences have indicated that HMGB1 is required for the development of neointima lesions following vascular injury [33,34]. Thus, HMGB1 released in the injured vasculatures is also suggested to be a key player in vascular remodeling. Although VSMC proliferation has been implicated in the progression of

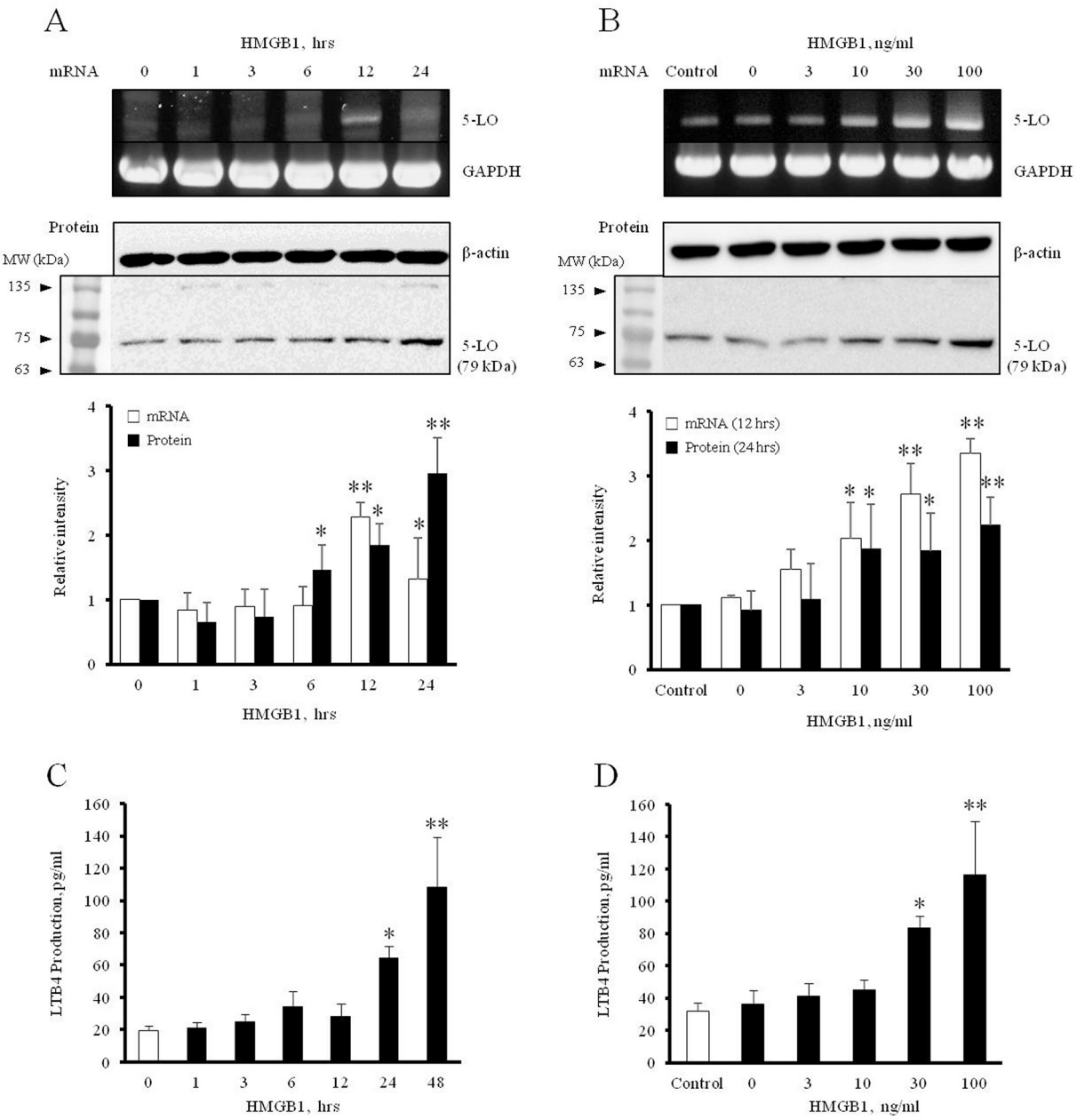

Fig. 4. Characteristics in 5-LO expression and leukotriene production in HMGB1-stimulated hVSMCs.
(A and B) hVSMCs were treated with HMGB1 (100 ng/ml) for 0 to 24 h, or HMGB1 (0 to 100 ng/ml) for 12 h (mRNA) and 24 h (Protein). The mRNA and protein levels of 5-LO were determined by RT-PCR and Western blotting, respectively. GAPDH and β-actin were used as internal controls. Blots are representative of 3–4 independent experiments. MW, molecular weight. Bottom: Relative intensities were expressed as the means ± SEMs of 3–4 independent experiments. *P < 0.05, **P < 0.01 vs. corresponding controls. (C and D) hVSMCs were treated with HMGB1 (100 ng/ml) for 0 to 48 h, and HMGB1 (0 to 100 ng/ml) for 24 h. LTB4 production in the culture media was measured by ELISA. Results were expressed as the means ± SEMs of 3 independent experiments. *P < 0.05, **P < 0.01 vs. corresponding controls.

vascular remodeling [35,36], the precise role of HMGB1 in VSMC proliferation is unclear. In our present study, stimulation of human VSMCs with HMGB1 induced surface expression of RAGE, which was accompanied by an increase in AGE-mediated cellular proliferation. These results suggested an important role of RAGE in VSMC on vascular remodeling in the injured vasculature.

RAGE is expressed in many cell types including VSMCs [37]. In VSMCs, the low basal expression of RAGE in normal vessels was markedly upregulated at the site of vascular injury [17,38], thereby mediating AGE-induced VSMC proliferation [39]. The cell surface expression of RAGE is partly controlled by NF-κB signaling [18,19]. Several independent studies have indicated that cellular responses mediated by pattern recognition receptors are characterized by synthesis of the 5-LO-derived lipid mediator LTB4 [15], and 5-LO metabolites have been suggested to participate in *in vivo* and *in vitro* responses to ligand for pattern recognition receptors including HMGB1 [40]. Likewise, in our present study, stimulation of human VSMCs with HMGB1 induced surface expression of RAGE, which was significantly attenuated in cells pretreated with zileuton, a 5-LO inhibitor [41], as well as in cells transfected with 5-LO siRNA. Moreover, 5-LO expression with resultant production of leukotrienes were markedly increased in VSMC stimulated with HMGB1. These results suggested a pivotal role for 5-LO signaling in RAGE expression in VSMCs exposed to HMGB1.

In association with the increased expression of RAGE in HMGB1-

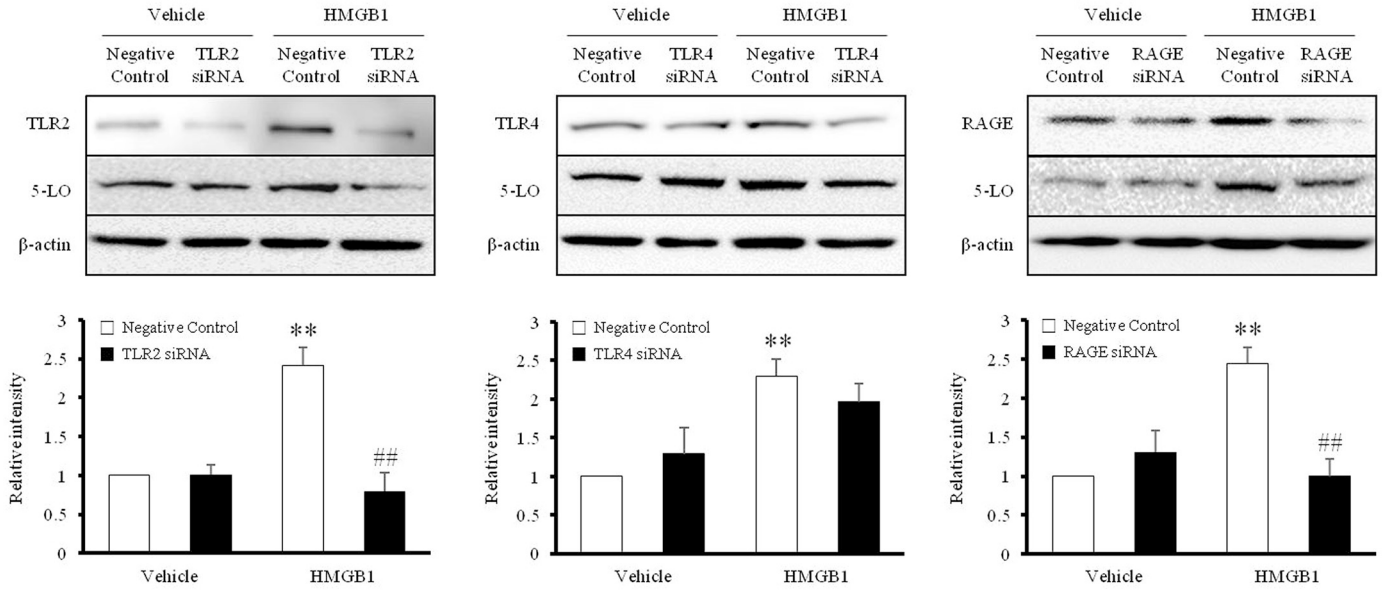

Fig. 5. Involvement of TLR2, TLR4 and RAGE in HMGB1-induced 5-LO expression in hVSMCs.
hVSMCs were transfected with siRNAs (200 nM) for TLR2, TLR4 and RAGE for 48 h, and then stimulated with HMGB1 for 24 h. The expression of 5-LO was determined by Western blotting using β-actin as an internal control. Blots are representative of 4–6 independent experiments. Relative band intensities to β-actin were quantified, and data were expressed as the means ± SEMs of 4–6 independent experiments. $^{**}P < 0.01$ vs. corresponding value in vehicle. $^{\#\#}P < 0.01$ vs. corresponding negative control.

stimulated VSMCs, AGE-induced proliferation was markedly increased in cells pretreated with HMGB1, which was also attenuated by inhibition of 5-LO signaling in VSMCs. Considering the fact that the phenotypic modulation of VSMCs is a key cellular event driving vascular remodeling, it is suggested that 5-LO-RAGE signaling axis in VSMCs plays a pivotal role in the development of vascular remodeling. Taken together, the results of present study suggest that the increased expression of 5-LO in HMGB1-exposed VSMCs drives the overexpression of RAGE, which facilitates AGE-mediated VSMC proliferation. Thus, the 5-LO-RAGE signaling axis in VSMCs might serve as a potential therapeutic target for vascular remodeling in the injured vasculature.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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