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RESEARCH PAPER

Glycyrrhizic Acid Attenuates Balloon-Induced Vascular Injury Through Inactivation of RAGE Signaling Pathways

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Abstract

Percutaneous coronary intervention is a well-established technique used to treat coronary artery disease, but the risk of coronary artery in-stent restenosis following percutaneous coronary intervention is still high. Previous studies revealed that high mobility group protein B1 (HMGB1) plays a critical role in neointima formation. In this study, we aimed to investigate the role of glycyrrhizic acid (GA), an HMGB1 inhibitor, in the process of neointima formation and the potential mechanisms. We investigated the role of GA in neointima formation through an iliac artery balloon injury model in rabbits. Proliferation, migration, and phenotype transformation of human vascular smooth muscle cells (VSMCs) were observed. Besides, inflammation and receptor for advanced glycosylation end products (RAGE) signaling pathways were studied. The results indicate that GA attenuated neointima formation and downregulated HMGB1 expression in injured artery in rabbits. HMGB1 promoted proliferation, migration, and phenotype transformation through the activation of RAGE signaling pathways in VSMCs, and blockade of HMGB1 by GA (1, 10, and 100 μM) could attenuate those processes and reduce proliferation of human VSMCs. In conclusion, the HMGB1 inhibitor GA might be useful to treat proliferative vascular diseases by downregulating RAGE signaling pathways. Our results indicate a new and promising therapeutic agent for restenosis.

Keywords: Glycyrrhizic acid; high mobility group protein B1; inflammation; vascular smooth muscle cell; receptor for advanced glycosylation end products

Background

Percutaneous coronary intervention is currently a popular and standard treatment for coronary artery disease. However, even with the new generation of drug-eluting stents, in-stent restenosis after percutaneous coronary intervention remains a challenge for interventional cardiologists [1].

Inflammatory reaction followed by excessive migration and proliferation of vascular smooth muscle cells (VSMCs) is thought to be a key factor involved in the process of restenosis after vascular injury [2]. High mobility group protein B1 (HMGB1), which is expressed in nearly all eukaryotic cells, can not only regulate DNA transcription and replication but also actively participates

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in initiating an inflammatory cascade as an early inflammatory cytokine. As a DNA chaperone, HMGB1 can interact with and enhance the activities of a number of transcription factors, including p53 and p73, and nuclear hormone receptors, including estrogen receptor. After release from or being actively secreted by cells, HMGB1 can bind with high affinity to several receptors, including receptor for advanced glycosylation end products (RAGE), Toll-like receptor 2 (TLR2), TLR4, triggering receptor expressed on myeloid cells 1 (TREM1), and CD24, mediating the response to cell migration, cell activation, cell proliferation, and cell differentiation [3]. Previous studies have found that HMGB1 plays an important role in cardiovascular diseases, including atherosclerosis [4, 5] and intimal hyperplasia [6], through TLR2, TLR4, and the RAGE pathway. In a previous study, we also found that HMGB1 could induce endothelial cell hyperpermeability via the TRL4/caveolin 1 pathway [7].

Glycyrrhizic acid (GA; also known as glycyrrhizin) [8], a triterpenoid saponin glycoside, is a major active constituent of the licorice plant *Glycyrrhiza glabra*. Pharmaceutical chemistry studies have shown that GA has various potential bioactivities, such as antiviral [9, 10], antioxidant [11], and anti-inflammatory [12] activities and endothelium protection [6]. GA has been used clinically for more than 20 years in China and Japan and has been confirmed to be a safe and nontoxic biomaterial [9]. Recently, GA has been recognized as an inhibitor of HMGB1 [9]. Although previous studies showed GA treatment inhibited the intimal hyperplasia of arteries after injury [13] in a mouse model or a rat model, the effect of GA in a rabbit model and the underlying mechanism are still unclear. In the present study, we aimed to investigate the role of GA in a rabbit model of balloon-induced iliac artery injury.

Materials and Methods

Animal Model

New Zealand White rabbits (male, 4 months old, 1.8–2.2 kg) were from the Hunan SJA Laboratory Animal Co. An iliac artery balloon injury model in rabbits was made as previously described with minor modification [14]. Briefly, pentobarbital (30 mg/kg marginal ear vein injection) was used for anesthesia. The right iliac artery of rabbits was isolated and punctured gently after incision of the right thigh. A 0.014 inch (diameter) guide wire (Terumo, Japan) was introduced and advanced to the aorta, and then a 2.5 mm×20 mm balloon (Medtronic, USA) was advanced into the iliac artery along this wire. The balloon was gradually withdrawn three times after inflation to a moderate resistance (the balloon was inflated to 12 atm). Sham operations were performed on the left iliac artery. The distal iliac artery puncture point was ligated after the operation. Rabbits that had been operated on received normal saline or GA (50 mg/kg/day) through ear vein intravenous injection. The rabbits were euthanized after 28 days of observation, and the iliac arteries were harvested for further examination. All the protocols were approved by the Animal Research Committee, Central South University, Hunan, China. The study was conducted in accordance with the *Basic & Clinical Pharmacology & Toxicology* policy for experimental studies [15].

Culture of VSMCs

The human VSMC cell line was cultured in Dulbecco's modified Eagle's medium (Gibco) containing 1% penicillin-streptomycin and 10% fetal bovine serum (Gibco) and was maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were harvested for further protein and messenger RNA (mRNA) expression analysis. Cell death was tested with SYTOX Green (Invitrogen) staining after treatment with 100 μM GA without serum or with 10% serum.

Cell Proliferation

Cell proliferation was studied with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma). Briefly, 3×10^4 cells per well were seeded into 96-well plates in 200 μL medium. After serum starvation for 24 h, cells were treated with different concentrations of GA (Macklin, China) (10 mg/mL in 10 mM NaOH at 37 °C, pH 7.35–7.45) and incubated for 12, 24, 48, or 72 h at 37 °C. Then 20 μL MTT solution (5 mg/mL) was used for the test. The absorbance was measured

at 490 nm with a microplate reader (Thermo Scientific).

Cell Migration

Cell migration was studied with a transwell chamber. Briefly, the upper chamber was seeded with serum-starved VSMCs (2×10^5), while the lower chamber was filled with Dulbecco's modified Eagle's medium containing HMGB1. Cells on the bottom membrane were fixed with 4% polyoxymethylene for hematoxylin and eosin staining 12 h later. The cell number was counted in five randomly selected squares per well with a fluorescence microscope (Nikon).

Reverse Transcription–Quantitative Polymerase Chain Reaction

Trizon (Cwbiotech, China) was used to collect total RNA according to the manufacturer's protocol. Total RNA was reverse transcribed into complementary DNA (cDNA) with a cDNA synthesis kit (Thermo). Real-time polymerase chain reaction (PCR) was performed with a 7300 real-time PCR system with gene-specific primers and SYBR Green PCR master mix (Bio-Rad). The PCR thermal cycling protocol was as follows: one cycle of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and extension at 72 °C for 1 min. The following primer sequences were used: IL-1β, forward 5′-CTGAGCTCGCCAGTGAAATG-3′ and reverse 5′-GCTGTAGTGGTGGTCGGAGATT-3′; IL-6, forward 5′-CAGCCACTCACCTCTTCAGA-3′ and reverse 5′-GTTGGGTCAGGGGTGGTTAT-3′; IL-8, forward 5′-ACATACTCCAAACCTTTCCACC-3′ and reverse 5′-AAAACTTCTCCACAACCCTCTG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5′-CCATGGGTGGAATCATATTGGA-3′ and reverse 5′-TCAACGGATTTGGTCGTATTGG-3′. Relative quantification was done by the $2^{-\Delta\Delta Ct}$ method, with the GAPDH level as a reference.

Western Blotting Analysis

The Western blotting assay was performed as previously described. Briefly, radioimmunoprecipitation assay buffer (Cwbiotech) containing protease and phosphatase inhibitor cocktail (Cwbiotech) was used for cell lysis. An aliquot of lysate was then centrifuged at 12,000*g* at 4 °C for 15 min. A bicinchoninic acid protein assay kit (Cwbiotech) was used to verify the protein concentration. Equal amounts of protein (25 μg) were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto poly(vinylidene fluoride) membranes, followed by incubation with primary antibodies and then horseradish peroxidase–labeled secondary antibodies. Quantitative analysis of Western blotting was done with Gel-Pro Analyzer. The dilutions of the primary antibodies were as follows: smooth muscle protein 22α (SM22α) antibody (Abcam) 1:2,000; p38 antibody (Proteintech) 1:1,000; phospho-p38 antibody (Cell Signaling Technology) 1:1000; HMGB1 antibody (Proteintech) 1:2,000; RAGE antibody (Proteintech) 1:1,000; p65 antibody (Proteintech) 1:2,000; and GAPDH antibody (Proteintech) 1:3,000.

Hematoxylin and Eosin Staining and Immunohistochemistry/ Immunofluorescence of Vascular Sections

Rabbit vascular tissues were fixed and then embedded in paraffin. Sections (5 μm) were deparaffinized and processed for hematoxylin and eosin staining or incubated with primary antibodies (CD68 antibody, Abcam, 1:1,000; HMGB1 antibody, Proteintech, 1:1,000) for immunohistochemistry/immunofluorescence staining. The media areas and the intima/media ratios of the iliac arteries were measured. An Olympus microscope (Tokyo) captured images and ImageJ quantified microscopic images from each section.

Statistical Analysis

The results are expressed as the mean ± the standard error of the mean. All data were statistically analyzed by the unpaired Student's *t* test and analysis of variance. $P<0.05$ was considered to represent a statistically significant difference. All statistical analyses were performed with SPSS Statistics version 17.0 or GraphPad Prism 6.

Results

Attenuation of Intimal Hyperplasia by GA in an Animal Model with Balloon-Induced Vascular Injury

Neointimal hyperplasia was evaluated morphologically 28 days after balloon injury. As shown in Figure 1, neointima area (0.87±0.26 mm^2 vs. 3.82±1.16 mm^2, P=0.016) and intima/media ratio (1.06±0.29 vs. 2.61±0.35, P=0.023) were significantly reduced in GA-treated animals as compared with those without GA treatment.

Inhibition of Proliferation and Migration of VSMCs by GA

To further investigate the role of GA in attenuating neointimal hyperplasia, a transwell migration assay was performed to investigate the effects of GA on the migration of VSMCs. As shown in Figure 2A, HMGB1 could promote VSMC migration, while GA concentration-dependently inhibited VSMC migration induced by HMGB1. Compared with the vehicle group, HMGB1 increased the migration of VSMCs (46.2% vs. 65.1%, P=0.02); however, application of GA can partly rescue the change in

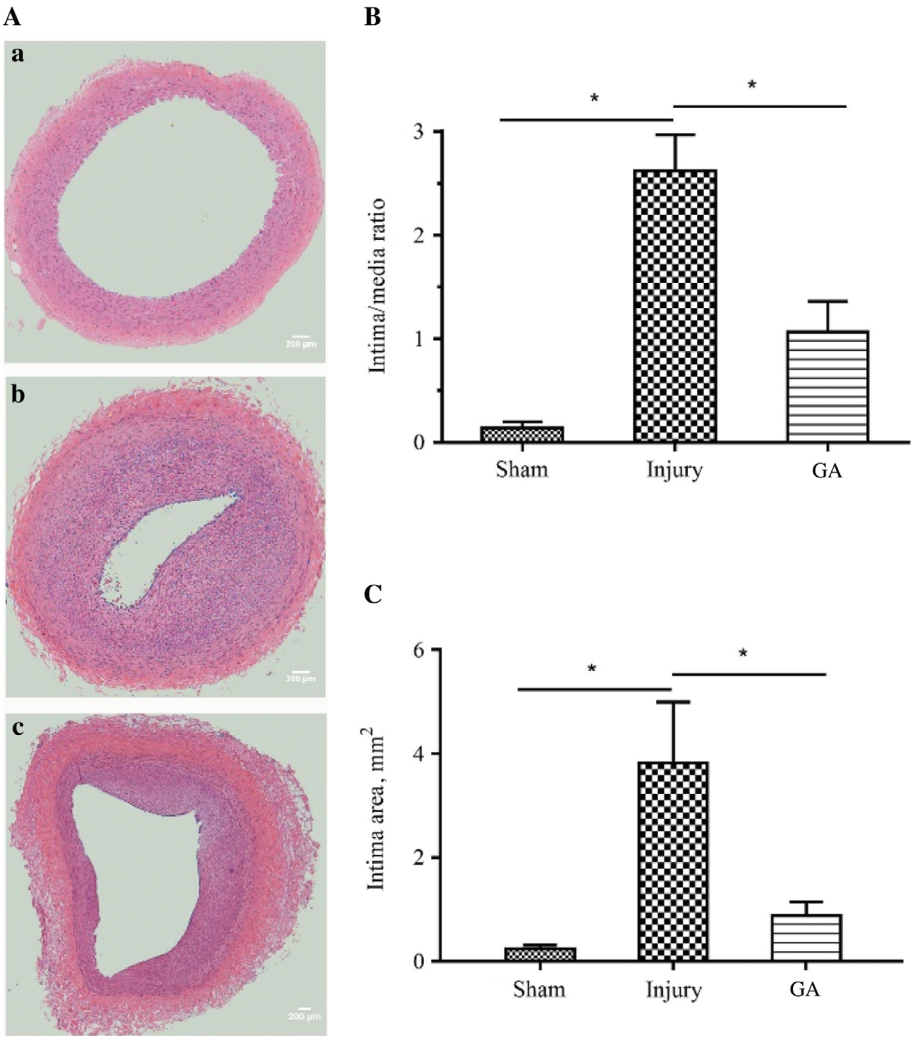

Figure 1: Glycyrrhizic Acid (GA) Attenuated Neointima Formation in a Balloon-Induced Vascular Injury Model. (A) Hematoxylin and eosin staining of injured and noninjured femoral arteries: (a) control artery; (b) injured artery; (c) injured artery treated with GA. Quantitative analysis of neointima area (B) and intima/media ratio (C) of sham-operation and injured iliac arteries from rabbits into which saline or GA was injected 28 days after balloon injury. Data shown are the mean ± the standard error of the mean (n=5). Scale bar, 200 µm. Asterisk, P<0.05 compared with control group.

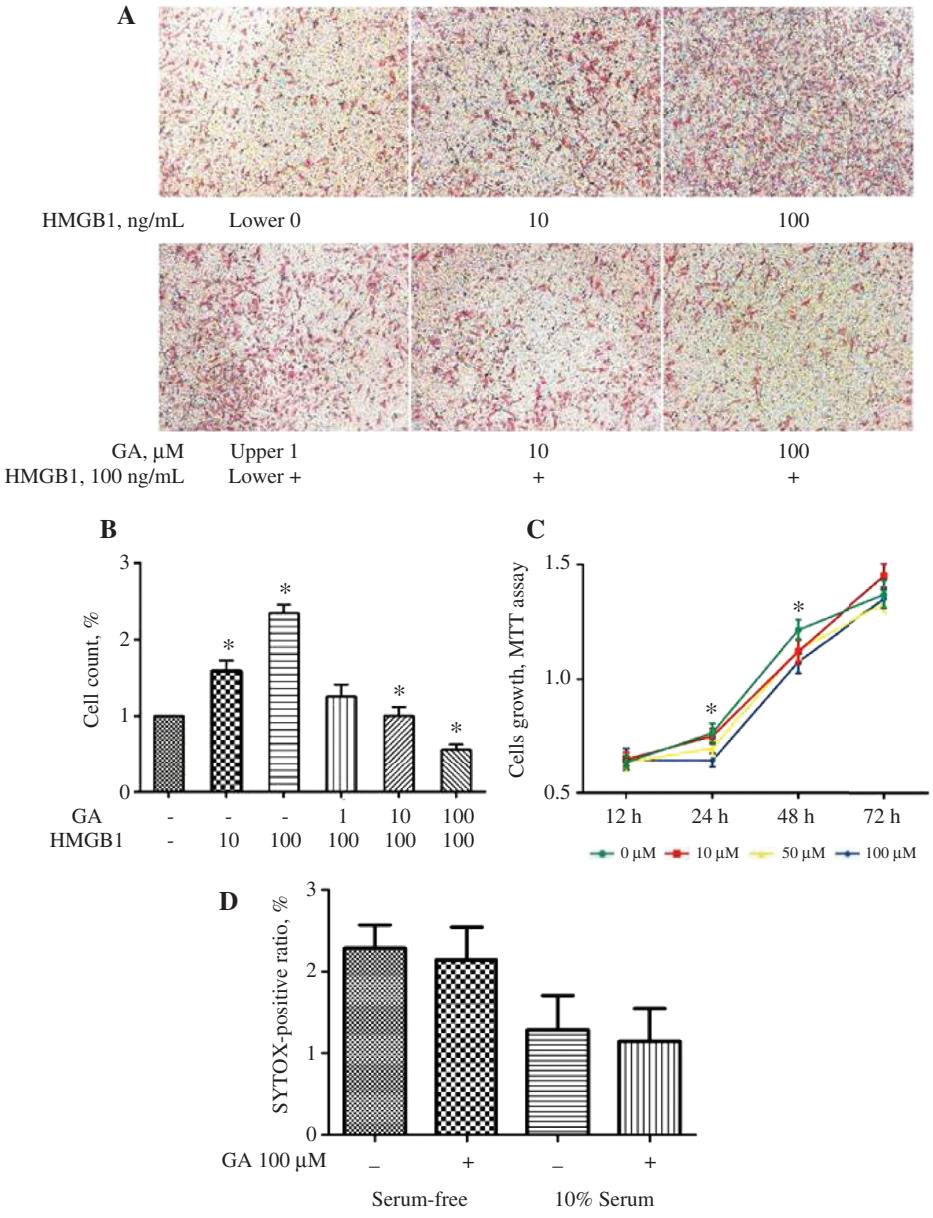

Figure 2: Effects of Glycyrrhizic Acid (GA) on High Mobility Group Protein B1 (HMGB1)-Induced Migration and Proliferation of Vascular Smooth Muscle Cells (VSMCs).
(A) GA inhibited VSMC migration induced by HMGB1 (HMGB1 concentration, 0, 10, and 100 ng/mL; GA concentration, 1, 10, and 100 μM). Lower and Upper refer to the lower and upper chambers of the transwell, respectively. (B) Statistical results obtained from the transwell migration assay ($n=5$). Data are shown as the mean ± the standard error of the mean. Asterisk, $P<0.05$ versus the control group. (C) Effects of GA on VSMC proliferation. Asterisk, $P<0.05$ for the 100 μM group versus the control group at 24 and 48 h. (D) SYTOX Green staining quantification was performed for VSMCs 24 h after treatment with 100 μM GA without serum or with 10serum. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

migration by 55%, 46%, and 31% at concentrations of 1, 10, and 100 μM, respectively. The results confirmed that GA inhibited the HMGB1-induced VSMC transmigration (Figure 2B).

In addition, VSMC proliferation was studied. It was found that VSMC proliferation under basal conditions increased slowly in a time-dependent manner. GA at a concentration of 100 μM markedly inhibited the proliferative capability of VSMCs at 24 h (Figure 2C). Besides, 100 μM GA did not increase death of VSMCs 24 h after incubation (Figure 2D).

Overall, these functional experiments suggest that GA suppressed proliferation and HMGB1 induced migration of VSMCs.

Inhibition of HMGB1-Induced VSMC Dedifferentiated Phenotype by GA

To investigate the effect of HMGB1 on VSMC phenotype, 10% serum was used to induce dedifferentiated VSMCs, while serum starvation was used to induce differentiated VSMCs. As shown in Figure 3A, the level of SM22α, a differentiation marker, was increased after serum starvation in a time-dependent manner, while the level of HMGB1 was significantly downregulated. We found that 10% serum caused reduced SM22α expression, while GA significantly upregulated SM22α expression, which suggests that treatment with GA significantly inhibited transition of VSMCs from a differentiated to a dedifferentiated phenotype. In addition, our results proved that angiotensin II at concentrations of 0.1 and 1 μM could significantly induce the expression of HMGB1 in VSMCs (P=0.04).

Suppression of Inflammation by GA in HMGB1-Induced Inflammation

There is mounting evidence that inflammation is an independent risk factor for the development of coronary heart disease [16, 17]. Therefore, we studied whether GA treatment affected the levels of proinflammatory cytokine mRNAs during HMGB1 treatment at 18 h. Our data revealed that HMGB1 increased the mRNA levels of the proinflammatory cytokines IL-1β and IL-6 (especially IL-6) (Figure 4A), while GA treatment downregulated the mRNA levels of IL-1β (45.5%) and IL-6 (47.6%) at 18 h (Figure 4B and C). We found that HMGB1

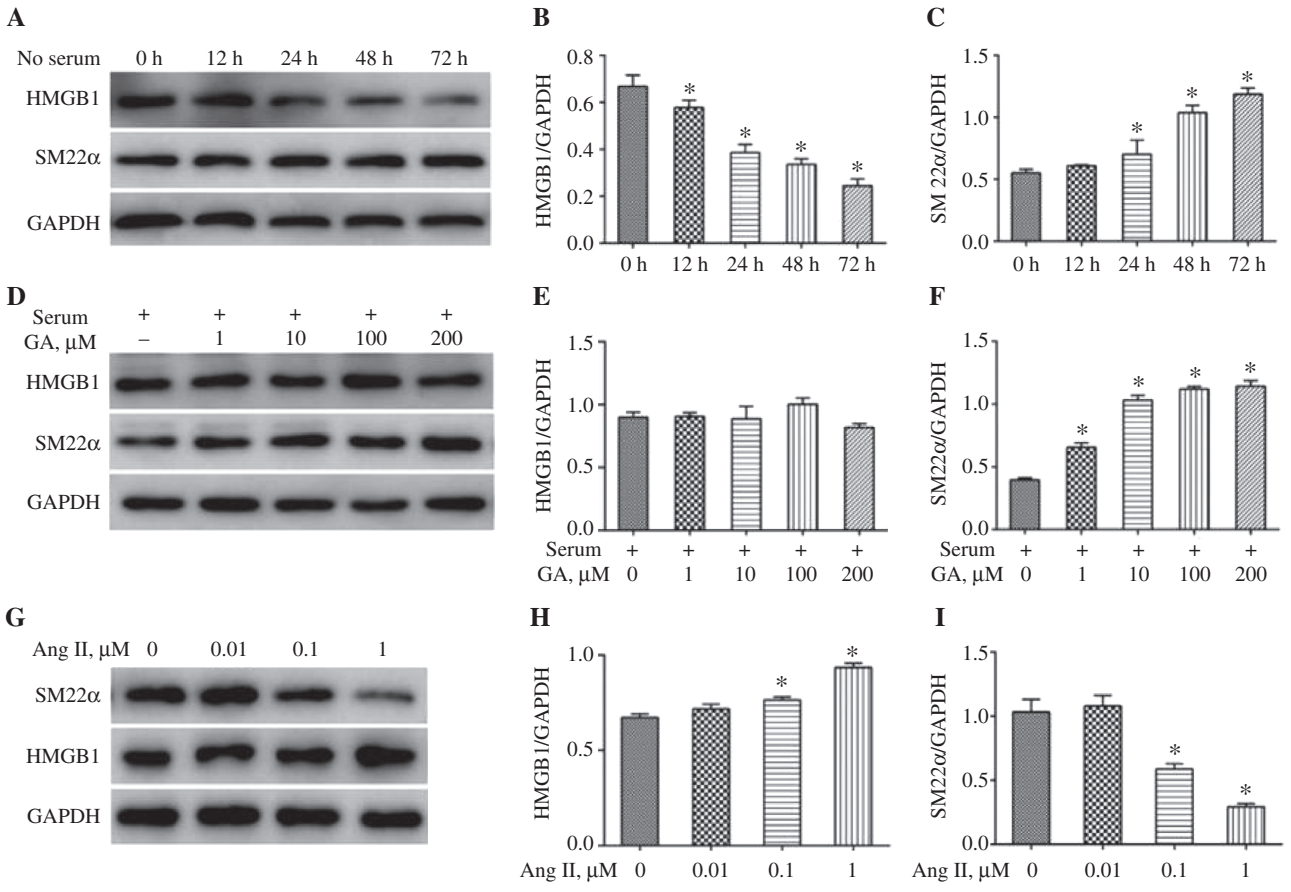

Figure 3: Effects of Serum Starvation, Glycyrrhizic Acid (GA), and Angiotensin II (Ang II) on the Phenotypic Transformation of Vascular Smooth Muscle Cells (VSMCs).
(A–C) The expression of smooth muscle protein 22α (SM22α) was induced and the expression of high mobility group protein B1 (HMGB1) was downregulated by serum starvation in a time-dependent manner. (D–F) SM22α expression was significantly upregulated by GA treatment. (G–I) Ang II significantly upregulated the expression of HMGB1 and downregulated the expression of SM22α in VSMCs at concentrations of 0.1 and 1 μM. All results are shown as the mean ± the standard deviation. Asterisk, P<0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

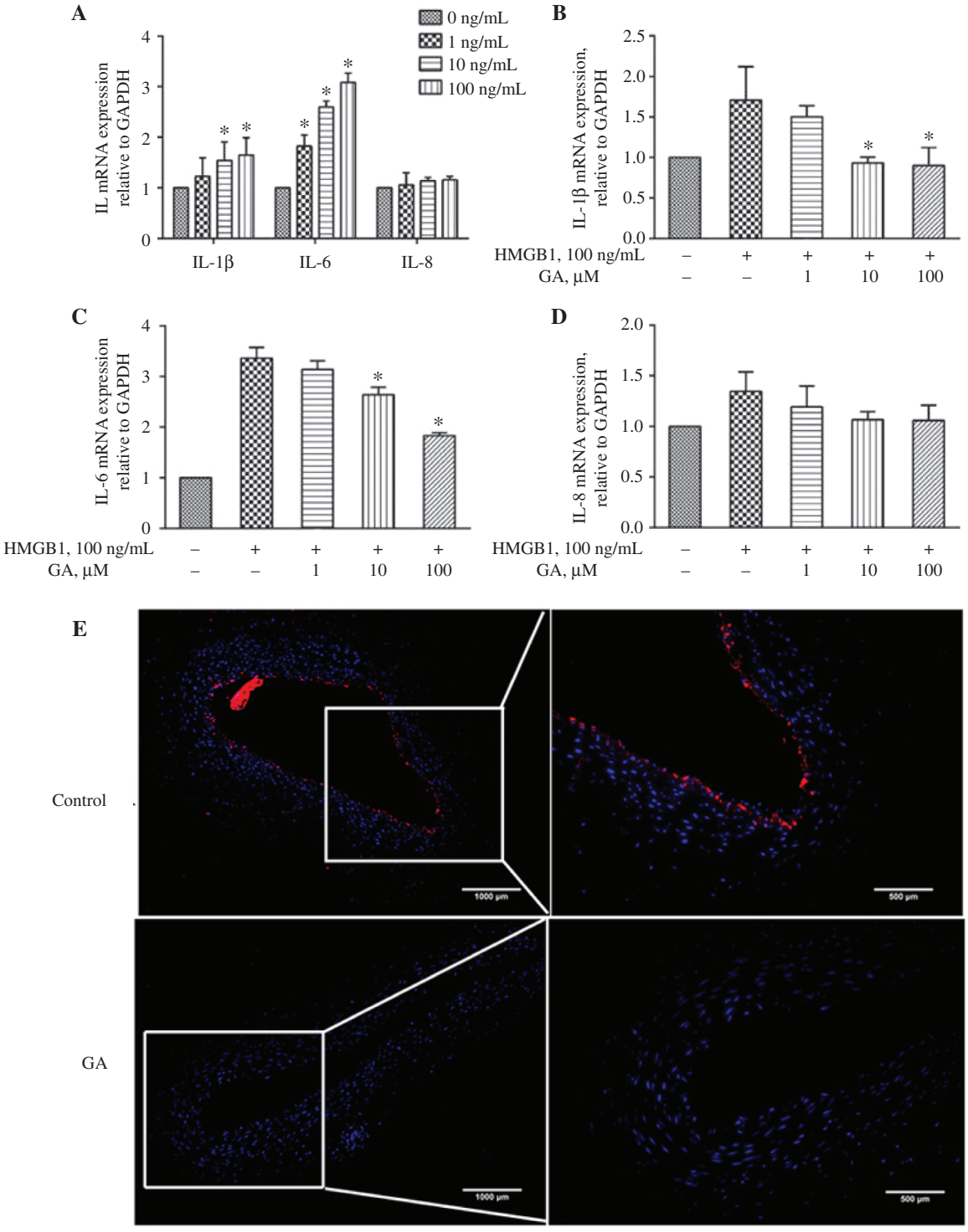

Figure 4: Messenger RNA (mRNA) Expression of IL-1β, IL-6, and IL-8 in the Presence and Absence of High Mobility Group Protein B1 (HMGB1) or Glycyrrhizic Acid (GA).
(A) The levels of IL-1β and IL-6 were significantly upregulated after treatment with HMGB1 (1–100 ng/mL). (B, C) GA suppresses the inflammatory response triggered by HMGB1 in vascular smooth muscle cells. (D) mRNA expression of IL-8 was not changed under treatment with HMGB1 or GA. (E) CD68 staining in injured vessels (red, CD68; blue, 4′,6-diamidino-2-phenylindole). The results are reported as the mean ± the standard error of the mean ($n=4$). Asterisk, $P<0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

treatment did not induce an obvious change in the level of IL-8 with or without GA treatment. To investigate the role of GA in inflammation in vivo, we studied the macrophage cells (CD68-positive cells) in injured vessels and found that GA reduced the number of macrophage cells in injured vessels (Figure 4E). All these results suggest that GA treatment attenuated the proinflammatory responses of the VSMCs.

Inhibition of the Effect of HMGB1 by GA on RAGE Signaling Pathways

RAGE is one of the main receptors of HMGB1, and therefore we investigated the effect of HMGB1 and GA on the expression of RAGE signaling pathway molecules. We found that phospho-p38 could be significantly induced by HMGB1 at both 10 and 100 ng/mL compared with the control group, and GA treatment at both 10 and 100 μM could ameliorate such an effect (Figure 5A, B, E, and F). In addition, Western blotting analysis revealed that HMGB1 increased the expression levels of RAGE and p65 after 24 h, while the HMGB1-induced increase of RAGE and p65 expression was significantly decreased by treatment with 10 and 100 μM GA (Figure 5A–D, G, and H). (P=0.04, compared with the control group).

Inhibition of HMGB1 expression by GA in Vascular Tissue at 28 days after Balloon Injury

To examine the exact role of HMGB1 in an animal model, we investigated the expression of HMGB1 in vascular tissue at 28 days after balloon injury. Immunohistochemical staining showed HMGB1 expression increased in injured arteries compared with control arteries (Figure 6A). In detail, the proportions of cytoplasmic HMGB1-positive cells was significantly higher in injured artery than in sham-operation artery ((91.33±3.61)% vs. (43.50±6.53)%; P=0.008). Accordingly, after GA treatment, the proportion of cytoplasmic HMGB1-positive cells was significantly downregulated ((30.33±8.06)%; P=0.006) (Figure 6C). We found that p65, a subunit of nuclear factor κB, was also significantly upregulated in balloon-injured vascular tissue and that GA

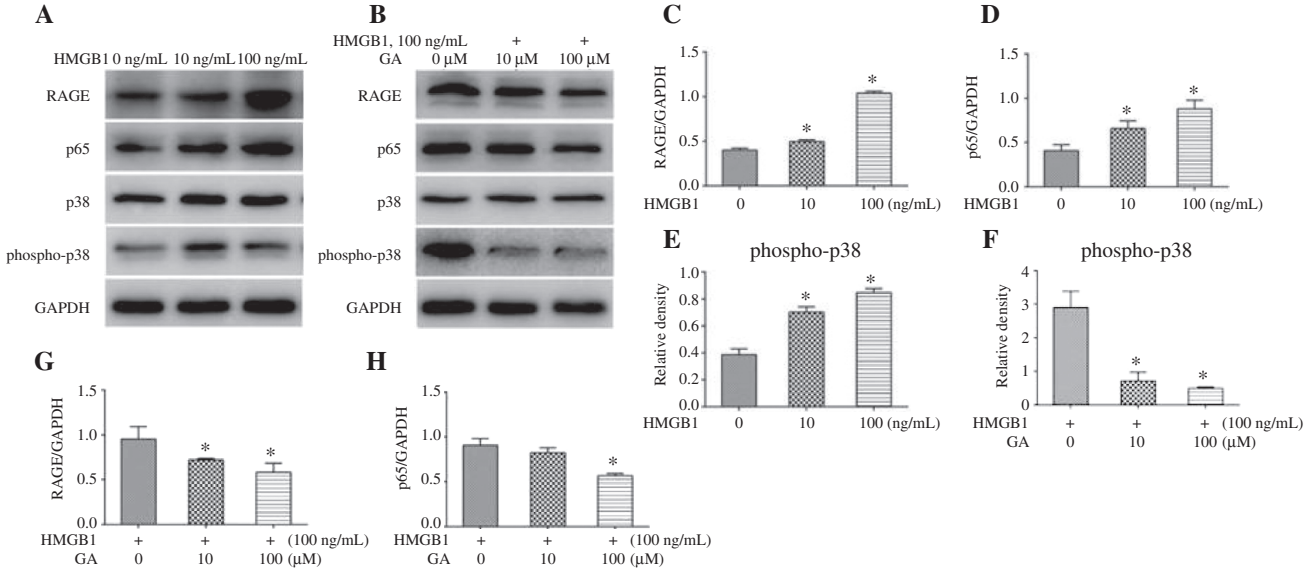

Figure 5: Glycyrrhizic Acid (GA) Inhibited the Receptor for Advanced Glycosylation end Products (RAGE) Signaling Pathway in Vascular Smooth Muscle Cells (VSMCs).
(A) High mobility group protein B1 (HMGB1) triggered the RAGE pathway in VSMCs at concentrations of 10 and 100 ng/mL for 1 or 24 h. Activation of the RAGE, p65, and p38 pathways was determined by Western blotting. (B) GA inhibited HMGB1-induced RAGE pathway activation. Human VSMCs were pretreated with 10 and 100 μM GA for 30 min, followed by stimulation with HMGB1 (100 ng/mL) for 1 or 24 h. Inhibition of the RAGE, p65, and p38 pathways was determined by Western blotting. (C–H) Quantification of pathway activation and inhibition in (A) and (B). The results are reported as the mean ± the standard error of the mean (n=4). Asterisk, P<0.05. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

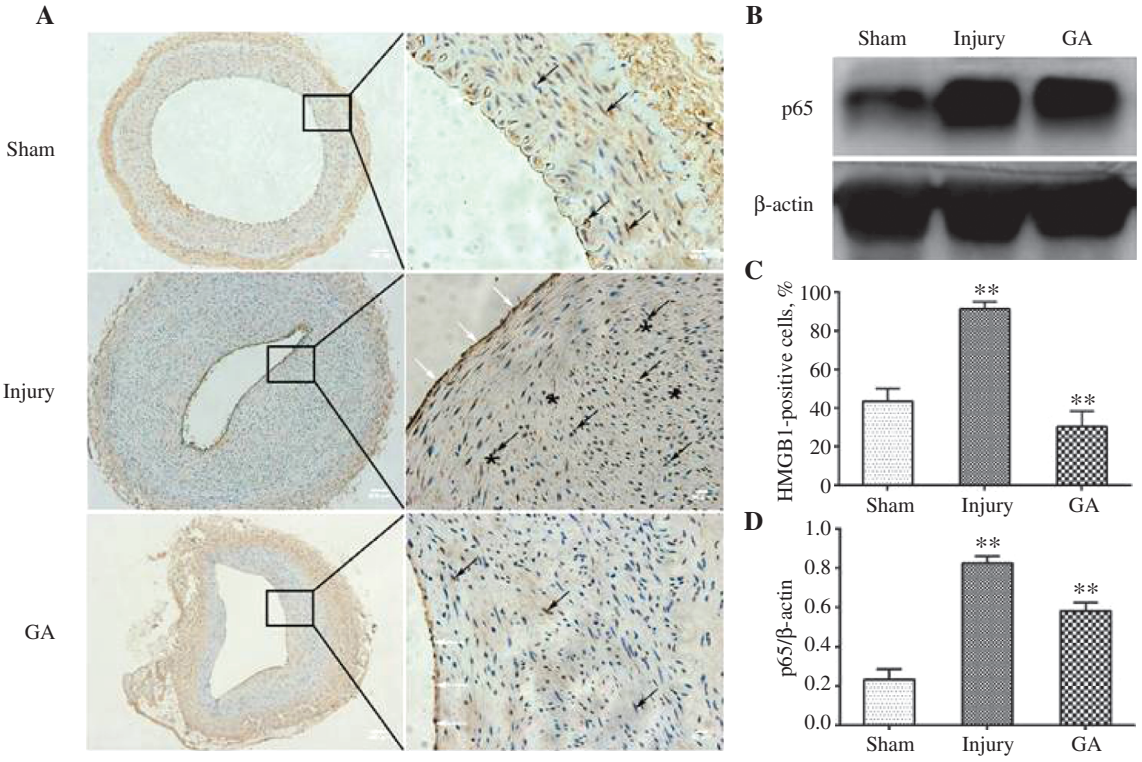

Figure 6: Glycyrrhizic Acid (GA) Decreased the Expression of High Mobility Group Protein B1 (HMGB1) and p65 in Injured Iliac Arteries.
(A) Representative microphotographs of immunohistochemical staining for HMGB1 (brown) of sections from control, balloon-injured, and GA-treated iliac arteries (arrows and asterisk). (B) Expression of p65 was examined by Western blotting. (C) HMGB1-positive cell number was quantified in sections from sham-operation and injured iliac arteries from control or GA-treated rabbits. (D) Quantification of p65 expression in Western blotting. $n=5$. Two asterisks, $P<0.01$ versus the control group.

can significantly inhibit the expression of p65 in balloon-injured vascular tissue ($P=0.005$).

Discussion

We found that GA can attenuate neointimal hyperplasia in balloon-induced iliac artery injury in rabbits even 28 days after injury. Besides, we found inflammation and RAGE signaling pathways may play key roles in this effect.

HMGB1, a nuclear localized non-histone DNA-binding protein, can be released into the cytosome in response to different kinds of stimuli [18], inducing various types of systemic inflammation through TLR2/TLR4 and RAGE [19, 20]. Consistent with previous studies, we found that HMGB1 expression increased in the local injured arteries, which indicated an immune response. Although we do not know the exact time for a such response, we found that HMGB1 expression increased even 28 days after injury in rabbit arteries. Accordingly, increased HMGB1 expression triggered inflammation, as shown in our in vitro study, and different concentrations of HMGB1 upregulated the mRNA expression of proinflammatory cytokines such as IL-1β and IL-6.

Accumulating evidence suggests that serum HMGB1 levels increase in coronary artery disease [5, 21, 22]. Our results showed that inhibition of HMGB1 by GA treatment significantly decreased the expression of HMGB1 in injured arteries. Moreover, the neointima thickness (neointima area and intima/media ratio) was significantly decreased in the GA-treated group compared with the non-treated group. Thus, HMGB1 might be a critical molecule promoting vascular neointima formation and medial hyperplasia, and the inhibition of HMGB1 by GA markedly reversed the vascular remodeling in balloon-induced vascular injury in rabbits. Through all the studies, we verified partly the injury-HMGB1-inflammation-hyperplasia axis.

GA can inhibit HMGB1 activities in vivo [23] in addition to its multiple therapeutic properties. In a previous study, GA was reported to attenuate pulmonary hypertension in the monocrotaline-induced pulmonary hypertension rat model via suppression of inflammatory cytokine production [24]. Besides, GA can also improve lipid profiles [25]. In our study, we came to a similar conclusion that HMGB1 plays a significant role in the inflammation reaction during restenosis and that GA can markedly suppress HMGB1-induced inflammation in a concentration-dependent manner. Moreover, in our in vivo study, we also found that the administration of GA at 50 mg/kg after iliac artery balloon injury markedly suppressed the expression of p65, further confirming the anti-inflammatory effects of GA.

HMGB1 is known to regulate cellular events through multiple cell-surface receptors: RAGE and TLRs. In this study, RAGE and p65 expression were significantly increased after HMGB1 treatment, while there was no significant changes in the expression of TRL4, which is different from the previous study.

Limitations

There are several limitations in our study. Firstly, the animal model used in this study could not fully represent the clinical scenario of in-stent restenosis. Although balloon injury or wire injury animal models (in health animal) were widely applied to simulate intimal hyperplasia in previous animal studies [26, 27], it is hard to accurately reflect the situation in the human body, especially under the background of atherosclerosis. Besides, stent implantation in an animal model will be a better choice in a future study. Secondly, we studied the role of GA in HMGB1-induced VSMC dedifferentiated phenotype under the condition of 10% serum; however, further studies are needed to test if HMGB1-induced VSMC dedifferentiated phenotype still works under intervention with angiotensin II.

Conclusions

The present study verified the beneficial effects of GA in balloon-injured iliac artery of rabbits, and the underlying mechanism may be the HMGB1-RAGE pathway and its downregulated signals. Therefore, our study indicates GA may potentially be a novel medicine to prevent injury-associated restenosis.

Ethics Statement

All protocols were approved by the Animal Research Committee, Central South University, Hunan, China.

Disclosure Statement

None.

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Author Contributions

Z. Zhu and Y. Guo wrote the manuscript, X. Li, S. Teng, and X. Peng performed animal studies, P. Zou performed molecular studies, and S. Zhou designed the project and revised the manuscript.

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