European Review for Medical and Pharmacological Sciences 2018; 22: 8447-8453

GAS5 promotes myocardial apoptosis in myocardial ischemia-reperfusion injury via upregulating LAS1 expression

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Abstract. – OBJECTIVE: This study aims at investigating whether GAS5 (grow arrest-specific 5) could promote cardiomyocyte apoptosis by upregulating LAS1 expression, thereby participating in the development of myocardial ischemia-reperfusion injury.

MATERIALS AND METHODS: The expression level of GAS5 in H9c2 cells after hypoxia/reoxygenation (H/R) treatment was detected by quantitative Real time-polymerase chain reaction (qRT-PCR). Myocardial injury markers in H9c2 cells were evaluated using relative commercial kits, including activities of LDH (lactate dehydrogenase), MDA (malondialdehyde), SOD (superoxide dismutase) and GSH-PX (glutathione peroxidase). Cell proliferation and apoptosis were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. The protein expressions of apoptosis-related genes and p38/MAPK pathway-related genes were detected by Western blot. The regulatory effects of GAS5 on the p38/MAPK pathway were assessed after treatment with p38/MAPK pathway inhibitor in H9c2 cells.

RESULTS: QRT-PCR results showed that the expression levels of GAS5 and LAS1 in H/R-treated H9c2 cells were remarkably upregulated compared to those of controls. GAS5 overexpression increased activities of LDH, MDA, SOD and GSH-PX in H/R-treated H9c2 cells. Meanwhile, GAS5 overexpression reduced cell proliferation and apoptosis of H/R-treated cells. Western blot results suggested that the pro-apoptosis genes Bax and cytochrome C were upregulated, whereas the anti-apoptosis gene Bcl-2 was downregulated after GAS5 overexpression. The overexpression of LAS1 in H9c2 cells obtained the same results as GAS5 overexpression. Furthermore, the expressions of p-p38 and p-ERK were upregulated by GAS5 overexpression. SB203580, the p38/MAPK pathway inhibitor, could reverse the inhibited proliferation and increase apoptosis induced by overexpression of GAS5.

CONCLUSIONS: GAS5 promotes myocardial apoptosis in myocardial ischemia-reperfusion injury by upregulating LAS1 expression via p38/MAPK pathway. GAS5 may be a potential therapeutic target for myocardial ischemia-reperfusion injury.

Key Words:
GAS5, LAS1, Ischemia-reperfusion, Myocardial apoptosis, P38/MAPK pathway.

Introduction

Coronary heart disease is one of the major diseases leading to human death. The death number from coronary heart disease worldwide accounts for about 10% of all deaths each year. Thrombolytic therapy, percutaneous transluminal coronary angioplasty, and coronary artery bypass grafting can quickly restore blood perfusion and oxygen supply in ischemic myocardium. However, reperfusion in ischemic myocardium might aggravate damages in myocardial ultrastructure, metabolism and function and even lead to irreversible myocardial ischemia-reperfusion injury (MIRI)[1]. Swelling and rupture of mitochondria induced by oxygen free radicals, calcium overload and mPTP opening further stimulate the release of the apoptosis genes. Subsequently, activated caspase cascade induces programmed apoptosis[2,3]. After MIRI, the RISK pathway (including PI3K/AKT, PKC, MAPKs, ERK1/2 and p38 pathways) is activated to inhibit the GSK3 pathway and regulate ATP-sensitive K^+ channel and mPTP function, thus exerting a cytoprotective effect[4]. However, the specific regulatory mechanisms of these pathways still need to be further explored.

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Long non-coding RNA (lncRNA) is a small, endogenous RNA with more than 200 nt in length. It regulates gene expression at epigenetic, transcriptional and post-transcriptional levels[5]. In recent years, studies have shown the biological functions of lncRNAs in many important regulatory processes, such as X chromosome silence, genomic imprinting, chromatin modification, transcriptional activation and interference[6]. Scholars[7,8] have found that many lncRNAs exert a vital regulatory role in cardiac hypertrophy, fibrosis, and ischemia/reperfusion (I/R) processes. For example, NovInc6[9] and Mhrt[10] are lowly expressed in the myocardium experiencing acute myocardial infarction and heart failure, respectively. LncRNA MDRL inhibits the incidence of myocardial infarction through regulating mitochondrial fusion and division by targeting miR-361[11]. In addition, lncRNA MALAT1[12] and Tie-l-AS[13] in the endothelium can regulate blood vessel growth and its function. LncRNA SENCR[14] can regulate the contraction of smooth muscle cells. It is of great importance to search for functional lncRNAs involving in I/R, which may be served as therapeutic targets for heart diseases.

GAS5 (grow arrest-specific 5) is a novel lncRNA discovered in recent years, which is associated with cell proliferation. GAS5 exerts an extremely important role in the apoptosis and cell cycle regulation of lymphocytes and peripheral blood T cells. The downregulation of GAS5 can inhibit apoptosis and promote cell proliferation[15]. Some studies have found that GAS5 is involved in the incidence and development of cervical cancer[16], gastric cancer[17] and prostate cancer as a tumor-suppressor gene[18]. GAS5 is also involved in the regulation of cell cycle and apoptosis. Relative studies have confirmed that GAS5 overexpression inhibits the activation of primary hepatic stellate cells and reduces collagen accumulation in fibrotic liver tissues[19]. To our best knowledge, no researches have been conducted to explore the role of GAS5 in regulating cardiomyocyte apoptosis.

Materials and Methods

Cell Culture and H/R Treatment

Rat embryonic cardiac myocytes H9c2 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). H9c2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). For constructing H/R model, H9c2 cells were incubated in serum-free DMEM and maintained in an anaerobic incubator with 5% CO_2 and 95% N_2 for 4 h. Subsequently, cells were incubated in DMEM containing 10% glycerol in an environment with 5% CO_2 and 95% air for another 3 h. 24 h after reoxygenation, cells were collected for the following experiments.

Cell Transfection

Cells in good growth condition were seeded into 6-well plates with serum-free medium. Transfection was performed when the confluence was up to 60% following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In brief, Lipofectamine 2000 and plasmid were diluted in serum-free medium and mixed at room temperature for 20 min. The mixture was then added in each well for incubation. Culture medium was replaced 4-6 h later. Transfection plasmids used in the study were constructed by GenePharma Co., Ltd. (Shanghai, China).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer (Hitachi, Tokyo, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the $2^{-\Delta Ct}$ method. Primers used in the study were as follows: GAS5 (F: 5'-CTTCTGGGCTCAAGTGATCCT-3', R: 5'-TTGTGCCATGAGACTCCATCAG-3'); LAS1 (F: 5′-CCACTGCGGCCGCTTGTTG-CGCACTAGGTACG-3′, R: 5′-CAAGTG-GATCCCTTGTCATCGTCATCTTTATAATC-CATAGCGGTAGAATATAATAGAA-3′); Bax (F: 5'-CACAACTCAGCGCAAACATT-3', R: 5'-ACAGCCATCTCTCTCCATGC-3'); Bcl2 (F: 5'-GAAGCACAGATGGTTGATGG-3', R: 5'-CAGCCTCACAAGGTTCCAAT-3'); Cytochrome C (F: 5′-TAAATATGAGGGTGTCGC-3′, R: 5′-AAGAATAGTTCCGTCCTG-3′); GAPDH (F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCCATCACAAAC-3'.

Detection of LDH, MDA, SOD, and GSH-PX

Transfected cells were collected for detecting activities of LDH (lactate dehydrogenase), MDA (malondialdehyde), SOD (superoxide dismutase) and GSH-PX (glutathione peroxidase) following the instructions of commercial kits.

CCK-8 (Cell Counting Kit-8) Assay

Transfected cells were seeded into 96-well plates at a density of $2\times10^3/\mu L$. 10 μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) was added in each well after cell culture for 0, 1, 2, and 3 days, respectively. 2 hours later, the absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Western Blot

Cells were lysed with RIPA (radioimmunoprecipitation assay) lysis buffer in the presence of a protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA) and total cellular protein was harvested. The protein concentration of each cell lysate was quantified using the BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA). An equal amount of protein sample was loaded onto a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gel and then transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) after being separated. After blocking with skim milk, membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C and then incubated with HRP (horseradish peroxidase) conjugated secondary antibody. Finally, an image of the protein band was captured by the Tanon detection system using enhanced chemiluminescence (ECL) reagent (Thermo, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Measurement data were analyzed by the *t*-test. $p < 0.05$ considered the difference was statistically significant.

Results

GAS5 Was Highly Expressed in H/R-Treated Cells

QRT-PCR results indicated that GAS5 is highly expressed in H/R-treated H9c2 cells than that of controls (Figure 1A). Subsequently, we detected the levels of myocardial injury markers in H/R-treated cells overexpressing GAS5. The results showed that GAS5 overexpression elevated activities of LDH, MDA, SOD and GSH-PX (Figure 1B-1E).

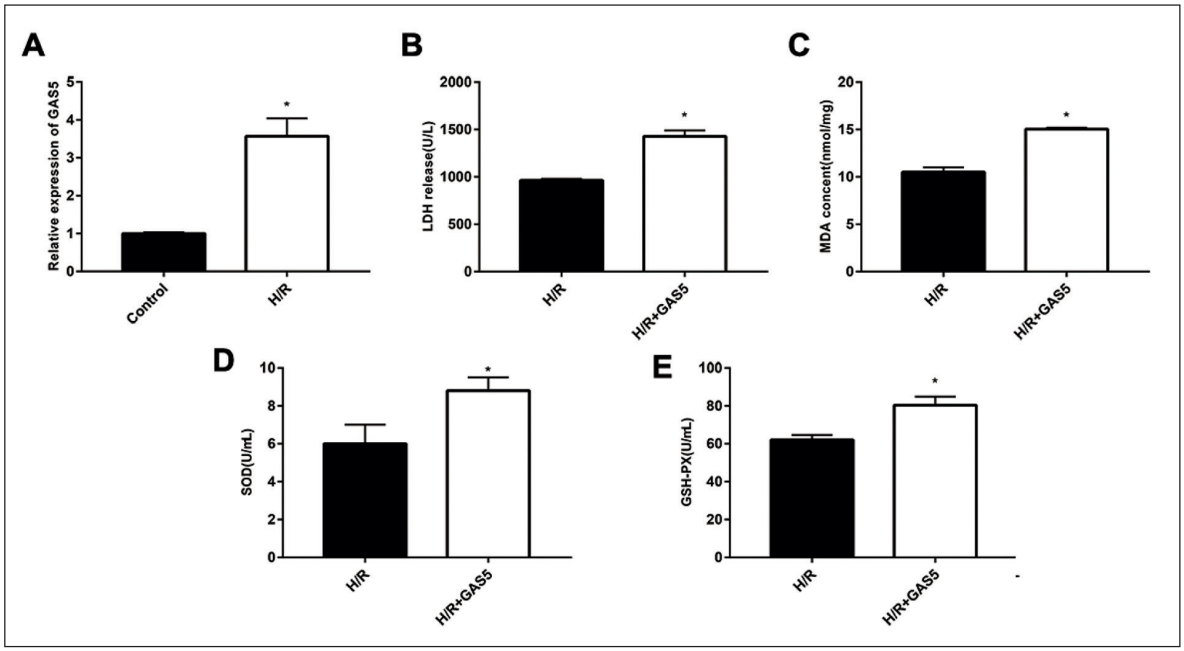

Figure 1. GAS5 was highly expressed in H/R-treated cells. ***A,*** GAS5 was highly expressed in H/R-treated H9c2 cells than that of controls. ***B-E,*** GAS5 overexpression elevated activities of LDH (***B***), MDA (***C***), SOD (***D***), and GSH-PX (***E***).

Overexpression of GAS5 Promoted Cardiomyocyte Apoptosis

We further investigated the regulatory effect of GAS5 on the proliferation of H/R cells. The CCK-8 assay showed that cell proliferation was attenuated after GAS5 overexpression (Figure 2A). Flow cytometry suggested that overexpression of GAS5 promoted apoptosis of H9c2 cells (Figure 2B). To further explore the mechanism of GAS5 in regulating apoptosis, we detected expression levels of the pro-apoptosis genes Bax and cytochrome C, as well as anti-apoptosis gene Bcl-2. QRT-PCR data showed that mRNA levels of Bax and cytochrome C increased, whereas Bcl-2 expression decreased after GAS5 overexpression (Figure 2C). Western blot showed the similar results (Figure 2D).

GAS5 Promoted Cardiomyocyte Apoptosis via Upregulating LAS1

Bioinformatics predicted that GAS5 may regulate LAS1 expression. QRT-PCR showed that the mRNA expression of LAS1 in H/R-treated cells was remarkably higher than that of controls (Figure 3A). After overexpression of GAS5 in H9c2 cells, LAS1 expression significantly increased (Figure 3B). The proliferation of H/R-treated cells decreased after LAS1 overexpression (Figure 3C). Flow cytometry revealed that the overexpression of LAS1 promoted cardiomyocyte apoptosis (Figure 3D). To further explore whether LAS1 exerts a role in regulating cell apoptosis, we detected the expression levels of Bax, cytochrome C and Bcl-2 after LAS1 overexpression. Both mRNA and protein levels of Bax and cytochrome C increased, but the Bcl-2 expression decreased (Figure 3E and 3F).

GAS5 Promoted Cardiomyocyte Apoptosis via p38/MAPK Pathway

The p38/MAPK pathway is a classical apoptosis pathway. Western blot results showed that the expression levels of p-p38 and p-ERK were remarkably increased after overexpression of GAS5 (Figure 4A). Subsequently, H/R-treated cells were incubated with the p38/MAPK pathway inhibitor SB203580. Flow cytometry showed

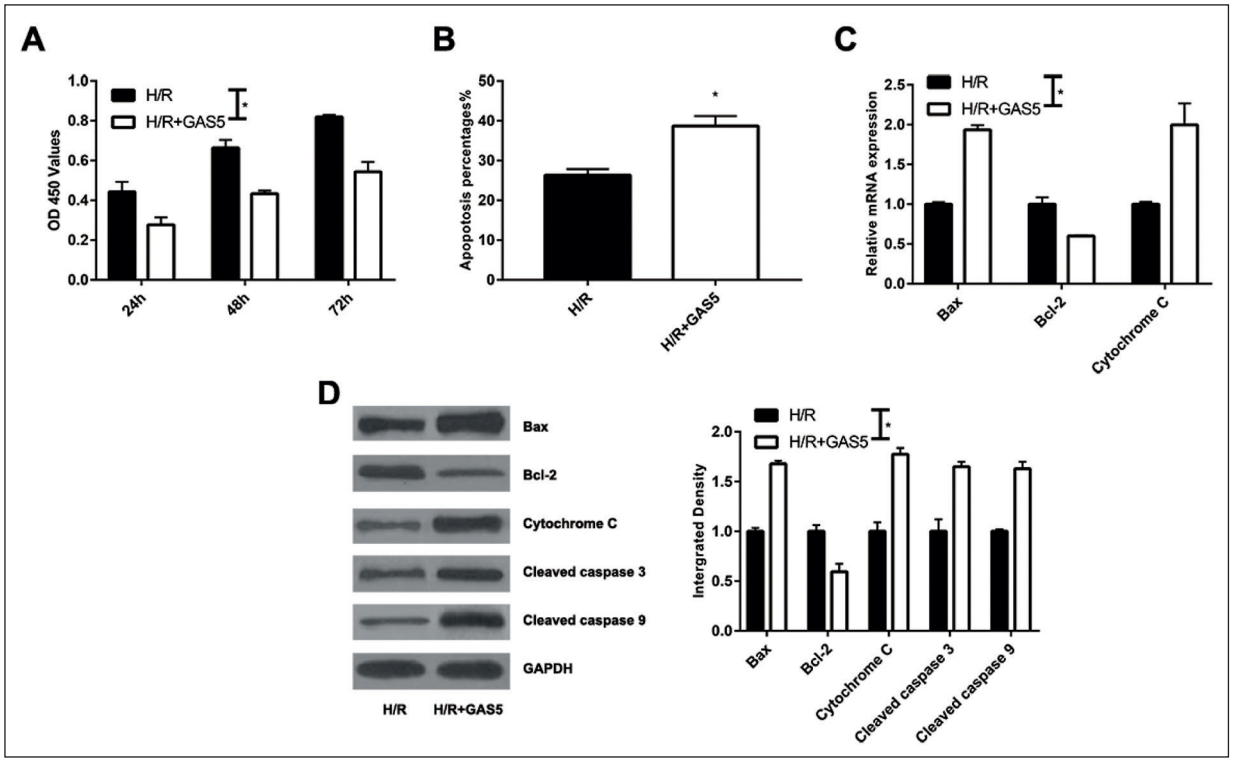

Figure 2. The overexpression of GAS5 promoted cardiomyocyte apoptosis. ***A,*** CCK-8 assay showed that cell proliferation was attenuated after overexpression of GAS5. ***B,*** Flow cytometry suggested that overexpression of GAS5 promoted apoptosis of H9c2 cells. ***C,*** QRT-PCR data showed that mRNA levels of Bax and cytochrome C increased, whereas Bcl-2 decreased after GAS5 overexpression. ***D,*** Western blot showed that protein levels of Bax and cytochrome C increased, whereas Bcl-2 decreased after GAS5 overexpression.

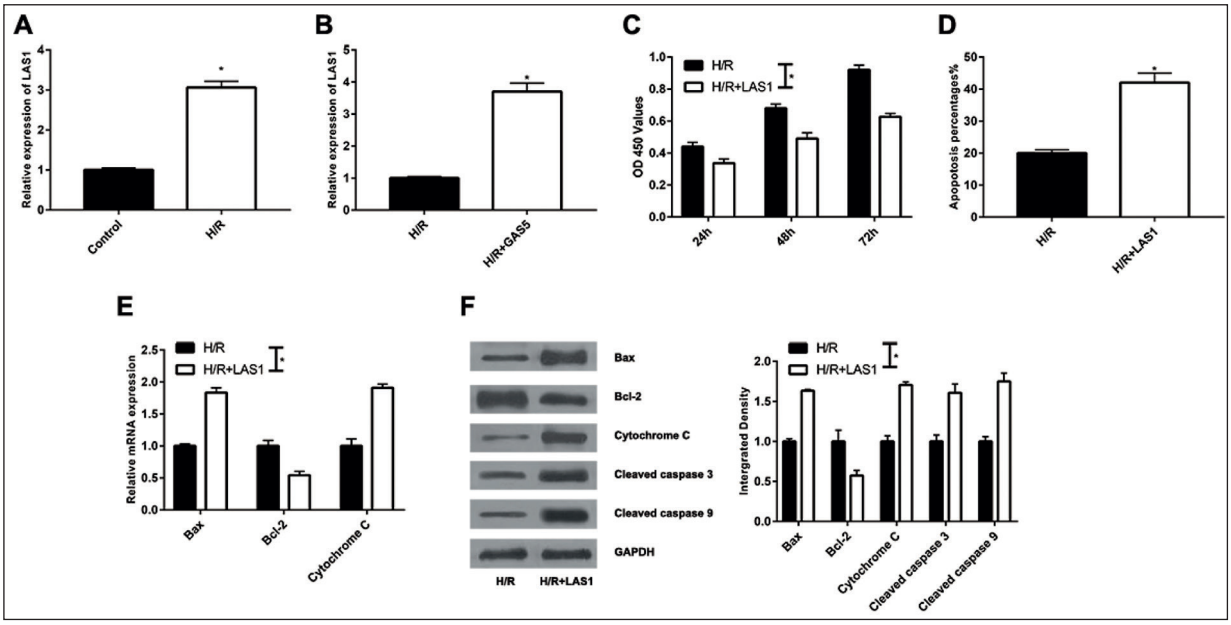

Figure 3. GAS5 promoted cardiomyocyte apoptosis *via* upregulating LAS1. ***A,*** QRT-PCR showed that the mRNA expression of LAS1 in H/R-treated cells was remarkably higher than that of controls. ***B,*** After overexpression of GAS5 in H9c2 cells, LAS1 expression significantly increased. ***C,*** Proliferation of H/R-treated cells decreased after LAS1 overexpression. ***D,*** Flow cytometry revealed that overexpression of LAS1 promoted cardiomyocyte apoptosis. ***E, F,*** Both mRNA and protein levels of Bax and cytochrome C increased, but Bcl-2 decreased.

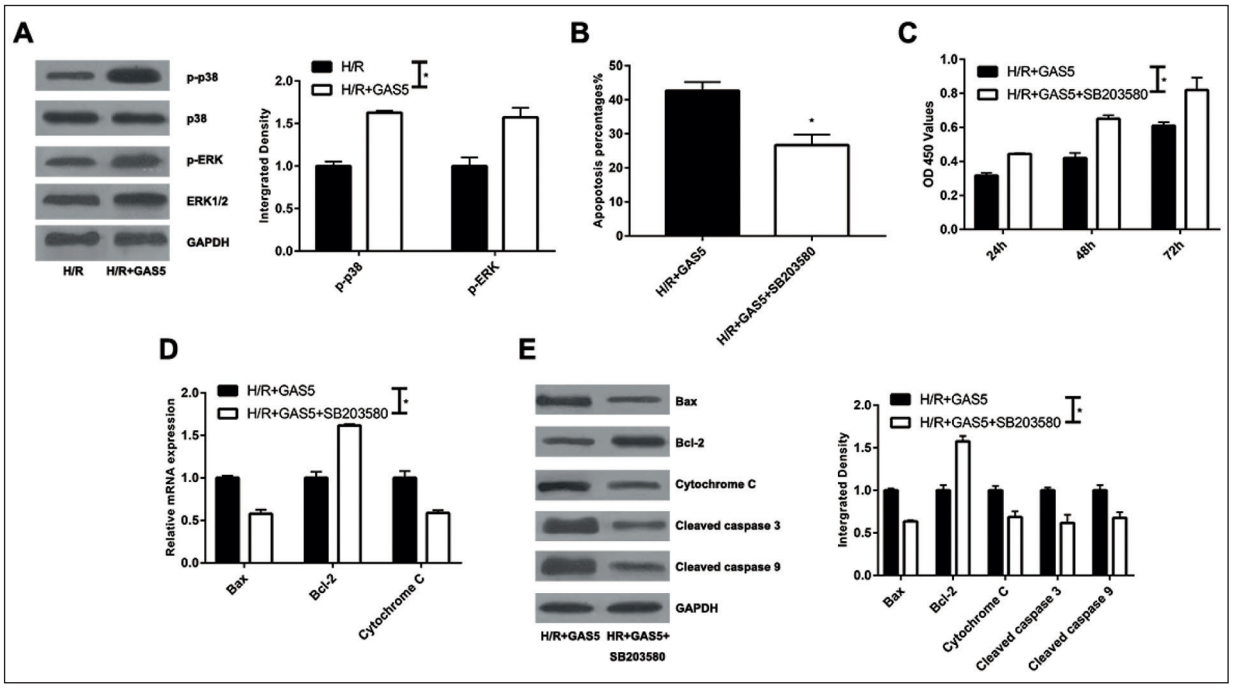

Figure 4. GAS5 promoted cardiomyocyte apoptosis *via* p38/MAPK pathway. ***A,*** Western blot results showed that the expression levels of p-p38 and p-ERK remarkably increased after overexpression of GAS5. ***B,*** Flow cytometry showed that SB203580 reversed the pro-apoptotic effects of GAS5. ***C,*** CCK-8 assay found that SB203580 can reverse the inhibitory effect of GAS5 on cell proliferation. ***D,*** The p38/MAPK pathway inhibitor could reverse the effect of GAS5 overexpression on regulating the mRNA levels of apoptosis genes. ***E,*** The p38/MAPK pathway inhibitor could reverse the effect of GAS5 overexpression on regulating the protein levels of apoptosis genes.

that SB203580 reversed the pro-apoptotic effects of GAS5 (Figure 4B). CCK-8 assay also found that SB203580 can reverse the inhibitory effect of GAS5 on cell proliferation (Figure 4C). To further reveal the role of the p38/MAPK pathway in the regulation of apoptosis, the expression levels of apoptosis genes were detected after cells were treated with p38/MAPK pathway inhibition. The results showed that the p38/MAPK pathway inhibitor could reverse the effect of GAS5 overexpression on regulating the mRNA levels of apoptosis genes (Figure 4D). Western blot obtained the similar results (Figure 4E).

Discussion

Coronary heart disease is one of the leading causes of death and disability worldwide. Treatments for coronary heart disease include drugs, percutaneous coronary intervention and coronary artery bypass grafting. Various methods of revascularization can reduce MIRI and necrosis by timely and effective restoration of myocardial blood perfusion. However, blood reperfusion after myocardial ischemia can further lead to myocardial cell injury or even necrosis, which is known as MIRI[20]. As a multifactorial pathophysiological process, MIRI involves various risk factors, including oxidative stress, inflammatory response, endothelial dysfunction and calcium overload[21,22].

LAS1 is an essential component in the synthesis of ergosterol. Ergosterol is the major constituent of yeast cell membrane involved in the regulation of membrane fluidity and permeability of yeast cells[21]. It is rarely reported whether LAS1 exerts a vital role in regulating MIRI development.

The p38/MAPR pathway is involved in cell growth, development and differentiation, which is closely related to the incidence of various diseases such as inflammatory diseases and tumors[24,25]. It is involved in the regulation of inflammatory responses. Studies have showed that the p38/MAPK pathway inhibitor SB203580 can remarkably reduce the production of inflammatory factors[26]. Meanwhile, the p38/MAPK pathway inhibitor can reverse myocardial function in myocardial ischemia mice[27].

We found that GAS5 is highly expressed in H/R-treated cells than that of controls. The overexpression of GAS5 elevated activities of LDH, MDA, SOD and GSH-PX in H/R cells. *In vitro* experiments showed that after overexpression of GAS5, the cell proliferation ability was weakened and apoptosis was induced. GAS5 overexpression also upregulated Bax and cytochrome C, whereas downregulated Bcl-2. LAS1 expression was upregulated in H/R-treated cells, which was positively regulated by GAS5. We found that the overexpression of LAS1 decreased cell proliferation and increased apoptosis. The LAS1 overexpression upregulated Bax and cytochrome C, whereas downregulated Bcl-2. Furthermore, the overexpression of GAS5 resulted in increased expressions of p-p38 and p-ERK, indicating the activation of the p38/MAPK pathway. SB203580, the p38/MAPK pathway inhibitor, reversed the effect of GAS5 on cell proliferation and apoptosis. SB203580 also reversed protein expressions of Bax, cytochrome C and Bcl-2 induced by GAS5 overexpression. We considered that GAS5 might be a potential target for treating MIRI.

Conclusions

We observed that GAS5 promoted myocardial apoptosis in myocardial ischemia-reperfusion by upregulating LAS1 expression *via* p38/MAPK pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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