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HOTAIR promotes inflammatory response after acute myocardium infarction by upregulating RAGE

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of HOTAIR in inflammatory response after acute myocardium infarction (AMI) and to investigate its underlying mechanism.

MATERIALS AND METHODS: The AMI model was first constructed in rats, and heart tissues were harvested. Expression levels of HOTAIR and receptor of advanced glycation end-products (RAGE) in rat heart were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The protein expression level of pEKR in rat heart was detected by Western blot. The levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in rats were determined by enzyme-linked immunosorbent assay (ELISA). The hypoxia-induced H9C2 cells were used to construct the MI model *in vitro*. Meanwhile, the expression levels of HOTAIR and RAGE in H9C2 cells were detected. The levels of TNF-α and IL-6 in the culture medium were determined by ELISA. Rescue experiments were conducted by co-transfecting pcDNA-HOTAIR and si-RAGE in H9C2 cells. Subsequently, the levels of pERK, TNF-α, and IL-6 were detected.

RESULTS: The mRNA expression levels of HOTAIR and RAGE in the AMI group were significantly higher than those of the control group. Western blot showed remarkably higher protein levels of RAGE and pERK in AMI rats when compared with those of controls. Similarly, results of ELISA indicated that the levels of TNF-α and IL-6 in AMI rats were significantly higher than those of controls. Meanwhile, overexpression of HOTAIR in H9C2 cells remarkably elevated the expression levels of HOTAIR and RAGE. In addition, upregulated pERK, TNF-α, and IL-6 were observed in H9C2 cells overexpressing HOTAIR, which could be reversed by RAGE knockdown.

CONCLUSIONS: HOTAIR promotes inflammatory response after AMI by upregulating RAGE expression.

Key Words:
AMI, Inflammatory response, HOTAIR, RAGE.

Introduction

Acute myocardium infarction (AMI), resulting from acute myocardial ischemic necrosis, is a severe disease with high morbidity. It is estimated that more than 3 million people suffer from acute ST-segment elevation myocardial infarction annually[1]. Meanwhile, the number of patients increases year by year. AMI is a series of pathophysiological processes involving cardiomyocyte apoptosis and inflammatory cytokines. The main characteristics of AMI include arrhythmia and heart failure[2,3]. The inflammatory response is one of the key steps in determining the progression of AMI. Massive necrosis of myocardial cells in infarcted tissue may lead to a rapid inflammatory reaction, immediately recruiting leukocytes and monocytes. Moreover, a large number of inflammatory factors are released, which may further aggravate myocardial damage and eventually lead to ventricular dilatation as well as cardiac remodeling[4]. Therefore, it is of great significance to explore the relevant mechanism of inflammatory response after AMI.

Long non-coding RNA (lncRNA) is a type of endogenous RNA with greater than 200 nt in length. LncRNAs, with no protein-coding function, was previously considered to be a "gene translation noise" without specific biological function[5]. Recent studies have found that lncRNAs exhibit high tissue-specificity in myocardial tissue. Meanwhile, a large number of cardiac-specific lncRNAs have unique regulatory and functional properties for poor myocardial remodeling, myocardial regeneration, and cardiac function. Therefore, lncRNAs may serve as potential target molecules and biomarkers for cardiac diseases[6]. HOTAIR was the first lncRNA discovered to regulate gene expression at transcrip-

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tional level[7]. In recent years, multiple studies have indicated that HOTAIR can regulate chromatin dynamics and gene expression, thereby acting as an oncogene in tumorigenesis and development[8]. In addition, studies have reported that HOTAIR is associated with AMI[9]. However, the specific mechanism of HOTAIR in the pathogenesis of AMI remains unclear.

Schmidt et al[10] and Neeper et al[11] first discovered receptor of advanced glycation endproducts (RAGE) in 1992. RAGE activates the downstream nuclear transcription factor NF-κB (Nuclear Factor κB) by binding to its receptors, thereby synthesizing various inflammatory factors and cell chemokines involved in the inflammatory response[12]. Previous researches have demonstrated that RAGE is highly expressed in patients with chronic inflammatory bowel disease, type 2 diabetes, chronic kidney disease, subclinical atherosclerosis, and chronic coronary heart disease[13]. Meanwhile, higher expression of RAGE indicates a higher risk of coronary heart disease. However, no studies have clearly reported the regulatory effect of RAGE on the pathogenic progression of AMI. Moreover, the relationship between HOTAIR and RAGE are rarely referred as well. Therefore, the aim of this study was to investigate whether HOTAIR could affect the inflammatory response after AMI by acting on RAGE. Our findings might provide new suggestions for clinical diagnosis and treatment of inflammatory response after AMI.

Materials and Methods

Construction of AMI Model in Rats

Rats were first anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. After tracheotomy and mechanical ventilation (expiration ratio: 1:1, frequency: 65 times/min, tidal volume: 8 mL), rat heart was exposed for the ligation of the ascending aorta. Subsequently, the color of ligated myocardium was observed, and pale myocardium indicated successful construction of the AMI model. Then, the incision was sutured layer by layer. A total of 2.0×10^5 U penicillin was intramuscular injected 30 min before the surgery and the first three days after surgery to prevent infection, respectively. Rats in the control group were only cut open without ligation. This study was approved by the Animal Ethics Committee of Taizhou People's Hospital Animal Center.

Cell Culture and Transfection

H9C2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). One day before transfection, the cells were seeded into 6-well plates at a density of 4×10^4 cells/per well. When the confluence was up to 80%, the cells were transfected with corresponding plasmids in accordance with the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 hours after transfection.

Hypoxia Induction in Cells

H9C2 cells were cultured in serum-free DMEM and maintained in an anaerobic incubator (5% CO_2 and 95% N_2) for hypoxia induction. After 12 h, cell transfection was performed for subsequent experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in heart tissues and cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) in accordance with PrimeScript RT reagent Kit (TaKaRa, Otsu Shiga, Japan). RNA concentration was detected by using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR™ Premix Ex Taq (TaKaRa, Otsu Shiga, Japan). Relative gene expression was calculated by the $2^{-\Delta Ct}$ method. Primers used in the study were as follows: HOTAIR (human) forward: 5'-CAGTGGGGAACTCTGACTCG-3', reverse: 5'-GTGCCTGGTGCTCTCTTACC-3'; RAGE (human) forward: 5'-GAATCCTCCCCAATGGTTCA-3', reverse: 5'-GCCCGACACCGGAAAGT-3'; GAPDH (human) forward: 5'-CATGCCGCCTGGAAACCTGCCA-3', reverse: 5'-TGGGCTGGGTGGTCCAGGGGTTTC-3'; HOTAIR (rat) forward: 5'-GGGTGGCTCACTCTTCTGGC-3', reverse: 5'-TGGCCTTGCCCGGGCTTGTC-3'; RAGE (rat) forward: 5'-CAGGGTCACAGAAACCGG-3', reverse: 5'-ATTCAGCTCTGCACGTTCCT-3'; GAPDH (rat) forward: 5'-ACCACAGTCCATGCCATCAC-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3'.

Western Blot

Radio-immunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was employed to extract total protein of tissues and transfected cells. After centrifugation, the supernatant was collected and the protein concentration was determined by the bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA). Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with Tris-Buffered Saline with Tween 20 (TBST), the membranes were incubated with corresponding secondary antibody. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Cell supernatant was first collected and then incubated with primary antibodies at 37°C for 30 min. After washing with phosphate-buffered saline (PBS), the samples were incubated with corresponding secondary antibodies, followed by color development in the dark. Finally, the TMB solution was added for development termination. Optical density (OD) value was detected at the wavelength of 450 nm by a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 was used for all statistical analysis (IBM, Armonk, NY, USA). Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used for comparison between two groups. $p < 0.05$ was considered statistically significant.

Results

HOTAIR and RAGE Were Highly Expressed in AMI Rats

After construction of the AMI model in rats, heart tissues were collected and harvested for RNA and protein extraction. QRT-PCR results showed that the mRNA expression level of HOTAIR in the AMI group was significantly higher than that of the control group (Figure 1A). Meanwhile, both the mRNA and protein levels of RAGE were remarkably higher in the AMI group when compared with those of the control group (Figure 1B and 1C). These results indicated that the expression levels of HOTAIR and RAGE were upregulated in AMI rats.

HOTAIR Upregulated RAGE Expression

After transfecting with pcDNA-HOTAIR in H9C2 cells, we found that the mRNA level of HOTAIR remarkably increased, indicating good transfection efficacy (Figure 2A). Furthermore, HOTAIR overexpression significantly elevated the mRNA and protein levels of RAGE (Figure 2B and 2C). This suggested that HOTAIR overexpression could upregulate RAGE expression in H9C2 cells.

HOTAIR Promoted an Inflammatory Response in Cardiomyocytes

Compared with the control group, a higher expression level of pERK was observed in the AMI group (Figure 3A). Besides, the expression levels of TNF-α and IL-6 were markedly elevated in the AMI group than those of the control group (Figure 3B). Subsequently, we performed hypoxia treatment in H9C2 cells and constructed the MI model *in vitro*. Similarly, the expression levels of pERK, TNF-α, and IL-6 were also significantly increased in hypoxia-induced H9C2 cells (Figure 3C and 3D). We might conclude that HOTAIR promoted cardiomyocyte inflammation.

HOTAIR Promoted an Inflammatory Response by Regulating RAGE

To further explore the mechanism of HOTAIR in promoting an inflammatory response, hypoxia-induced H9C2 cells were transfected with pcDNA-HOTAIR or co-transfected with pcDNA-HOTAIR and si-RAGE, respectively. Results indicated that HOTAIR overexpression remarkably increased the expression levels of pERK, TNF-α, and IL-6, which were partially reversed by RAGE knockdown (Figure 4A and 4B). Our findings demonstrated that HOTAIR promoted an inflammatory response in cardiomyocytes by regulating RAGE.

Discussion

AMI has become a serious disease that endangers human health[14], and its incidence is increasing year by year. AMI and hypoxia may cause in-

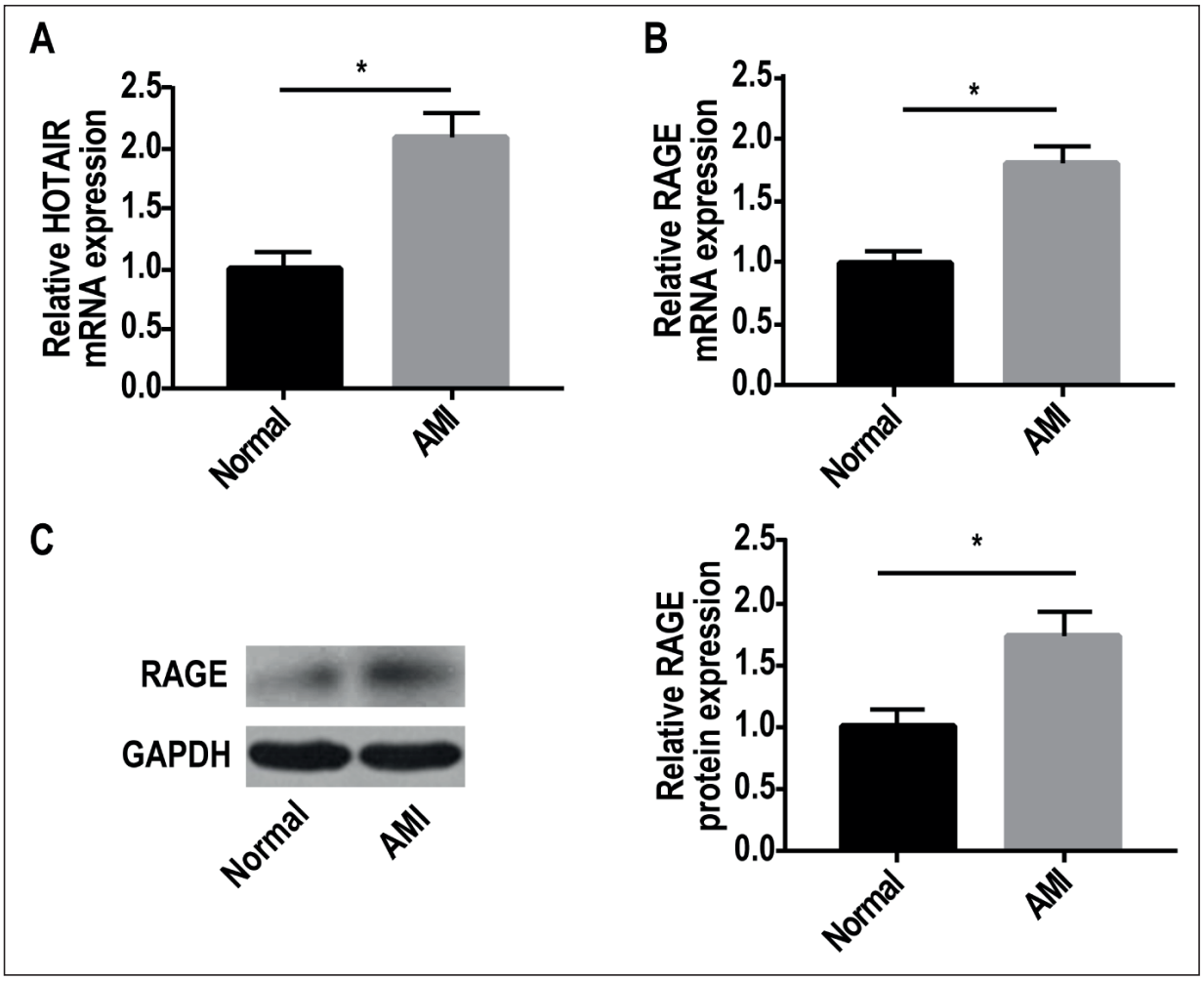

Figure 1. HOTAIR and RAGE were highly expressed in AMI rats. ***A,*** QRT-PCR results showed that the mRNA level of HOTAIR in the AMI group was significantly higher than that of the control group. ***B, C,*** Both mRNA and protein levels of RAGE were markedly higher in the AMI group when compared with the control group.

sufficient myocardial energy supply, myocardial cell metabolism alterations, ion channel and oxidative stress, eventually leading to cell necrosis and apoptosis. Non-infectious inflammatory responses can further aggravate myocardium damage. Although inflammation after AMI contributes to myocardium repair and reconstruction, an excessive inflammation may have a negative impact on cardiomyocyte and cardiac function[15]. Therefore, exploration of the occurrence and development of inflammatory response after AMI is of great significance for finding effective diagnostic indicators and therapeutic targets.

LncRNAs can regulate the gene expression at epigenetic, transcriptional and post-transcriptional levels, thus participating in pathological and physiological processes. Meanwhile, lncRNAs have been found to be differentially expressed in a variety of diseases, including neurodegenerative diseases, cardiovascular and cerebrovascular diseases, and tumors[16-18]. Human HOTAIR is located on chromosome 12q13. Studies on genetic regulation have found that HOTAIR regulates downstream oncogenes and tumor-suppressor genes by recruiting protein complexes[19]. Moreover, HOTAIR can serve as an oncogene in tumor development[20]. Previous studies have reported that HOTAIR is up-regulated in patients with endometrial cancer and is associated with epithelial-mesenchymal transition[21]. In addition, multiple researches have indicated that HOTAIR is an essential mediator of AMI[22]. In the present investigation, we found that HOTAIR was highly expressed in AMI rats, which is similar to previous studies.

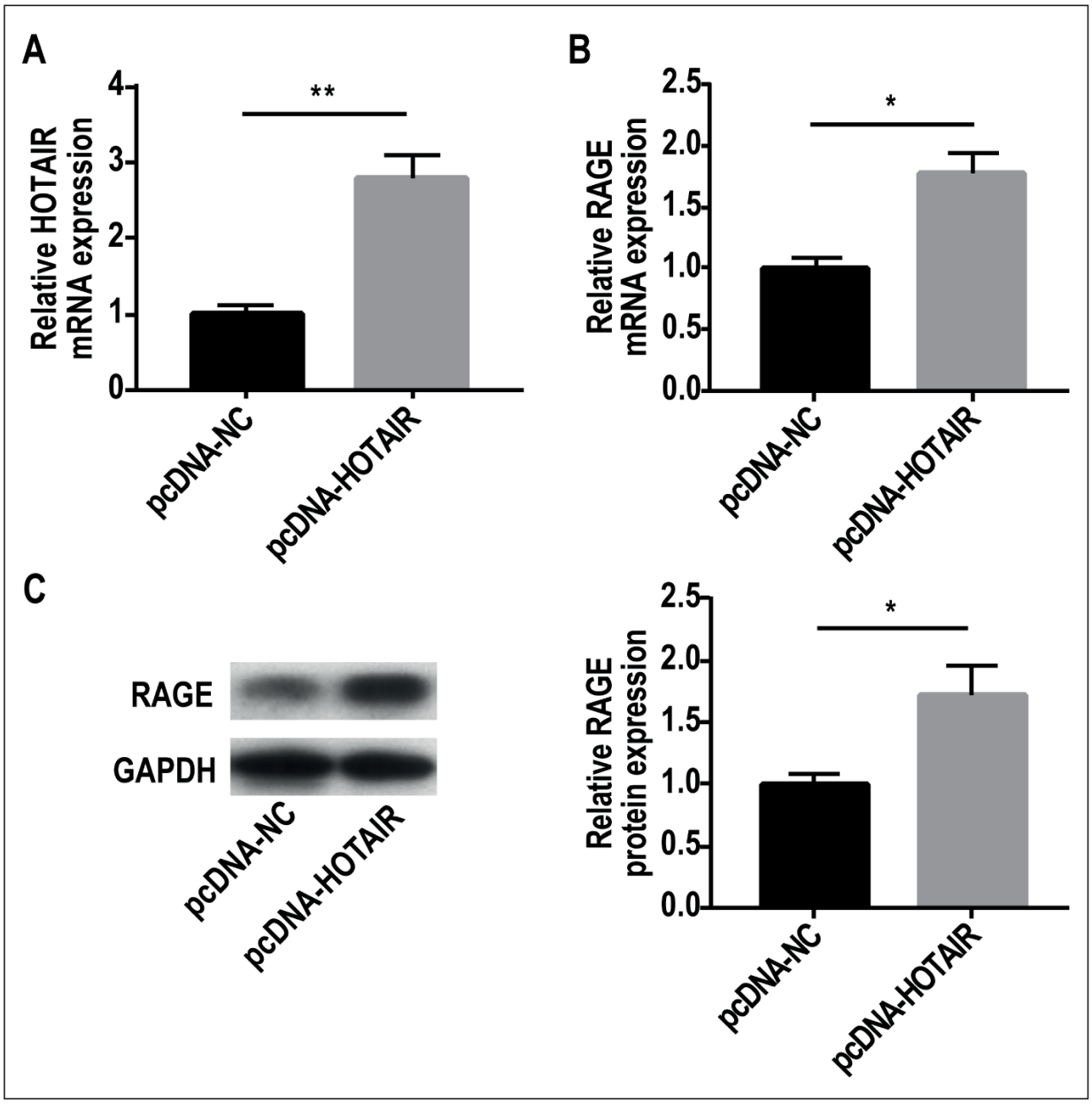

Figure 2. HOTAIR upregulated RAGE expression. ***A,*** Transfection of pcDNA-HOTAIR significantly upregulated the mRNA level of HOTAIR in H9C2 cells. ***B, C,*** Both mRNA and protein levels of RAGE were remarkably elevated by HOTAIR overexpression.

As one of the most crucial specific receptors for AGEs, RAGE is a multi-ligand receptor belonging to the immunoglobulin family. RAGE is expressed in different types of cells and is generally lowly expressed in tissues. The serum level of RAGE can be upregulated in the presence of a corresponding high-level ligand, which is closely related to inflammatory responses[23]. Scholars have found that under hypoxic reperfusion injury, the myocardial damage was markedly less in RAGE knockout mice and RAGE inhibitors injected mice. Meanwhile, the infarct size was significantly smaller than those of controls[24]. In recent years, RAGE and its downstream related signaling pathways have been extensively studied. Inhibition of the RAGE-NF-κB/Nrf2 pathway influences downstream inflammatory response, oxidative stress, and apoptosis, ultimately protecting the nervous system[25]. Some researchers have also found that RAGE is asso-

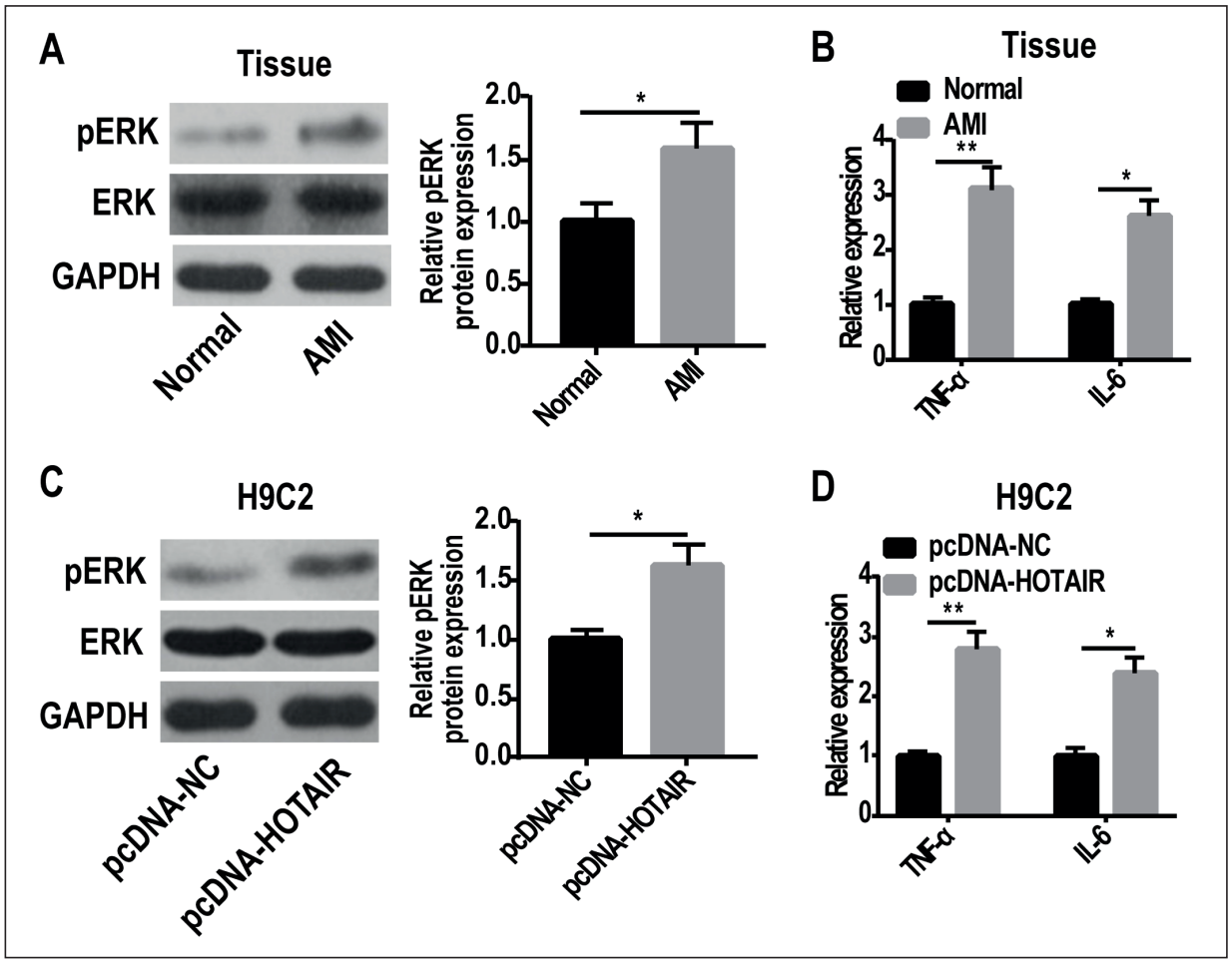

Figure 3. HOTAIR promoted inflammatory response in cardiomyocytes. ***A,*** Higher expression level of pERK was observed in the AMI group compared with that of the control group. ***B,*** Expression levels of TNF-α and IL-6 were markedly elevated in the AMI group than the control group. ***C, D,*** Expression levels of pERK, TNF-α, and IL-6 increased remarkably in hypoxia-induced H9C2 cells.

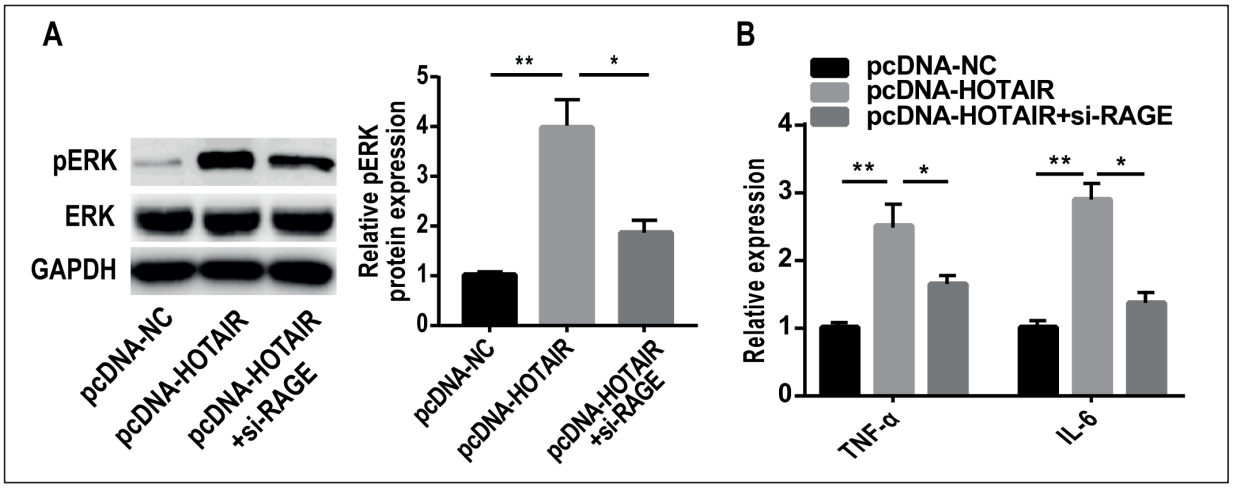

Figure 4. HOTAIR promoted inflammatory response in cardiomyocytes by regulating RAGE. ***A, B,*** HOTAIR overexpression significantly increased the expression levels of pERK, TNF-α, and IL-6 in H9C2 cells, which were partially reversed by RAGE knockdown.

ciated with poor prognosis of breast cancer. Targeting inhibition of RAGE by siRNA can reduce the excessive inflammatory infiltration, alleviate the tumor cell proliferation and metabolism, and inhibit the breast cancer cell invasion[26]. In addition, RAGE protects persistent atrial fibrillation by binding to AGEs and activating NF-κB[27]. We found that RAGE was regulated by HOTAIR, thereafter alleviating inflammatory response of cardiomyocytes after AMI.

Conclusions

We showed that HOTAIR promotes inflammatory response after AMI by upregulating RAGE. Our findings may provide new directions for the treatment of inflammation after AMI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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