ALDOA protects cardiomyocytes against H/R-induced apoptosis and oxidative stress by regulating the VEGF/Notch 1/Jagged 1 pathway

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Abstract

Myocardial infarction (MI) is a myocardial necrosis disease caused by continuous ischemia and hypoxia. Abnormal expression of aldolase A (ALDOA) has been reported in cardiac hypertrophy, heart failure and other cardio-cerebrovascular diseases. The present study aims to explore the effects of ALDOA on hypoxia/reperfusion (H/R)-induced oxidative stress, and investigate the underlying mechanisms. ALDOA was expressed at a low level in blood samples from MI patients and H/R-induced H9C2 cardiomyocytes. Overexpression of ALDOA suppressed H/R-induced oxidative stress and apoptosis. Using co-immunoprecipitation and protein blots, we demonstrated that ALDOA modulates the Notch 1–Jagged 1 signalling pathway by upregulating VEGF. Taken together, our data reveal that ALDOA protects cardiomyocytes from H/R-induced oxidative stress through the VEGF/Notch 1/Jagged 1 axis, and should be investigated as a therapeutic target for the treatment of MI in future.

Keywords Apoptosis · Aldolase A · Cardiomyocytes · Oxidative stress

Introduction

Myocardial infarction (MI) is a cardiovascular disease of myocardial necrosis caused by continuous ischemia and hypoxia [1]. Oxidative stress is implicated in the pathogenesis of MI [2]. MI dramatically influences the cardiovascular, respiratory and digestive systems of patients, and even leads to devastating injury [3]. To date, the common therapeutic strategies for MI include intensive care, drug therapy, antiarrhythmic activity and reperfusion therapy [4]. Despite dramatic improvements in MI treatment, the incidence and prevalence of MI continue to increase [5]. Therefore, it is of value to illuminate the potential effects of pathogenesis-related genes on MI and investigate the underlying mechanisms.

Aldolase A (ALDOA) plays pivotal roles in energy balance, gluconeogenesis and glycolysis [6]. Abnormal expression of ALDOA results in cardiac hypertrophy, heart failure and other cardio-cerebrovascular diseases [7]. Previous studies revealed that ALDOA is dramatically upregulated in hypertrophic hearts [8, 9], and silencing of ALDOA inhibits cardiac hypertrophy in vivo [8]. Furthermore, ALDOA contributes to the myocardial stress-gene response [10]. Overexpression of ALDOA strengthens resistance to myocardial injury in rats [6]. A recent study revealed that ALDOA is differentially expressed in patients during MI compared to control [11]. However, the role of ALDOA in MI and the underlying mechanisms are poorly understand.

Vascular endothelial growth factor (VEGF) is a key mediator of angiogenesis, and participates in cell growth, apoptosis and the immune-inflammatory response [12]. Anoxia, surgery and myocardial ischemia reperfusion injury trigger the VEGF cascade and activate the Notch signalling pathway, thus promoting apoptosis and oxidative stress [13]. Inhibition of VEGF protects mice against renal ischemic–reperfusion (I/R)-induced injury [14]. Activation of the VEGF/Notch 1 signalling pathway attenuates cellular senescence in H9C2 cardiomyocytes [15]. Furthermore, it has been reported that ALDOA decreases the mRNA level

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of VEGF, and downregulation of VEGF leads to a dramatic exacerbation of MI [16, 17].

In the current study, we detected ALDOA expression levels in blood samples from MI patients and hypoxia/reperfusion (H/R)-induced H9C2 cells, and assessed the effects of ALDOA on H/R-induced oxidative stress and apoptosis. Furthermore, the underlying mechanism of ALDOA in the mediation of H/R-induced MI was also explored.

Materials and methods

Blood sample collection

The blood samples from 24 patients with myocardial infarction and 28 healthy subjects who were admitted more than 24 h after the onset of chest pain were collected from Xi'an No 5 hospital (Shaanxi, China) between August 2017 and December 2018. These participants included 22 males and 30 females, aged from 40 to 56 years. Myocardial infarction was diagnosed based on the Framingham Heart Study. Serum samples were separated by centrifugation at $1000 \times g$ for 10 min. Each serum supernatant was collected and stored at $-80\ °C$ until use. The study was approved by the ethics committee of Xi'an No 5 hospital, and informed consent was obtained from all subjects.

Cell culture

The H9C2 cell line was purchased from the Chinese Type Culture Collection (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) at $37\ °C$, 5% CO_2. The H/R model of cardiomyocytes was constructed by oxygen-glucose deprivation/recovery (OGD/R) assay. In brief, ALDOA-treated H9C2 cell line was cultured in serum-free DMEM at $37\ °C$ in a hypoxia chamber with 94% N_2, 5% CO_2 and 1% O_2 (OGD) for 10 h, then glucose and 10% FBS (Gibco, Carlsbad, CA, USA) were added to the culture plate. Cells were then cultured under normal growth conditions for an additional 12 h.

Transfection experiments

HepG2-ALDOA (ALDOA), pcDNA-VEGF (VEGF), si-VEGF and negative control (NC) were synthesised by Ribobio (Guangzhou, China). H9C2 cells were plated into a 6-well plate at a concentration of 5×10^5 cells/ml and cultured for 24 h at $37\ °C$. Subsequently, H9C2 cells were transfected with 0.15 μg of these plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. All luciferase assays were repeated in three independent experiments. The cells were harvested by trypsinisation 48 h post transfection and firefly and Renilla luciferase activity

were measured using the Dual-Luciferase Reporter Assay System (Promega). All firefly luciferase values were normalised to Renilla luciferase activity.

RNA extraction and RT-qPCR

Total RNA was isolated from serum or H9C2 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), followed by reverse transcription reaction using the Revert Aid cDNA Synthesis kit (Transgen Biotech; Beijing). The RT-qPCR was performed using qPCR SYBR-Green Master Mix (Takara, Dalian, China) in a DNA Engine Opticon™ system (MJ Research, Waltham, MA). β-actin was used as the references for the DNA template. The $2^{-\Delta\Delta C_t}$ method was applied to quantify gene expression. Each sample was analysed in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were separated by centrifugation at $1000 \times g$ for 10 min. The level of ROS generation, malondialdehyde (MDA) and superoxide dismutase (SOD) activity were detected using appropriate ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's protocols. The absorbance was measured at 450 nm under a microplate reader (Olympus, Tokyo, Japan). The concentration of ALDOA was obtained from a calculation based on the standard curve.

Annexin V assay

Transfected cells were plated into a 6-well plate at a concentration of 5×10^5 cells/ml and cultured for 24 h at $37\ °C$. Then, 6 μl of annexin-V-FITC was added to the trypsinised cells. After storing at room temperature for 25 min away from light, 400 μl PBS/well was added. Apoptosis was subsequently assessed using a fluorescence microscope (Olympus, Tokyo, Japan).

Western blotting

H9C2 cells were lysed with RIPA lysis buffer (Solarbio, Beijing, China) containing a protease inhibitor mixture (Solarbio, Beijing, China). The concentration of proteins were determined with the BCA assay (Pierce, Rockford, IL). Lysates were denatured with loading buffer at $100\ °C$ for 5 min. Then, equal protein amounts were loaded on SDS-PAGE (12%) and transferred onto PVDF membranes, eliminating non-specific staining with blocking reagents. The member was probed with primary Abs (1:1000) at $4\ °C$ overnight, followed by a PBS wash, incubation with an HRP-labelled secondary Abs (1:2000) and development using ECL (Luminata Forte, Millipore,

USA). The protein bands were quantified using Image J (National Institutes of Health, USA).

Co-immunoprecipitation

H9C2 cells were lysed with RIPA lysis buffer (Solarbio, Beijing, China). Protein A/G-agarose was diluted to the working concentration of 50% with PBS. Then, the solution was added to samples (100 μl/ml) and shaken for 10 min at 4 °C. After centrifugation at 14,000×g for 10 min, the supernatant was collected in a new centrifugal tube. The concentration of proteins were determined with the BCA assay (Pierce, Rockford, IL). The primary Abs were added and incubated at 4 °C overnight. Samples were then centrifuged at 14,000×g for 5 min and the supernatant was collected for subsequent studies.

Statistical analysis

The expression of ALDOA in blood samples and H9C2 cells was evaluated with Mann–Whitney test. The effects of ALDOA on H/R-induced oxidative stress and apoptosis were analysed by the Kruskal–Wallis test, followed by Dunn's Test. The experiments performed in this study were repeated at least three times and data are expressed as standard error of mean (SEM). $p < 0.05$ was considered significant. The software used was SPSS version 22 (SPSS Inc, Chicago, IL).

Results

ALDOA is downregulated in MI patients and H/R-induced cardiomyocytes

As shown in Fig. 1a, the expression of ALDOA in blood samples of MI patients were dramatically lower than in the healthy group. Furthermore, the mRNA expression of ALDOA was decreased in H/R-induced H9C2 cells compared with the control (Fig. 1b). ALDOA mimic transfection effectively overexpressed ALDOA (Fig. 1b).

ALDOA suppresses H/R-induced oxidative stress and apoptosis

We next investigated the effects of ALDOA on oxidative stress and apoptosis in H/R-induced H9C2 cells. The results reveal that treatment with H/R significantly promoted apoptosis (Fig. 2a), elevated the protein expression of cleaved caspase-3 and Bax, and inhibited Bcl-2 protein expression (Fig. 2b). Overexpression of ALDOA suppressed H/R-induced apoptosis (Fig. 2b). ELISA showed that H/R treatment led to increased ROS and MDA levels, and decreased SOD activity (Fig. 2c–e).

ALDOA positively regulates VEGF protein expression

Accumulating evidence demonstrates that VEGF plays vital role in H/R-induced apoptosis and oxidative stress [18, 19]. Our results show that the protein and mRNA expression of VEGF were inhibited by H/R treatment, and ALDOA
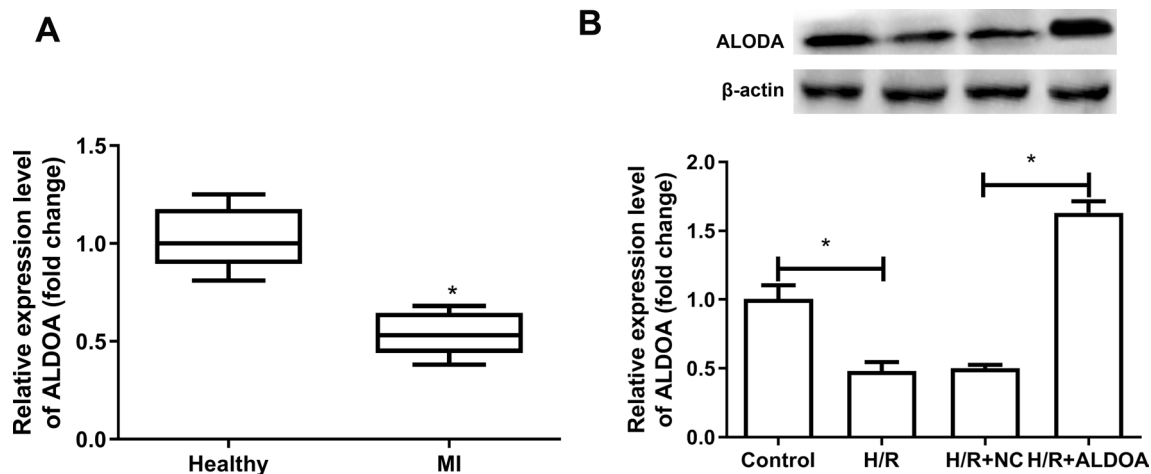

Fig. 1 Expression of ALDOA detected in the H/R-induced H9C2 cells and blood samples from patients with MI. Serum samples were separated by centrifugation at 1000×g for 10 min. Each serum supernatant was collected and stored at − 80 °C until use. **a** The expression of ALDOA in MI blood samples was measured by RT-qPCR. *$p < 0.05$ compared with healthy. **b** The expression of ALDOA in the H/R-induced H9C2 cells were determined by RT-qPCR. *$p < 0.05$ compared with control. The results are presented as mean ± SEM from three independent experiments
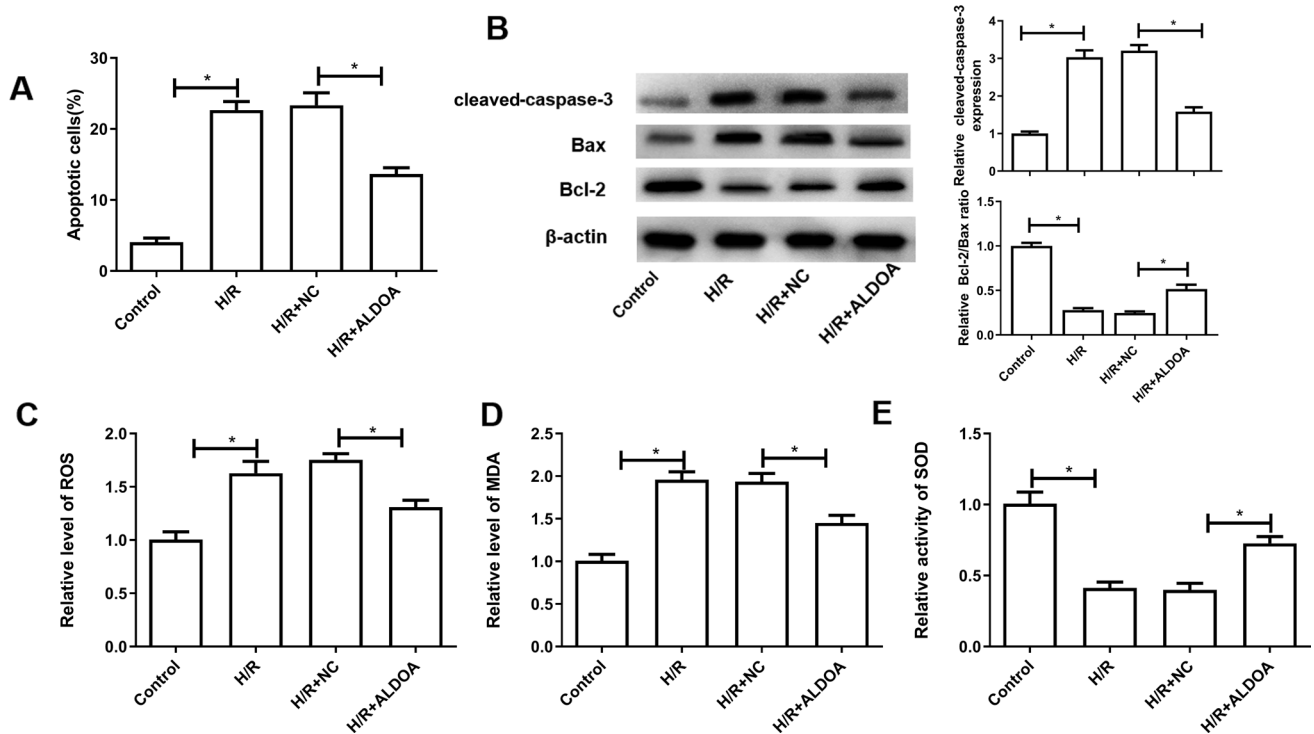
Fig. 2 ALDOA reduces H/R-induced oxidative stress and apoptosis. After treatment with H/R, cells were respectively transfected with the NC or ALDOA. **a** The apoptotic rate was detected by the Annexin *V* assay. **b** The protein expression of cleaved caspase-3, Bcl-2 and Bax were determined by western blotting. **c**–**e** ROS generation, MDA levels and SOD activity were detected by ELISA. The results are presented as mean ± SEM from three independent experiments. *$p < 0.05$

reversed the inhibitory effect of H/R on VEGF expression (Fig. 3a, b). The luciferase reporter assay showed that HEK293T cells co-transfected with WT VEGF 3′UTR luciferase reporter plasmids and ALDOA has a significant decrease in luciferase activity, whereas the mutant groups had no influence on luciferase activity (Fig. 3c). Moreover, VEGF was found to co-precipitate with ALDOA (Fig. 3d). The expression of VEGF was lower in blood samples of MI patients than healthy participants (Fig. 3e). The Pearson correlation analysis indicated that a positive correlation exists between ALDOA and VEGF (Fig. 3f).

ALDOA activates the Notch pathway by regulating VEGF

VEGF has been shown to be involved in regulating the Notch signalling pathway in endothelial cells and human pluripotent stem cells [20, 21], but whether ALDOA modulates the Notch pathway through VEGF is unclear. The efficiency of pcDNA-VEGF and VEGF siRNA is shown in Fig. 4a. VEGF dramatically promoted Notch 1 and Jagged 1 protein expression, and si-VEGF treatment showed the opposite results (Fig. 4b–d). Moreover, the protein expression of p-STAT3/STAT3 and p-JAK2/JAK2 underwent no significant change with VEGF or si-VEGF transfection (data not showed).

VEGF modulates the Notch pathway and reverses the effects of ALDOA on H/R-induced oxidative stress and apoptosis

We next studied the involvement of VEGF in H/R-induced H9C2 cells. The results show that VEGF siRNA further elevated the H/R-induced expression of cleaved caspase-3, Bax, ROS and MDA (Fig. 5a–d), and inhibited Bcl-2 protein expression and SOD activity (Fig. 5e). Moreover, transfection with VEGF siRNA abolished the inhibitory effect of ALDOA on H/R-induced H9C2 cells (Fig. 5c–e). Similarly, treatment with the Notch inhibitor carvacrol also abolished the inhibition effect of ALDOA on oxidative stress and apoptosis in H/R-induced H9C2 cells. (Fig. 5a–e).

Discussion

ALDOA is a key regulator of cytokine signal transduction and plays important roles in cell proliferation, invasion and other molecular events [22]. Previous studies have revealed that ALDOA is expressed in acute coronary syndrome at a low level [23, 24], and that treatment with ALDOA effectively moderates the severity of chronic heart failure [25]. Moreover, aberrant expression of ALDOA is closely related
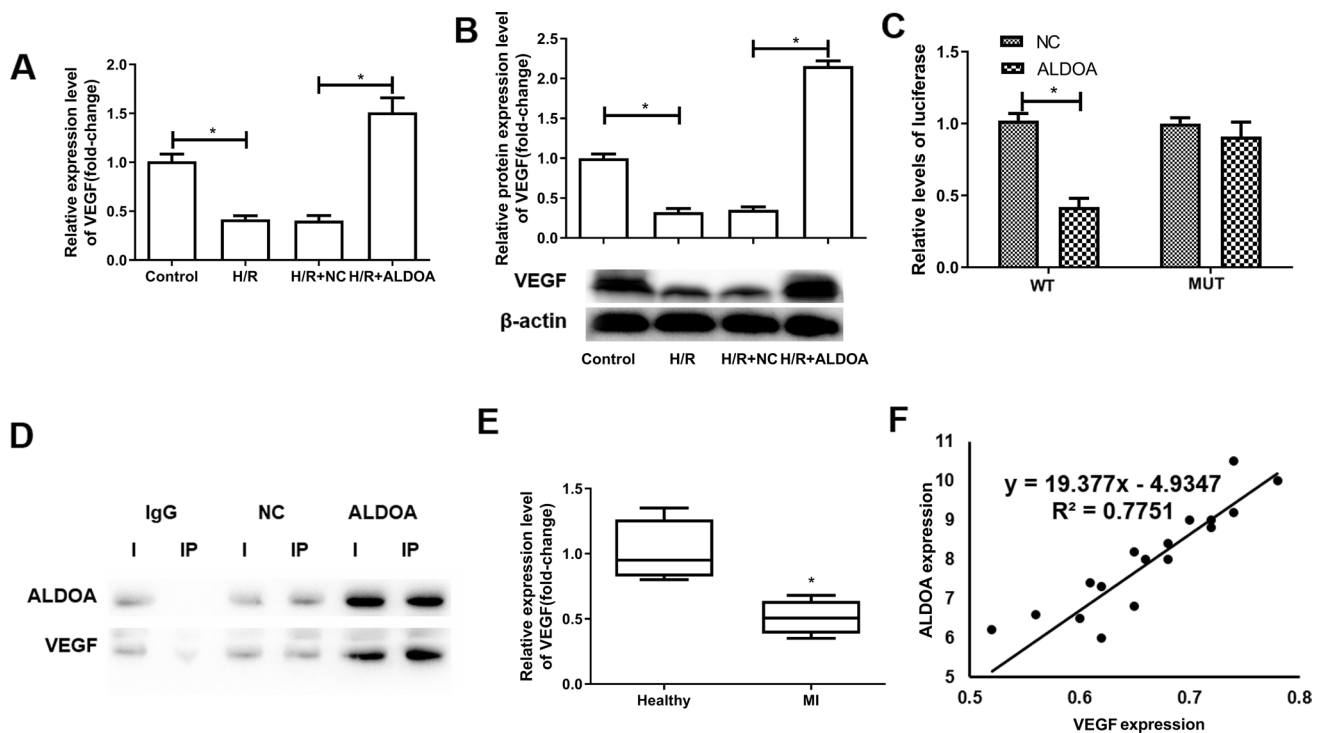
Fig. 3 ALDOA directly regulates VEGF. Cells were respectively transfected with the NC mimic or ALDOA mimic. Samples were collected after transfection for 48 h. The mRNA (**a**) and protein (**b**) expression of VEGF were detected in H9C2 cells. **c** The luciferase activity of the VEGF 3′UTR luciferase reporter vector was determined using the luciferase reporter assay. **d** The ALDOA–VEGF interaction was investigated by co-immunoprecipitation. **e** The levels of VEGF in MI patients were detected by RT-qPCR. **f** The relationship between ALDOA and VEGF was determined by Pearson analysis. The results are presented as mean ± SEM from three independent experiments. $*p < 0.05$

to the occurrence of hypertension, cardiac hypertrophy and ventricular arrhythmia [8]. High expression of ALDOA suggests the incidence of cardiac inflammation and fibrosis [26, 27]. Knockdown of ALDOA inhibits isoproterenol-induced cardiomyocyte hypertrophy [8]. A recent study showed that ALDOA is dramatically decreased in MI rats [11]. In accordance with these results, we confirmed that ALDOA is downregulated in MI patients and H/R-induced H9C2 cells, and has a protective effect against MI.

Oxidative stress in cardiomyocytes during the development of cardiac injury has increasingly drawn attention [28]. Previous studies have reported that oxidative stress is associated with the level of ROS, MDA and SOD activity in angiocardiopathy [29, 30]. Accumulation of ROS causes cardiac inflammation and apoptosis, ultimately resulting in myocardial injury [31]. MDA contributes to lipid peroxidation and mitochondrial metabolism [32]. Moreover, restoring the balance of SOD alleviates cardiac function in mice with heart failure [33]. The occurrence of oxidative stress aggravates myocardial necroptosis [34]. In the current study, ALDOA was found to decrease ROS and MDA levels, and protected cardiomyocytes against H/R-induced apoptosis and oxidative stress. As a part of multi-enzyme glycolytic complexes that attached to mitochondria [35], ALDOA may contribute to the biogenesis of ATP [36], and thus decrease the oxidative stress [37].

The involvement of the Notch pathway in oxidative stress has been described in previous studies [38, 39]. Activation
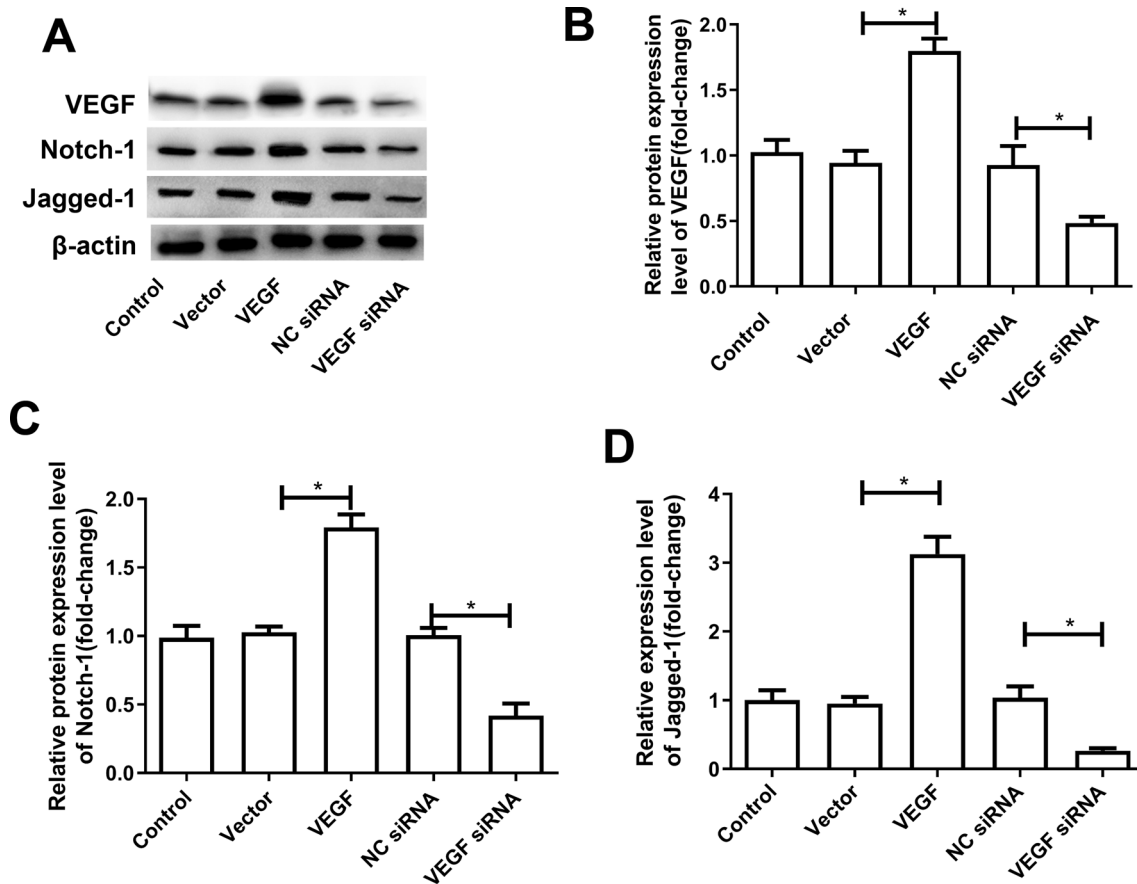
Fig. 4 VEGF regulates the Notch pathway in H9C2 cells. After stimulation with H/R, cells were respectively transfected with the NC, VEGF siRNA (si-VEGF) or pcDNA-VEGF (VEGF). **a** The protein expression of VEGF, Notch 1 and Jagged 1 were detected using western blotting. **b–d** The protein expression of VEGF, Notch 1 and Jagged 1 were quanlifited. *$p < 0.05$

of Notch 1 contributes to production of ROS and promotes mitochondrial oxidative phosphorylation [40]. Translation of VEGF has a promoting effect on the Notch 1 signalling pathway [20]. Moreover, Notch 1 protects the heart from I/R injury by counteracting oxidative/nitrate stress and increasing endothelial NOS phosphorylation [41]. A recent study showed that inhibiting VEGF blocks the Jagged 1/Notch 1 signalling pathway, counteracting the antisenescence effects on Dox-induced cardiomyocytes [42]. The data in the current study show that ALDOA upregulates VEGF and

triggers the Notch 1 pathway, inducing a cardioprotective effect in H/R-induced H9C2 cells.

In conclusion, our study revealed that ALDOA is expressed at low levels in blood samples from MI patients and H/R-induced H9C2 cells, and overexpression of ALDOA attenuates H/R-induced oxidative stress and apoptosis. Moreover, VEGF contributes to the activation of the Notch 1 signalling pathway, and ALDOA modulates H/R-induced oxidative stress and apoptosis by the VEGF/Notch 1 pathway. ALDOA may act as a potential target for the development of therapeutics against MI.
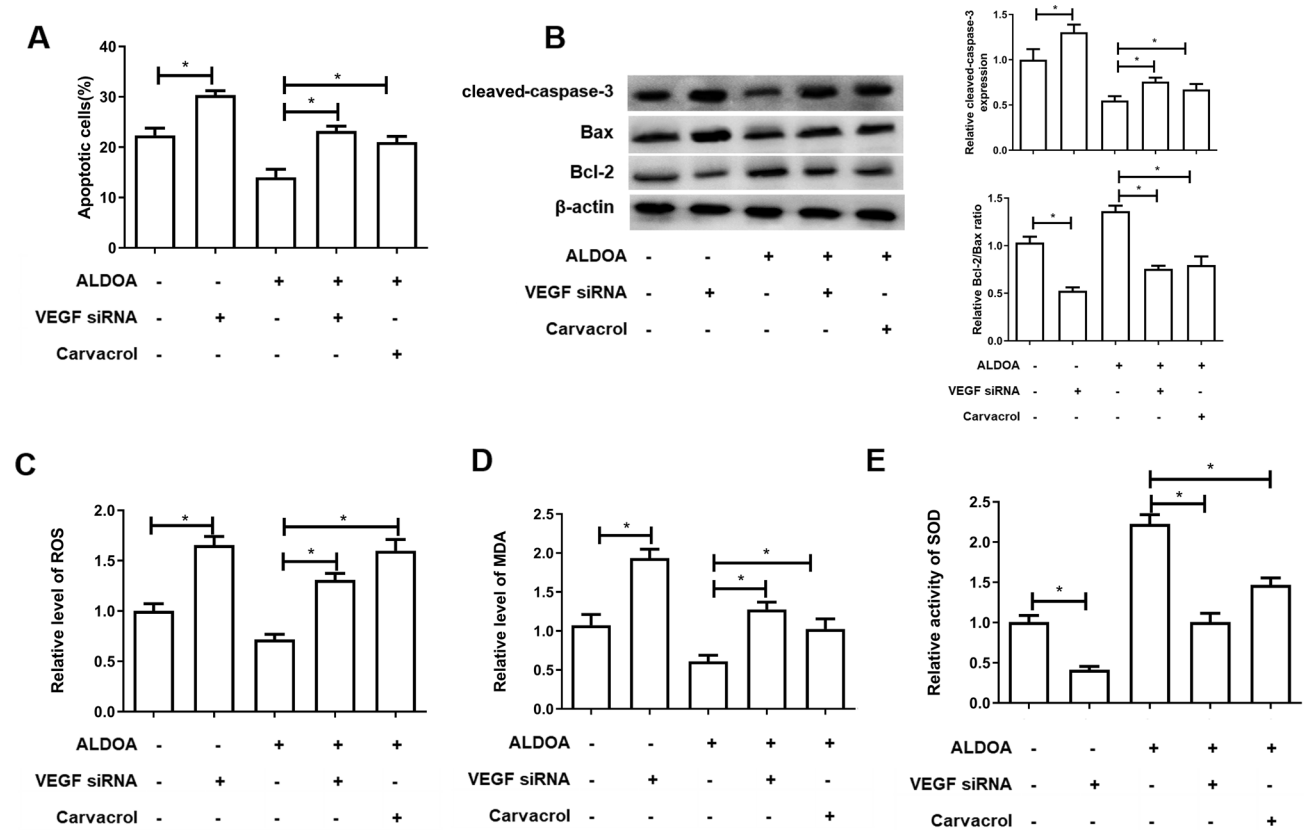
Fig. 5 VEGF siRNA reversed the effects of ALDOA on H/R-induced oxidative stress and apoptosis via the Notch pathway. H9C2 cells were transfected with specific plasmid vectors, and samples were collected 48 h after transfection. **a** The apoptotic rate was detected by the Annexin *V* assay. **b** The protein expression of cleaved caspase-3, Bcl-2 and Bax was determined by western blotting. **c–e** ROS generation, MDA levels and SOD activity were detected by ELISA. The results are presented as mean ± SEM from three independent experiments. *$p < 0.05$

Compliance with ethical standards

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