

# BRD4 Inhibition Protects Against Myocardial Ischemia/Reperfusion Injury by Suppressing Inflammation and Oxidative Stress Through the PI3K/AKT Signaling Pathway

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**Abstract:** This study aims to investigate the effect and the related mechanisms of bromodomain-containing protein 4 (BRD4) inhibition on myocardial ischemia/reperfusion (I/R) injury. In vivo and in vitro myocardial I/R models were constructed. Expression of BRD4 was examined by RT-qPCR and Western blot. I/R injury was evaluated by analyzing cardiac function and the activity of biochemical markers of myocardial injury. Inflammation and oxidative stress were determined by measuring the levels of myeloperoxidase, TNF- $\alpha$ , IL-6, malondialdehyde, and superoxide dismutase. The activation of the PI3K/AKT signaling pathway was tested by the phosphorylation of p85 and AKT. We found BRD4 was significantly increased in the myocardial tissues after myocardial I/R injury. BRD4 inhibition suppressed the indices of cardiac function and the biochemical markers of myocardial injury. I/R-induced inflammation and oxidative stress were suppressed by shBRD4 in vivo and in vitro. In addition, BRD4 inhibition significantly increased the relative protein expression levels of p-p85, p-AKT T308, and p-AKT S473. In conclusion, this study for the first time demonstrated the protective effect of BRD4 inhibition on myocardial I/R injury in vivo and in vitro, and this effect was related to the suppression of inflammation and oxidative stress through the activation of the PI3K/AKT signaling pathway.

**Key Words:** BRD4, myocardial ischemia/reperfusion injury, inflammation, oxidative stress, PI3K/AKT signaling pathway

(*J Cardiovasc Pharmacol*<sup>TM</sup> 2021;78:839–846)

## INTRODUCTION

Ischemic heart disease (IHD) is a significant cause of morbidity and mortality worldwide.<sup>1</sup> The return of blood supply is effective to reduce the size of ischemic myocardium<sup>2</sup>;

however, myocardial ischemia/reperfusion (I/R) can cause injury to the heart in myocardial ischemic disease.<sup>3</sup> Better understanding of the molecular mechanisms underlying myocardial I/R injury and the investigation of essential therapeutic targets in IHD are urgently required.

Bromodomain-containing protein 4 (BRD4) belongs to the bromodomain and extraterminal (BET) family, which is known to bind acetylated lysine and change the structure of chromatin through bromodomain.<sup>4,5</sup> BRD4 has been reported to play important roles in various biological processes such as growth, apoptosis, fibrosis, and inflammation.<sup>6–8</sup> Previous studies demonstrated that the change of BRD4 expression is associated with the pathological process of cardiomyocyte hypertrophy. For instance, BRD4 inhibition suppresses fibrosis, inflammation, and oxidative stress in Ang II-induced hypertrophy in cardiomyocytes as well as in mouse models with aortic banding operation.<sup>9</sup> BRD4 stimulates the activation of cardiac fibroblasts.<sup>10</sup> Anand et al suggested that BRD4 acts as a coactivator of pathologic gene transactivation during cardiomyocyte hypertrophy.<sup>11,12</sup> Recently, Sun et al found that BRD4 expression is altered in a rat model of acute myocardial infarction and BRD4 suppression attenuated cardiomyocyte apoptosis in myocardial infarction.<sup>13,14</sup> However, the studies on the effect and the mechanisms of BRD4 on myocardial I/R injury are few.

In this study, in vivo and in vitro myocardial I/R models were constructed, and the expression of BRD4 after I/R injury was detected. Furthermore, the effect of BRD4 inhibition on myocardial I/R injury and the underlying mechanisms were explored.

## MATERIALS AND METHODS

### Establishment of a Rat Model of Myocardial I/R Injury

This study was approved by the Ethics Committee of People's Hospital of Rizhao, and all animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. 40 male Sprague–Dawley rats (Hunan Slac Jingda Laboratory Animal Co, Ltd, Changsha, Hunan, China) were divided into 4 groups (n = 10 in each group): sham group, I/R group, I/R + shCtrl group, and I/R + shBRD4 group. To establish the myocardial I/R model, the rats were anesthetized with chloral hydrate, intubated and artificially ventilated with a respirator, and then needle electrodes were inserted into the limbs of rats. The heart was exposed through a left thoracotomy in the fourth intercostal space. The left

Received for publication June 11, 2021; accepted August 28, 2021.

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The authors report no conflicts of interest.

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anterior descending coronary artery of rats was ligated with 6-0 silk suture using a snare occluder. After successful ligation of the blood vessel, we observed cyanosis in the left anterior ventricular wall and the electrocardiogram showed ST elevation. After ischemia for 40 minutes, coronary artery was reperfused by releasing the knot, followed by reperfusion of 180 minutes. Rats in the sham group underwent the similar protocols without ligation. Rats in the I/R + shCtrl group and I/R + shBRD4 group received either BRD4 shRNA (shBRD4) or the negative control (shCtrl) 1 week before I/R surgery. The indices of cardiac function, including heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), and rates of maximum positive/negative left ventricular pressure development ( $\pm dp/dt$  max), were measured by RM6000 8 channel physiological recorder (NIHON KOHDEN CORP, Japan).

### Cell Culture

H9C2 cells were purchased from the American Type Culture Collection (Manassas, VA.). Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc, Waltham, MA) containing 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10% fetal bovine serum (Invitrogen), at 37°C with 5% CO<sub>2</sub> and 95% air. Oxygen–glucose deprivation (OGD) was performed on H9C2 cells to induce myocardial I/R injury in vitro. The cells were cultured in glucose-free DMEM (Invitrogen) without serum and maintained at 37°C with 5% CO<sub>2</sub> and 95% N<sub>2</sub> (vol/vol) for 10 hours. Then, the medium was replaced with the complete medium, and the cells were cultured at 37°C with 5% CO<sub>2</sub> and 95% air.

### Transfection

Cells were seeded into the 6-well plates at a density of  $5 \times 10^4$  cells/well. shBRD4 and shCtrl were transfected into H9C2 cells using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were collected for experiments.

### RT-qPCR

Total RNA was isolated from the rat myocardial tissues and H9C2 cells using TRIzol reagent (Invitrogen). Reverse transcription synthesis of complimentary DNA (cDNA) was conducted using the Genesee II First Strand cDNA Synthesis Kit (Genesee, Guangzhou, China), followed by PCR reaction on an Applied Biosystems PCR7900 instrument (Thermo Fisher Scientific, WA) with Genesee qPCR SYBR Green Master Mix (Genesee). PCR reaction was performed at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. GAPDH was selected as an endogenous control gene of BRD4. BRD4 relative expression level was calculated using the method of  $2^{-\Delta\Delta Ct}$ .

### Western Blot

Total proteins were extracted from the rat myocardial tissues and H9C2 cells using protein lysis buffer (Thermo Fisher Scientific, Inc.) with a protease inhibitor (Thermo Fisher Scientific, Inc). Protein concentration was measured by a BCA kit (Pierce, Rockford, IL) according to the manufacturer's instructions. 40  $\mu$ g of protein extracts were separated using

10% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences, Bucks, United Kingdom). After blocking with 5% BSA in 0.5% Tween 20 in phosphate-buffered saline at 4°C overnight, the membranes were incubated with anti-BRD4 polyclonal antibody (1:500; cat. no. ab75898; Abcam, Cambridge, MA), anti-GAPDH monoclonal antibody (1:2000; cat. no. ab181602; Abcam), anti-phospho-p85 polyclonal antibody (1:500; cat. no. ab182651; Abcam), anti-P85 polyclonal antibody (1:500; cat. no. 4292; Cell Signaling Technology, Beverly, MA), anti-AKT 1/2 monoclonal antibody (1:800; cat. no. ab182729; Abcam), anti-AKT (phospho S473) monoclonal antibody (1:400; cat. no. ab81283; Abcam), and anti-AKT (phospho T308) monoclonal antibody (1:400; cat. no. ab38449; Abcam) at 37°C for 2 hours. Membranes were washed with TBST and incubated with secondary antibody labeled with horseradish peroxidase (HRP; 1:1000; cat. no. ab97051; Abcam) at 37°C for 1 hour, followed by an enhanced chemiluminescence detection kit (Amersham Biosciences).

### ELISA

The activities of lactate dehydrogenase (LDH), creatine kinase MB isoenzyme (CK-MB), and cardiac troponin I (cTnI); the levels of TNF- $\alpha$  and IL-6 in rat serum and cellular supernatant; as well as the level of myeloperoxidase (MPO) in rat myocardial tissues were measured by ELISA using commercial kits purchased from Shanghai Enzyme-linked Biotechnology Co, Ltd (Shanghai, China). Malondialdehyde (MDA) and superoxide dismutase (SOD) activities were measured using the Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology) and the Superoxide Dismutase Activity Assay Kit (BioVision, Milpitas, CA) following the manufacturer's instructions. In brief, 50  $\mu$ L of the diluted standard substance and samples was added to the wells, and then 50  $\mu$ L of biotin labeled antibody was added to the standard wells and sample wells. The plates were incubated at 37°C for 60 minutes. After washing with Wash Solution for 4 times, 80  $\mu$ L of streptavidin HRP was added to each well, and the mixture was shaken gently and incubated at 37°C for 30 minutes. After washing, 50  $\mu$ L of Substrate A and B were added to each well, and incubated at 37°C for 15 minutes in the dark. Finally, 50  $\mu$ L of Stop Buffer was added to each well, and the optical density at 450 nm was read using a microtiter plate reader within 15 minutes.

### Statistical Analysis

The data were analyzed using SPSS statistical software and expressed as mean  $\pm$  SD. Randomized data met the normally distribution, and homogeneity of variance were tested by one-way analysis of variance followed by Tukey's post hoc test to assess potentially significant differences between groups. A *P*-value of less than 0.05 was considered significant.

## RESULTS

### Expression of BRD4 in the Myocardial Tissues of a Rat Myocardial I/R Model

Expression of BRD4 in rat myocardial tissues was determined by RT-qPCR and Western blot. As shown in

Figure 1A, the mRNA and protein levels of BRD4 were significantly increased in the I/R group compared with the sham group. As demonstrated by RT-qPCR and Western blot results, BRD4 relative mRNA and protein expression levels were significantly decreased in the I/R+shBRD4 group compared with the I/R+shCtrl group.

### Effect of shBRD4 on Cardiac Dysfunction Induced by Myocardial I/R

To investigate the effect of shBRD4 on myocardial I/R injury in vivo, rat cardiac function was examined. As shown in Table 1, the indices of cardiac function, including HR, MAP, LVSP, and  $\pm dp/dt$  max, were significantly decreased in the I/R group compared with the sham group. shBRD4 improved cardiac function as evidenced by the increased HR, MAP, LVSP, and  $\pm dp/dt$  max in the I/R+shBRD4 group compared with the I/R+shCtrl group.

### Effect of shBRD4 on the Biochemical Markers of Myocardial Injury in Vivo

The activities of LDH, CK-MB, and cTnI in rat serum were examined by ELISA kits, and the results were shown in Figure 1B. LDH, CK-MB, and cTnI activities were significantly increased in the I/R group compared with the sham group, but the increased activities were significantly suppressed by shBRD4.

### Effect of shBRD4 on Inflammation in a Rat Myocardial I/R Model

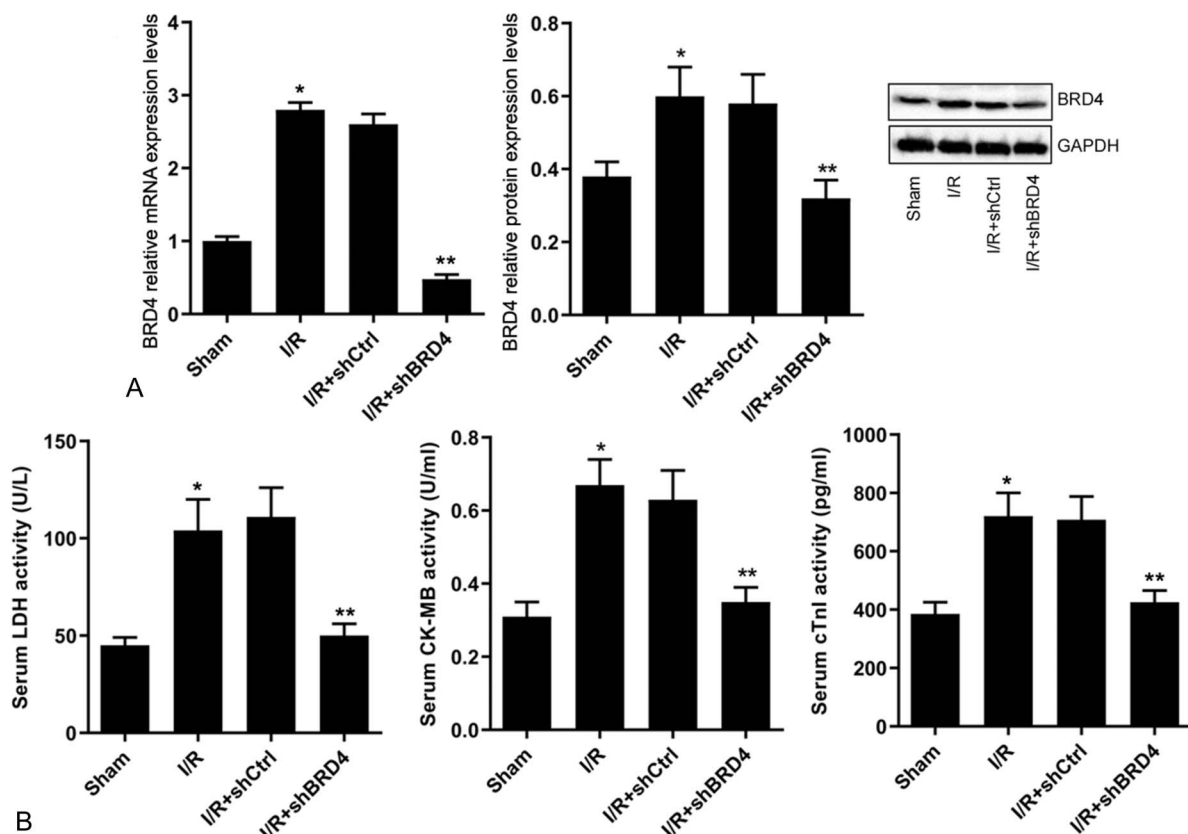
To investigate the effect of shBRD4 on inflammation in a rat myocardial I/R model, the activity of MPO in the myocardial tissues, as well as the levels of TNF- $\alpha$  and IL-6 in rat serum were examined. We found I/R led to the increased activity of MPO in the myocardial tissues and the elevated levels of TNF- $\alpha$  and IL-6 in rat serum. However, this effect was reversed by shBRD4 (Fig. 2A).

### Effect of shBRD4 on Oxidative Stress in a Rat Myocardial I/R Model

To investigate the effect of shBRD4 on oxidative stress in a rat myocardial I/R model, the activities of MDA and SOD in the myocardial tissues were evaluated. Compared with the sham group, MDA activity was significantly increased and SOD activity was significantly decreased in the I/R group. However, shBRD4 suppressed MDA activity and induced SOD activity (Fig. 2B).

### Effect of shBRD4 on PI3K/AKT Signaling Pathway in a Rat Myocardial I/R Model

To investigate the effect of shBRD4 on PI3K/AKT signaling pathway in a rat myocardial I/R model, the expression of p-p85 and p-AKT in rat myocardial tissues



**FIGURE 1.** Expression of BRD4 in rat myocardial tissues and the effect of shBRD4 on myocardial injury in vivo. A, mRNA and protein levels of BRD4 in rat myocardial tissues. B, Effect of shBRD4 on the biochemical markers of myocardial injury. \* $P < 0.05$  versus sham group; † $P < 0.05$  versus I/R + shCtrl group.  $n = 10$  rats per group.

**TABLE 1.** Effect of shBRD4 on Cardiac Function of Rats After Myocardial I/R

	Sham	I/R	I/R+shCtrl	I/R+shBRD4
HR	450.77 ± 33.98	280.02 ± 55.35*	271.32 ± 50.11	382.32 ± 28.13†
MAP (mm Hg)	92.45 ± 8.97	76.41 ± 10.88*	82.28 ± 11.44	88.72 ± 7.12†
LVSP (mm Hg)	149.43 ± 11.35	100.21 ± 10.21*	106.65 ± 12.05	133.89 ± 14.01†
+dp/dt max (mm Hg/s)	5489.35 ± 1011.24	2755.43 ± 822.19*	2714.09 ± 813.99	4219.55 ± 1054.12†
-dp/dt max (mm Hg/s)	4022.98 ± 929.71	2314.51 ± 500.02*	2334.65 ± 525.45	3522 ± 887.24†

n = 10 rats per group. +dp/dt max, rates of maximum positive left ventricular pressure development; -dp/dt max, rates of maximum negative left ventricular pressure development. \*P < 0.05 versus sham group. †P < 0.05 versus I/R + shCtrl group.

was examined by Western blot. As shown in Figure 3, compared with the sham group, the protein levels of p-p85, p-AKT T308, and p-AKT S473 were significantly decreased in the I/R group. However, shBRD4 significantly increased the protein levels of p-p85, p-AKT T308, and p-AKT S473.

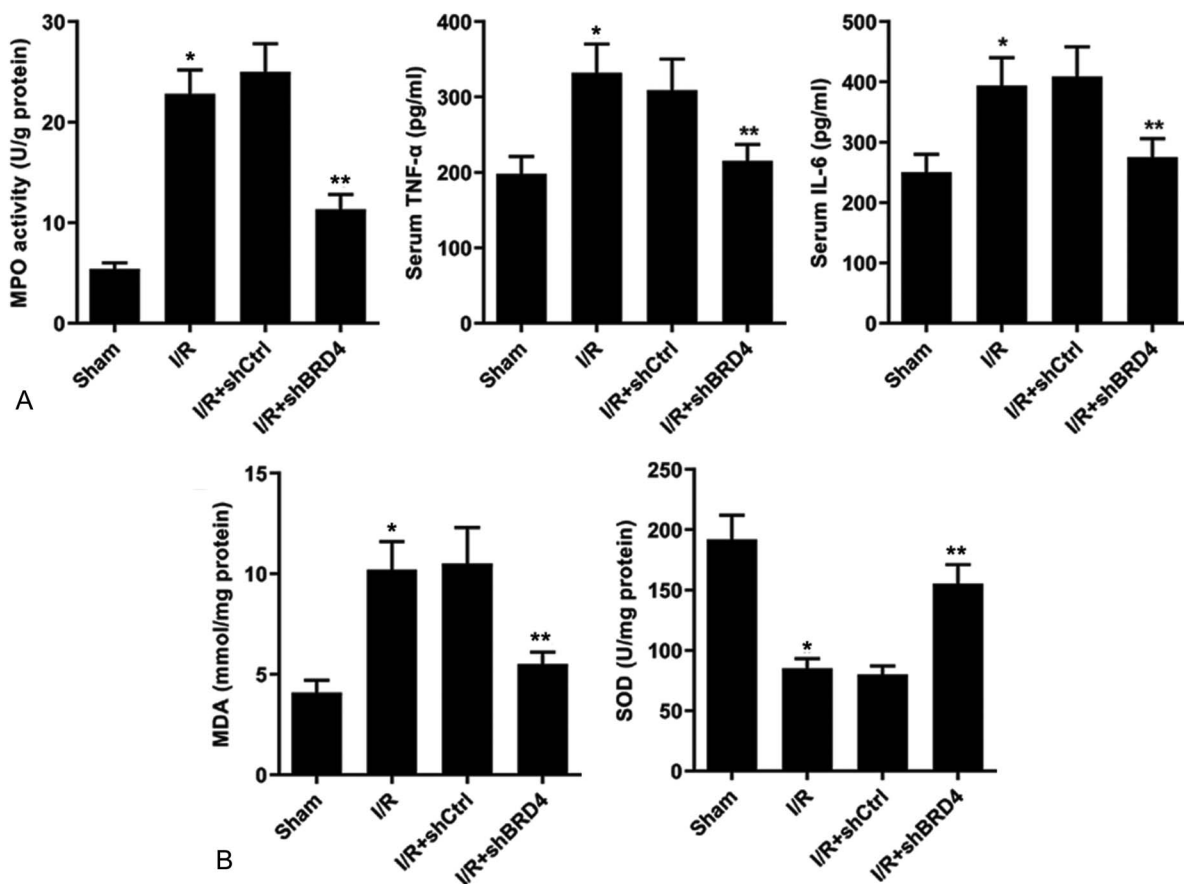
**Expression of BRD4 in OGD-Treated H9C2 Cells**

We detected the expression of BRD4 in OGD-treated H9C2 cells and normal cells. The results showed that the expression levels of BRD4 were significantly increased in OGD-

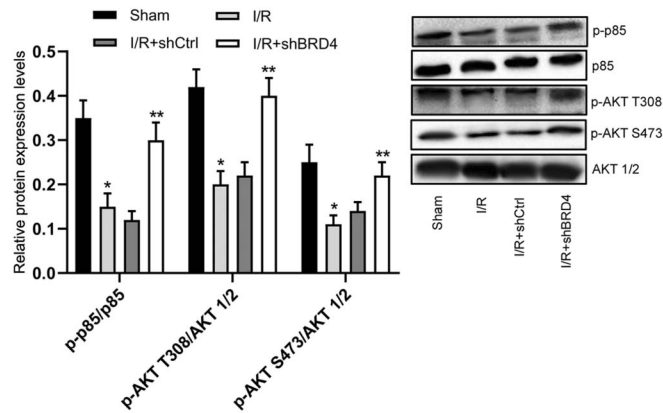
treated H9C2 cells compared with normal cells. BRD4 shRNA was transfected into OGD-treated H9C2 cells to knockdown BRD4. As demonstrated by RT-qPCR and Western blot, transfection of shCtrl did not affect BRD4 expression, but BRD4 mRNA and protein levels were significantly decreased by transfection of shBRD4 in OGD-treated H9C2 cells (Fig. 4A).

**Effect of shBRD4 on OGD-Induced Cell Damage in Vitro**

To investigate the effect of shBRD4 on OGD-induced cell damage, LDH and CK-MB activities in



**FIGURE 2.** Effect of shBRD4 on inflammation and oxidative stress in a rat myocardial I/R injury model. A, Effect of shBRD4 on inflammatory reaction in vivo. B, Effect of shBRD4 on the indicators of oxidation and antioxidation. \*P < 0.05 versus sham group; †P < 0.05 versus I/R + shCtrl group. n = 10 rats per group.



**FIGURE 3.** Effect of shBRD4 on PI3K/AKT signaling pathway in a rat myocardial I/R injury model. \* $P < 0.05$  versus sham group; † $P < 0.05$  versus I/R + shCtrl group.  $n = 10$  rats per group.

cellular supernatant was measured. We found OGD-treated H9C2 cells had higher LDH and CK-MB activities than normal H9C2 cells, but the increased LDH and

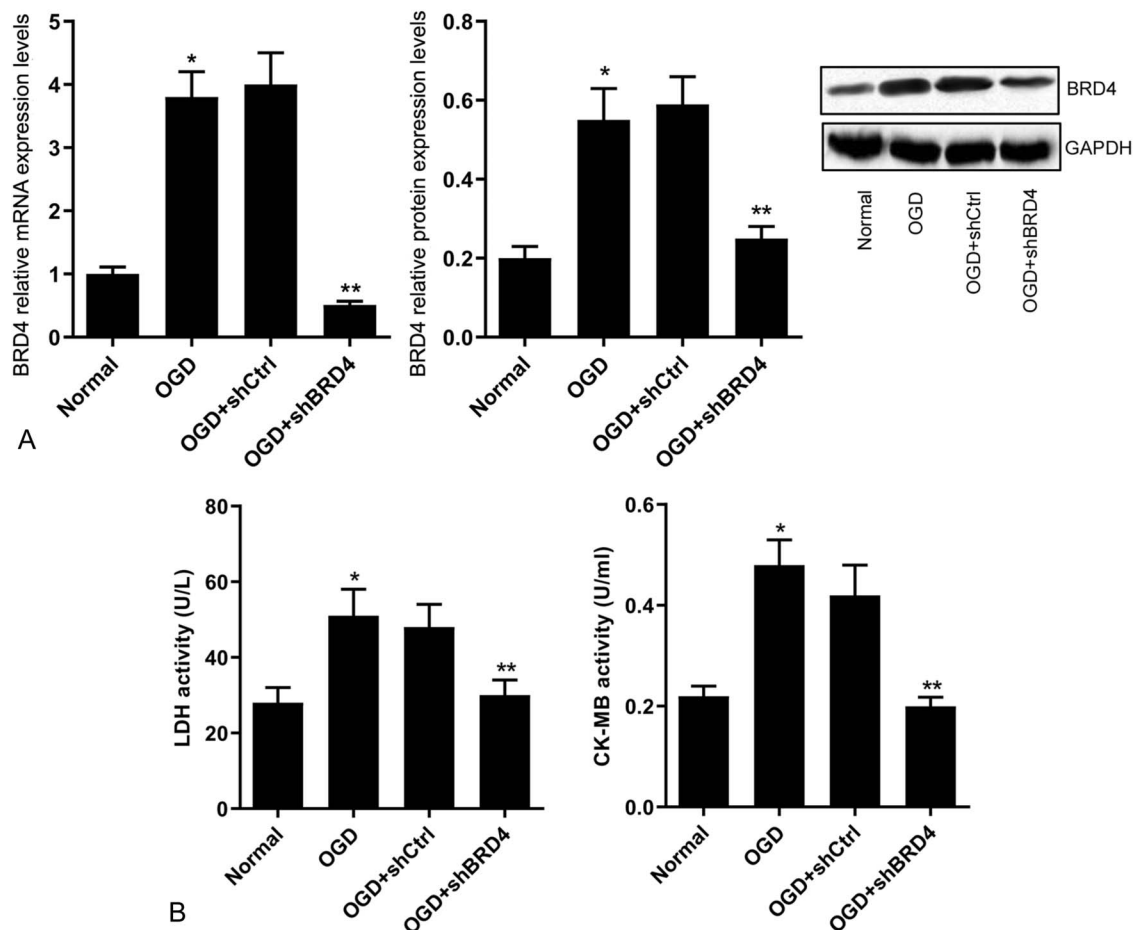
CK-MB activities could be suppressed by shBRD4 (Fig. 4B).

### Effect of shBRD4 on OGD-Induced Inflammation and Oxidative Stress in Vitro

To investigate the effect of shBRD4 on inflammation, the levels of TNF- $\alpha$  and IL-6 were detected by ELISA. The results showed that OGD treatment significantly increased the levels of TNF- $\alpha$  and IL-6. However, transfection with shBRD4 significantly suppressed the elevated TNF- $\alpha$  and IL-6 levels induced by OGD (Fig. 5A). MDA and SOD activities were measured to determine the effect of shBRD4 on oxidative stress in H9C2 cells. As shown in Figure 5B, the activity of MDA was significantly increased and the activity of SOD was significantly decreased in the OGD group compared with the normal group. shBRD4 transfection significantly decreased MDA activity and increased SOD activity.

### Effect of shBRD4 on the PI3K/AKT Signaling Pathway in OGD-Treated H9C2 Cells

To investigate the effect of shBRD4 on the PI3K/AKT signaling pathway in OGD-treated H9C2 cells, the relative



**FIGURE 4.** Expression of BRD4 in OGD-treated H9C2 cells and the effect of shBRD4 on OGD-induced cell damage. A, mRNA and protein levels of BRD4 in OGD-treated H9C2 cells. B, Effect of shBRD4 on the activity of cardiac enzymes. \* $P < 0.05$  versus OGD group; † $P < 0.05$  versus OGD + shCtrl group.  $n = 9$  independent samples per group.

protein levels of p-p85 and p-AKT were examined by Western blot. As shown in Figure 5C, OGD treatment inhibited the PI3K/AKT signaling pathway, as demonstrated by significantly decreased relative protein levels of p-p85, p-AKT T308, and p-AKT S473 in OGD cells compared with normal cells. However, the relative protein levels of p-p85, p-AKT T308, and p-AKT S473 were significantly increased in OGD cells transfected with shBRD4.

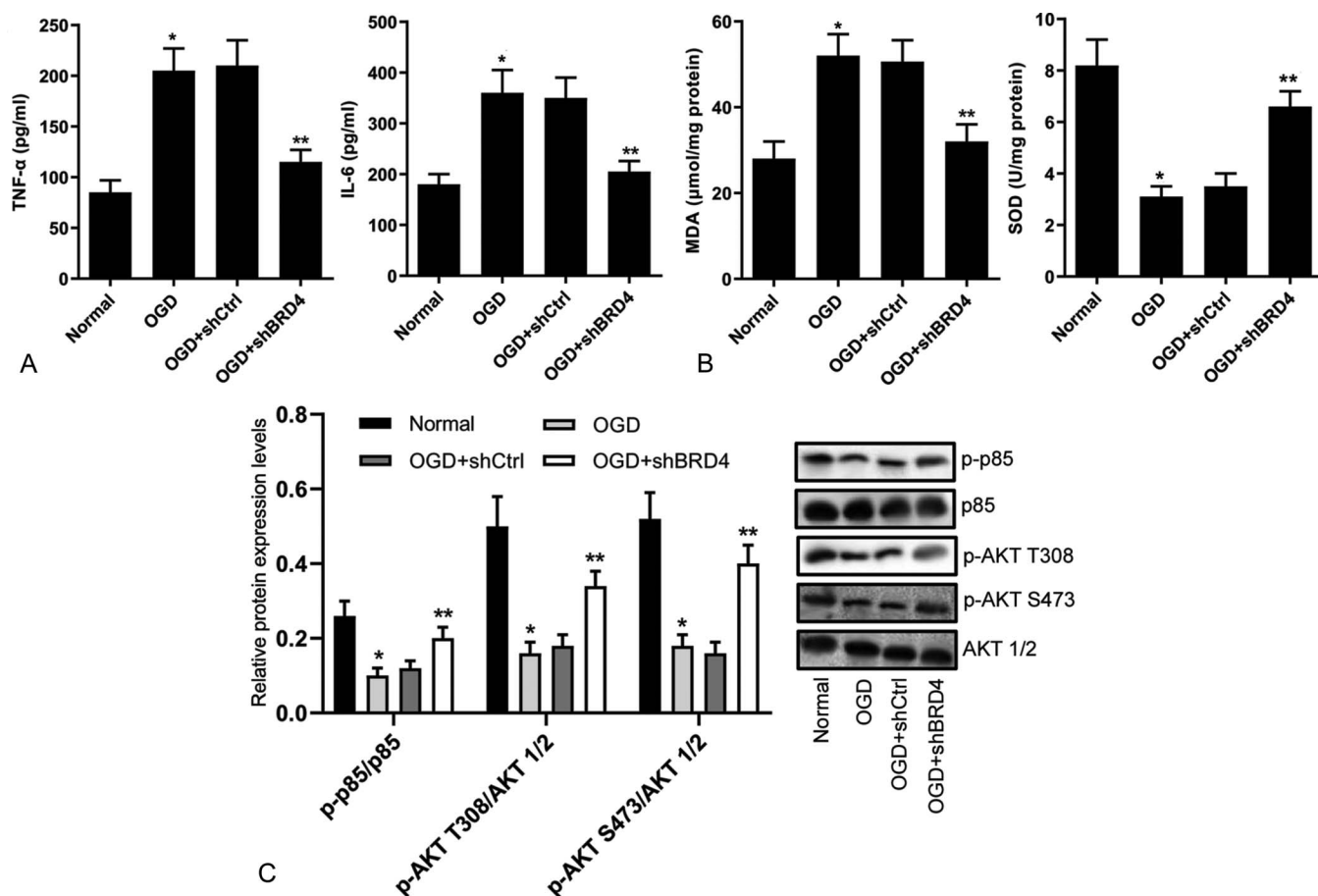
### DISCUSSION

BRD4 has been suggested to be a promising therapeutic target in a wide range of diseases, such as cancers, renal diseases, and skin diseases.<sup>15-17</sup> Recently, accumulating evidence suggests that BRD4 plays important roles in the regulation of cardiovascular physiological and pathological processes. Studies by Sun et al have reported that BRD4 expression was significantly increased in a rat model of acute myocardial infarction as well as in cardiomyocytes under hypoxia.<sup>13,14</sup> In this study, we established a rat model of myocardial I/R injury in vivo. In addition, I/R injury was established in vitro by performing OGD on H9C2 cell cultures. Both the in vivo and in vitro results showed that BRD4 expression was significantly increased after myocardial I/

R injury. These findings were consistent with the results in acute myocardial infarction and indicated that BRD4 might be involved in the progression of myocardial I/R injury.

Many studies have revealed the contribution of BRD4 in renal ischemia reperfusion injury.<sup>18-21</sup> In this study, we firstly evaluated the effect of BRD4 inhibition on myocardial I/R injury in vivo and in vitro. Using a rat model of myocardial I/R, we found that I/R-induced cardiac function injury could be reduced by BRD4 inhibition. LDH, CK-MB, and cTnI are the biochemical markers of myocardial injury, and their expression levels are significantly increased during myocardial I/R.<sup>22,23</sup> In this study, LDH, CK-MB, and cTnI activities were measured, and the results showed that they were significantly increased in the in vivo and in vitro myocardial I/R models. Furthermore, we found BRD4 inhibition suppressed the elevation of LDH, CK-MB, and cTnI, suggesting that BRD4 inhibition protects against myocardial I/R injury in vivo and in vitro.

Myocardial ischemia and reperfusion leads to a complex series of inflammatory reactions that cause further damage.<sup>24,25</sup> TNF- $\alpha$  and IL-6 are important cytokines in inflammation, and they are critical mediators in the pathogenesis of myocardial I/R injury.<sup>26,27</sup> The activated neutrophils can produce pro-oxidative and proinflammatory MPO. BRD4 inhibition has been



**FIGURE 5.** Effect of shBRD4 on inflammation, oxidative stress and PI3K/AKT signaling pathway in vitro. A, Effect of shBRD4 on the expression of inflammatory factors. B, Effect of shBRD4 on the level of MDA and the activity of SOD. C, Effect of shBRD4 on PI3K/AKT signaling pathway. \* $P < 0.05$  vs. OGD group; † $P < 0.05$  versus OGD + shCtrl group. n = 9 independent samples per group.

demonstrated to reduce neutrophil recruitment and activation and decrease inflammatory gene expression in primary tubule cells.<sup>19</sup> Zhu et al reported that the elevated IL-1 $\beta$ , TNF- $\alpha$ , and phosphorylated NF- $\kappa$ B levels in Ang II-incubated cardiomyocytes could be reduced by shBRD4.<sup>9</sup> In a mouse model of heart failure, BRD4 inhibition with JQ1 blocks the transactivation of proinflammatory transcription factors.<sup>28</sup> Oxidative stress also plays a pivotal role in myocardial I/R injury. MDA level and SOD activity are 2 indicators of oxidation and antioxidation. Liu et al<sup>18</sup> reported that the inhibition of BRD4 blocked FoxO4-mediated oxidative stress in renal I/R injury. Despite BRD4 has been implicated to be involved in inflammation-related diseases, the effect of BRD4 on inflammation and oxidative stress of myocardial I/R injury remains unknown. This study demonstrated that I/R-induced inflammation and oxidative stress could be suppressed by BRD4 shRNA in vivo and in vitro. Our findings were consistent with the study by Sun et al,<sup>13</sup> which revealed that BET protein inhibition suppresses inflammatory reaction in the cardiomyocytes of in vivo acute myocardial infarction model.

The PI3K/AKT signaling pathway is a key regulator involved in many physiological and pathological conditions, such as cell growth and survival, apoptosis, metabolism, and motility. Several studies have shown that the PI3K/AKT signaling pathway plays a protective role in myocardial I/R injury. Zhang et al found ischemic preconditioning-induced serum exosomes (IPC-Exo) could alleviate myocardial I/R injury by activating the PI3K/AKT signaling pathway. However, treatment with LY294002 significantly reversed the effect of IPC-Exo on hemodynamics, inflammatory factor production, and apoptosis in the I/R model.<sup>29</sup> Cui et al<sup>30</sup> showed that glutamine can protect cardiomyocytes from I/R injury by activating the PI3K/Akt signaling pathway. In this study, we investigated the effect of BRD4 on the PI3K/AKT signaling pathway in the myocardial I/R model. Activation of the PI3K/AKT signaling pathway was tested by the phosphorylation of P85, as well as the phosphorylation of AKT at both Thr-308 and Ser-473.<sup>31</sup> Consistent with the previous reports, our study demonstrated that the PI3K/AKT signaling pathway was suppressed in myocardial I/R; however, BRD4 inhibition resulted in the activation of the PI3K/AKT signaling pathway. These results indicated that BRD4 inhibition protects against myocardial I/R injury through the activation of the PI3K/AKT signaling pathway.

A limitation in this study is that the hemodynamic values were measured at 180 minutes after reperfusion. We did not measure the values at baseline (before intervention), immediately before ligation, in the last minute of ischemia, and at a specified time after the start of reperfusion.

In conclusion, we for the first time demonstrated the protective effect of BRD4 inhibition on myocardial I/R injury in vivo and in vitro. BRD4 inhibition could suppress inflammation and oxidative stress in myocardial I/R injury, and this effect was related to the activation of the PI3K/AKT signaling pathway. BRD4 is suggested to be a potential therapeutic target in the treatment of myocardial I/R injury.

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