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ORIGINAL ARTICLE

HINT2 deficiency deteriorates oxidative stress in a mouse model of myocardial infarction

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Abstract

Aims Myocardial infarction (MI) is one of the serious diseases with great mortality over the world. Myocardial mitochondrial oxidative stress has been implicated as a key player in MI. The histidine triad nucleotide-binding protein 2 (HINT2) is a nucleotide hydrolase and transferase located in mitochondria. HINT2 has multiple functions such as regulating mitochondrial lipid metabolism and respiration and glucose homeostasis. Although HINT2 has been shown to protect against MI, the underlying mechanisms were not fully elucidated. In this study, the effects of HINT2 on oxidative stress during MI were explored.

Methods and results MI mouse models in both wild-type and HINT2-deficient mice were established. The expression of HINT2 in HINT2-deficient mice was determined by quantitative real-time PCR and western blot. The levels of oxidative stress were measured, including the levels of malondialdehyde (MDA), nitric oxide (NO), superoxide dismutase (SOD), and glutathione (GSH). The myocardial functions, as indicated by left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular ejection fraction (LVEF), and left ventricular fractional shortening (LVFS), were monitored. Both mRNA and protein expressions of HINT2 in the myocardial tissues were significantly down-regulated in MI mice starting at 6 h post-MI. MI induced oxidative stress 6 h post-MI in myocardial tissues of wild-type mice, as suggested by the enhanced MDA and NO levels and decreased SOD and GSH levels. The expression of HINT2 was negatively correlated to the MDA and NO levels and positively correlated to the SOD and GSH levels. HINT2-deficient MI mice had significantly elevated levels of MDA and NO and significantly decreased levels of SOD and GSH when compared with wild-type MI mice. HINT2-deficient MI mice had higher LVEDD and LVESD and lower LVEF and LVFS compared with wild-type MI mice, indicating that HINT2 deficiency exacerbated myocardial dysfunction.

Conclusions HINT2 deficiency causes deteriorative oxidative stress in MI mice, leading to exacerbated myocardial dysfunction.

Keywords Myocardial infarction; HINT2; Deficiency; Oxidative stress

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Introduction

Myocardial infarction (MI) causes great morbidity and mortality over the world.[1] Due to the sudden decrease in oxygen and blood supply, MI results in heart muscle damage and cardiomyocytes loss, which lead to myocardial dysfunctions and heart failure.[2]

In recent years, increasing evidences from both experimental and clinical studies have revealed the contribution of oxidative stress to MI and heart failure.[3,4] Oxidative stress is characterized as the excess production of reactive oxygen species (ROS) and nitric oxide (NO).[5] In oxidative stress, the lipid peroxidation in membranes is usually evaluated by the formation of malondialdehyde (MDA). Superoxide dismutase (SOD) and reduced glutathione (GSH) are enzymatic antioxidant defences.[6] Augmented myocardial oxidative stress and ROS generation, and deficit of antioxidant have been identified in MI.[7,8] The excessive ROS causes the peroxidation of lipid and protein, and DNA damage, which leads to cellular dysfunction and cell death.[9] In MI, both ischaemic injury

and reperfusion injury cause mitochondrial dysfunctions in heart cells.[10] In heart cells, the mitochondrial dysfunctions were described as increased lipid peroxidation, decreased mtDNA copy number and mtRNA transcripts, and reduced oxidative capacity.[11] Inhibition of oxidative stress ameliorates cardiac dysfunction after MI.[12]

The histidine triad nucleotide-binding protein 2 (HINT2) belongs to the histidine triad proteins family.[13] HINT2 performs various biological functions such as regulating lipid metabolism, glucose homeostasis, and nicotinamide adenine dinucleotide (NAD) homeostasis.[14] HINT2-deficient mice have defective lipid metabolism and dysfunctional respiration. Interestingly, overexpression of HINT2 in MI mice protects cardiac function and preserves metabolism, indicating the protective role of HINT2 in MI. However, whether HINT2 regulates oxidative stress during MI remains unknown. In the present study, the potential role of HINT2 in oxidative stress during MI was explored.

Materials and methods

Animals

Wild-type (WT) C57BL/6J mice with age of 6–8 weeks were purchased from Charles River Laboratories (Beijing, China). Sperms of Hint2-deficient mice (C57BL/6J-Hint2^{em1cyagen}) were purchased from Cyagen Biosciences Inc. (Suzhou, China) and used to breed Hint knockout mice. MI was induced in mice following the protocols described previously.[15] Briefly, the mice were anaesthetized with pentobarbital sodium (60 mg/kg). Next, hearts were exposed by bevelling the third and fourth ribs and the coronary artery was ligated. At different time points after MI, samples were harvested for analysis. This study was performed in strict accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (eight edition, NIH). The study was approved by the ethics committee of the Second Hospital of Hebei Medical University.

Quantitative real-time PCR

The quantitative real-time PCR (qRT-PCR) was performed following the protocol described previously.[11] Briefly, total RNA from left ventricle tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed to obtain cDNA by using the reverse transcriptase core kit (Eurogenec, San Diego, CA, USA). Real-time PCR was set up using RT^2 SYBR® Green qPCR Mastermixes (Qiagen, Valencia, CA, USA) and performed in QuantStudio™ 3 Real-Time PCR System with 50 ng cDNA. The sequences of primers used in the present study included the following: *Hint2* forward: 5′-AGCATCGCCAACCATCTTCTC-3′, reverse: 5′-GTCACGGAACACAAGGCACT-3′; and *GAPDH* forward: 5′-AATGGATTTGGACGCATTGGT-3′, reverse: 5′-TTTGCACTG GTACGTGTTGAT-3′. The mRNA expression was normalized to *GAPDH* using the comparative threshold cycle ($2^{-\Delta\Delta ct}$) method.

Western blot

The total proteins from left ventricle tissues were extracted using RIPA buffer (Abcam, Shanghai, China). Twenty micrograms of proteins was loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and then transferred to polyvinylidene fluoride membrane. After blocking, corresponding primary antibodies were added and incubated for overnight at 4°C. Next day, membranes were washed for three times and incubated with corresponding secondary antibodies. Immunoreactive bands were visualized by adding electrochemiluminescence substrate. The primary antibodies used in the present study included anti-HINT2 (1:1000) (#AT5B10, Genetex, Irvine, CA, USA), anti-Bax (1:1000) (ab32503, Abcam), anti-Bcl2 (1:1000) (ab182858, Abcam), anti-β-actin (1:2000) (ab8226, Abcam), and anti-GAPDH (1:2000) (ab8245, Abcam). The band intensity was analysed using the ImageJ software.

Echocardiographic evaluation

The left ventricular ejection fraction (LVEF), left ventricular end-diastolic diameter (LVEDD), left ventricular fractional shortening (LVFS), and left ventricular end-systolic diameter (LVESD) were analysed using an ultrasound instrument from a Vevo2100 Ultrasound system (VisualSonics, Toronto, Canada).

Oxidation detection

Left ventricle tissues were collected and homogenized. The levels of GSH (#703002), SOD (#706002), MDA (#700870), and NO (#780051) in the homogenate were measured using commercial kits (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis

All the data were presented as mean ± standard deviation (SD). One-way ANOVA followed Dunn's multiple comparisons test, two-way ANOVA followed Tukey's multiple comparisons test, or Student's *t*-test was used for comparison. The difference was significant when $P < 0.05$.

Results

Down-regulation of HINT2 in the heart tissues of myocardial infarction mice

To detect the expression of HINT2 in heart tissues after MI, the heart tissues from MI mice were harvested at different time points and the HINT2 mRNA and protein levels were determined by qRT-PCR and western blot, respectively. As shown in *Figure 1A*, starting from 6 h post-MI, a significant decrease of *HINT2* mRNA level was detected in MI mice and the decreasing was in a time-dependent manner. Consistently, decreased HINT2 protein was detected in heart tissues of MI mice (*Figure 1B*) when compared with sham mice. The difference was significant starting at 6 h post-MI (*Figure 1C*). These results demonstrated the down-regulation of HINT2 in MI.

Induction of oxidative stress in the heart tissues of myocardial infarction mice

Next, the levels of oxidation markers including GSH, SOD, MDA, and NO in heart tissues were measured. As shown in *Figure 2A*, starting at 6 h post-MI, a significantly increased level of MDA, a marker of oxidative stress, was detected in heart tissues of MI mice and the increasing of MDA was in a time-dependent manner. Similarly, NO, another oxidative stress marker, was significantly increased after MI (*Figure 2B*). In contrast, the levels of SOD (*Figure 2C*) and GSH (*Figure 2D*), two oxidation markers and antioxidants, were significantly decreased after MI. Collectively, these results showed MI-induced oxidative stress in heart tissues.

Correlation between HINT2 and oxidative stress

We continued to analyse the correlation between HINT2 and these oxidative stress markers. It was found that HINT2 had a negative correlation with MDA (*Figure 3A*) and NO (*Figure 3B*). In contrast, HINT2 had a positive correlation with the antioxidants SOD (*Figure 3C*) and GSH (*Figure 3D*).

Expression of HINT2 in HINT2-deficient mice

Next, we determined the expression of HINT2 in both WT and HINT2-deficient mice. No HINT2 mRNA was detected in heart

Figure 1 HINT2 was down-regulated in the myocardial tissues following myocardial infarction in wild-type mice. Quantitative real-time PCR and western blot were used to analyse the mRNA (A) and protein (B, C) levels of HINT2 in left ventricle tissues at different time points after myocardial infarction (MI). Six mice were used in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the sham group.

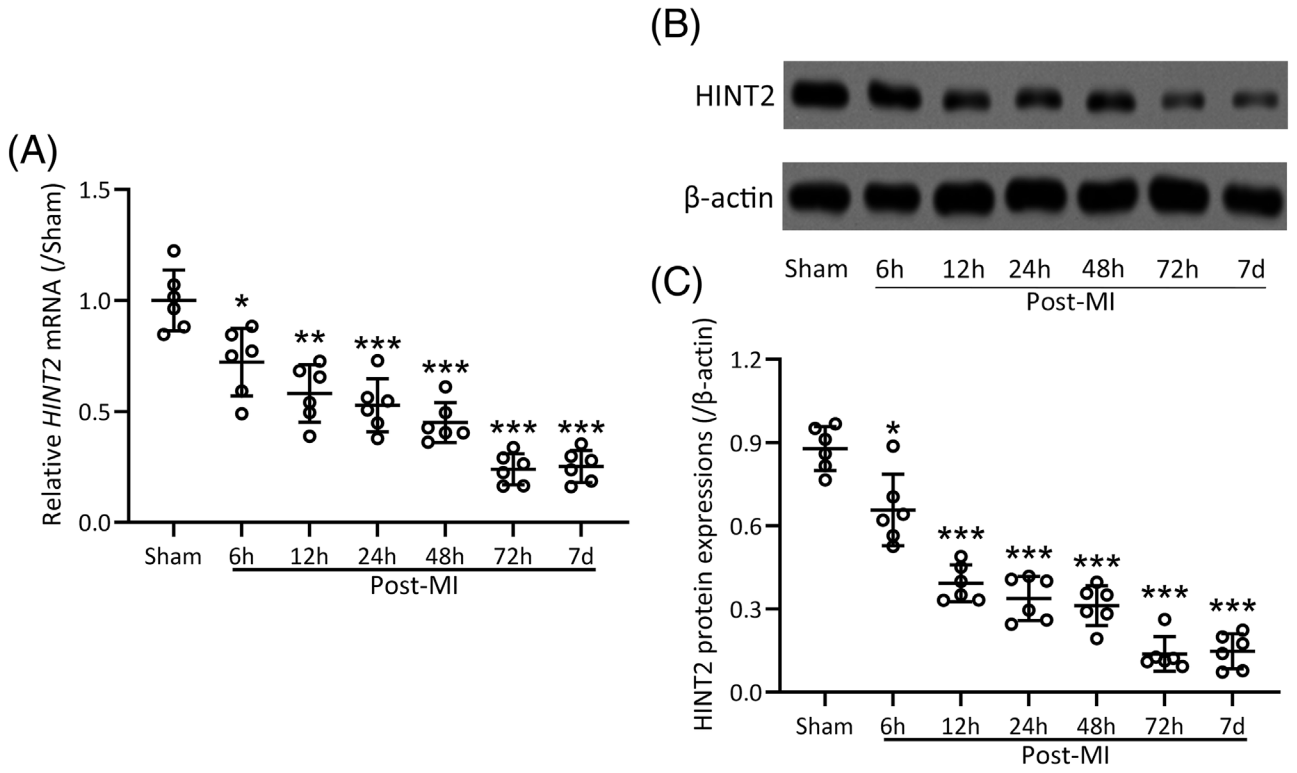
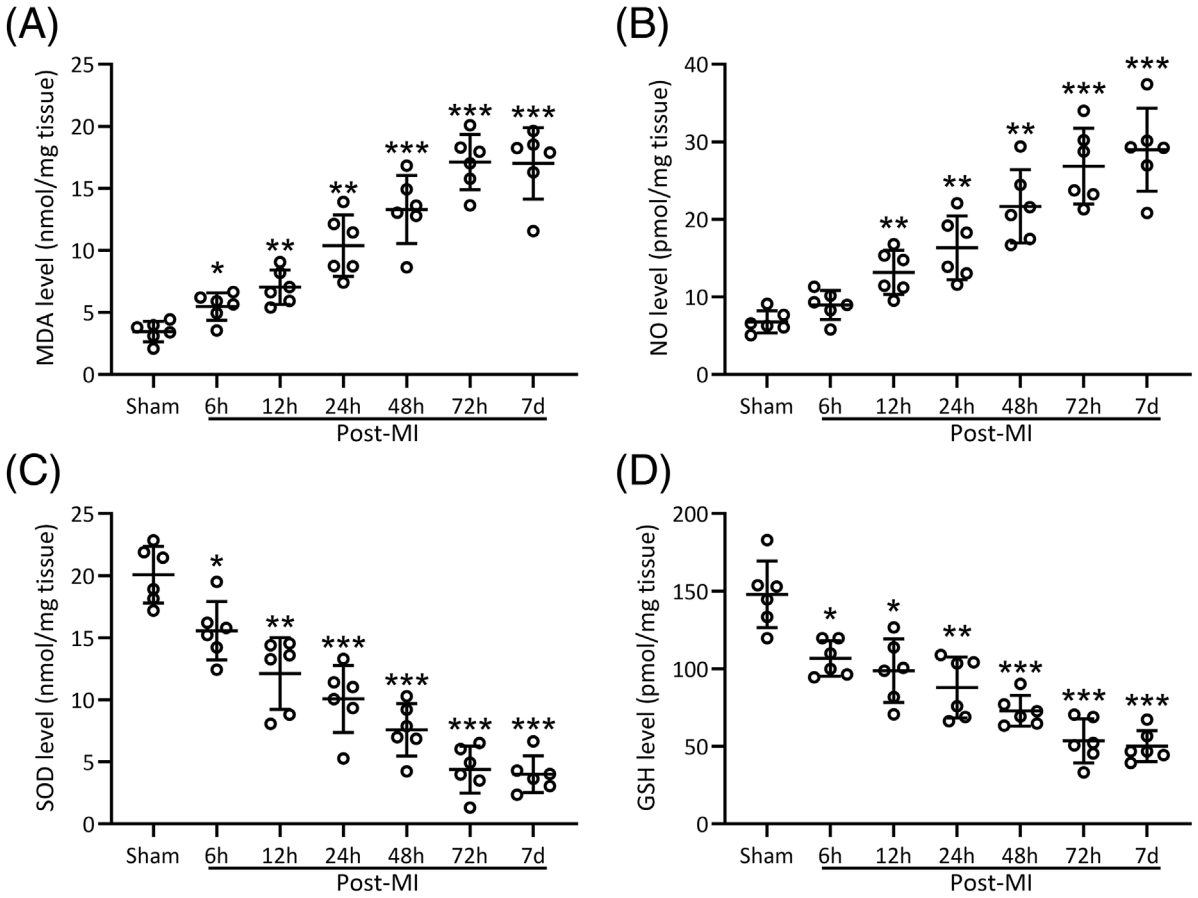


Figure 2 Myocardial infarction (MI) induced oxidative stress in myocardial tissues of wild-type mice. The levels of oxidative stress indicators malondialdehyde (MDA) (A), nitric oxide (NO) (B), superoxide dismutase (SOD) (C), and glutathione (GSH) (D) in left ventricle tissues were examined at different time points after MI. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sham group.



tissues of HINT2-deficient mice (*Figure 4A*). Consistently, clear HINT2 protein was detected in WT mice, whereas there was no HINT2 protein detected in heart tissues of HINT2-deficient mice (*Figure 4B,C*).

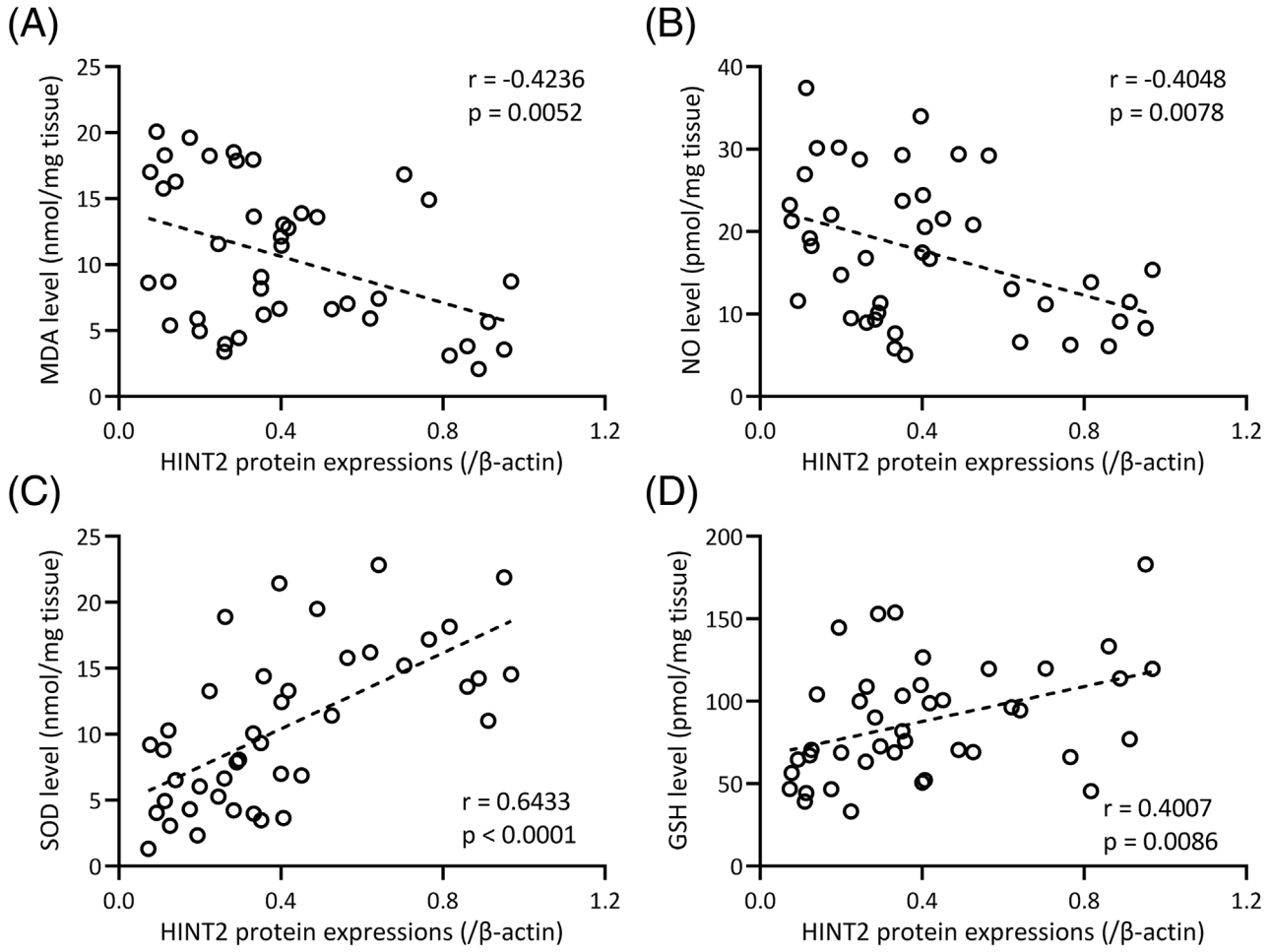
HINT2-deficient myocardial infarction mice had exacerbated myocardial dysfunction

The cardiac function was further compared between WT and HINT2-deficient mice. Compared with sham mice, WT and HINT2-deficient MI mice had significantly increased LVEDD (*Figure 5A*) and LVESD (*Figure 5B*) and had significantly decreased LVEF (*Figure 5C*) and LVFS (*Figure 5D*), which indicated impaired myocardial function in MI mice. Furthermore, HINT2-deficient MI mice had significantly higher LVEDD (*Figure 5A*) and LVESD (*Figure 5B*) and had significantly lower LVEF (*Figure 5C*) and LVFS (*Figure 5D*) than WT MI mice. These results were summarized in *Table S1* and indicated that HINT2 deficiency resulted in exacerbated cardiac dysfunction in MI mice. In addition, we detected up-regulated expression of pro-apoptotic factor Bax and decreased expression of anti-apoptotic factor Bcl2 in myocardial tissues of HINT2-deficient MI mice (*Figure S1a–c*), suggesting enhanced apoptosis in these mice.

HINT2 deficiency exacerbated oxidative stress in myocardial infarction mice

We continued to compare the oxidative stress between WT and HINT2-deficient MI mice. We measured the levels of oxidative stress markers in left ventricle tissues. We detected significantly elevated levels of MDA (*Figure 6A*) and NO (*Figure 6B*) and significantly reduced levels of SOD (*Figure 6C*) and GSH (*Figure 6D*) in left ventricle tissues of WT and HINT2-deficient MI mice when compared with sham WT and HINT2-deficient mice, respectively. In addition, the levels of MDA (*Figure 6A*) and NO (*Figure 6B*) in left ventricle tissues of HINT2-deficient MI mice were significantly higher than those of WT MI mice, whereas the levels of SOD (*Figure 6C*) and GSH (*Figure 6D*) in left ventricle tissues of

Figure 3 Pearson's correlation analysis was carried out to measure the correlations between HINT2 protein expressions and oxidative stress indicators malondialdehyde (MDA) (A), nitric oxide (NO) (B), superoxide dismutase (SOD) (C), and glutathione (GSH) (D) in left ventricle tissues from wild-type mice. *n* = 42 from 7 groups.



HINT2-deficient MI mice were significantly lower than those of WT mice. Collectively, these results demonstrated that HINT2-deficient MI mice had exacerbated oxidative stress in heart tissues.

Discussion

In the present study, we demonstrated that HINT2 was down-regulated in MI mice, which was negatively correlated to oxidative stress. HINT2-deficient MI mice had exacerbated myocardial dysfunction and oxidative stress. These results indicated that HINT2 may display a protective role against MI.

The key finding in the present study is that the exacerbated oxidative stress and myocardial dysfunctions were detected in HINT2-deficient MI mice, which suggested that HINT2 was protective against MI and was also related to oxidative stress regulation. Oxidative stress has been shown to contribute to the pathophysiology of MI. In MI, the high level of ROS produced by cardiomyocytes and infiltrating inflammatory cells is very detrimental, which disrupts membranes and proteins, and induces apoptosis. These activities worsen the myocardial injury and contribute to further myocardial dysfunction. In cells, mitochondria are the major site of ROS production. It has been revealed that mitochondrial ROS (mtROS) could directly induce the expression of pro-inflammatory cytokines. In diverse pathological conditions such as cancer and inflammatory diseases, increased mtROS production is detected.[16] MI can disrupt the oxidative phosphorylation in mitochondria, which impairs ATP generation and the intracellular homeostasis of calcium and ROS.[17]

Our study implied the protective role of HINT2 in MI, which was consistent to the previous study of Fan *et al*.[18] They utilized adenovirus to overexpress HINT2 in MI mice and found that HINT2 overexpression maintained the mitochondrial

Figure 4 Quantitative real-time PCR and western blot were used to analyse the mRNA and protein levels of HINT2 in left ventricle tissues between wild-type (WT) and HINT2^{KO} mice (A–C). The data were shown in mean ± SD. Three mice were used in each group. ***$P < 0.001$ compared with the WT group.

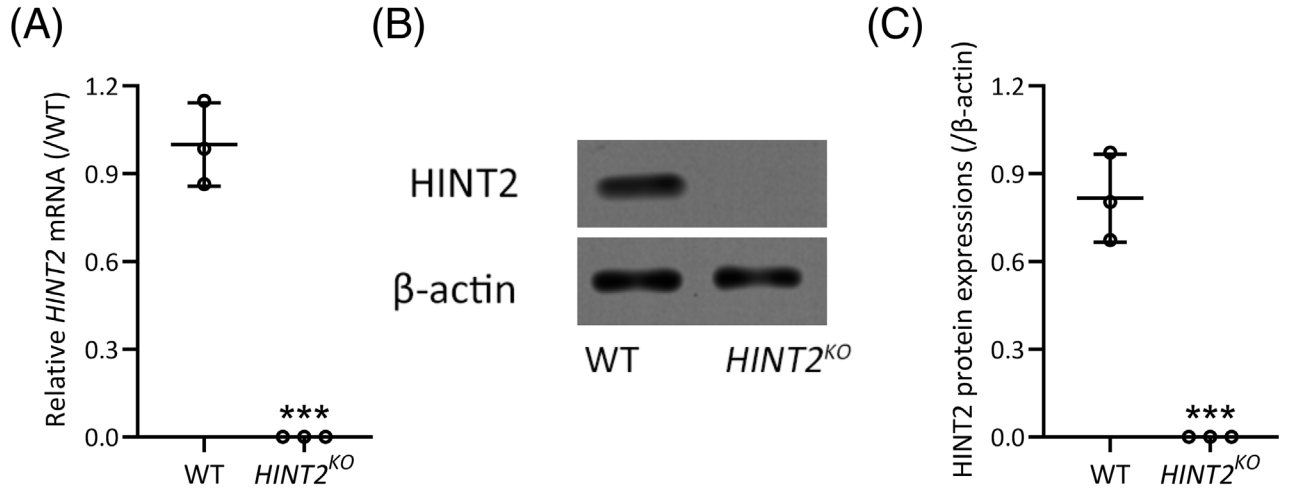


Figure 5 Effects of HINT2 deficiency on left ventricular end-diastolic diameter (LVEDD) (A), left ventricular end-systolic diameter (LVESD) (B), left ventricular ejection fraction (LVEF) (C), and left ventricular fractional shortening (LVFS) (D) 7 days post-myocardial infarction (MI) from echocardiography. Eight mice were used in each group. WT, wild-type. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

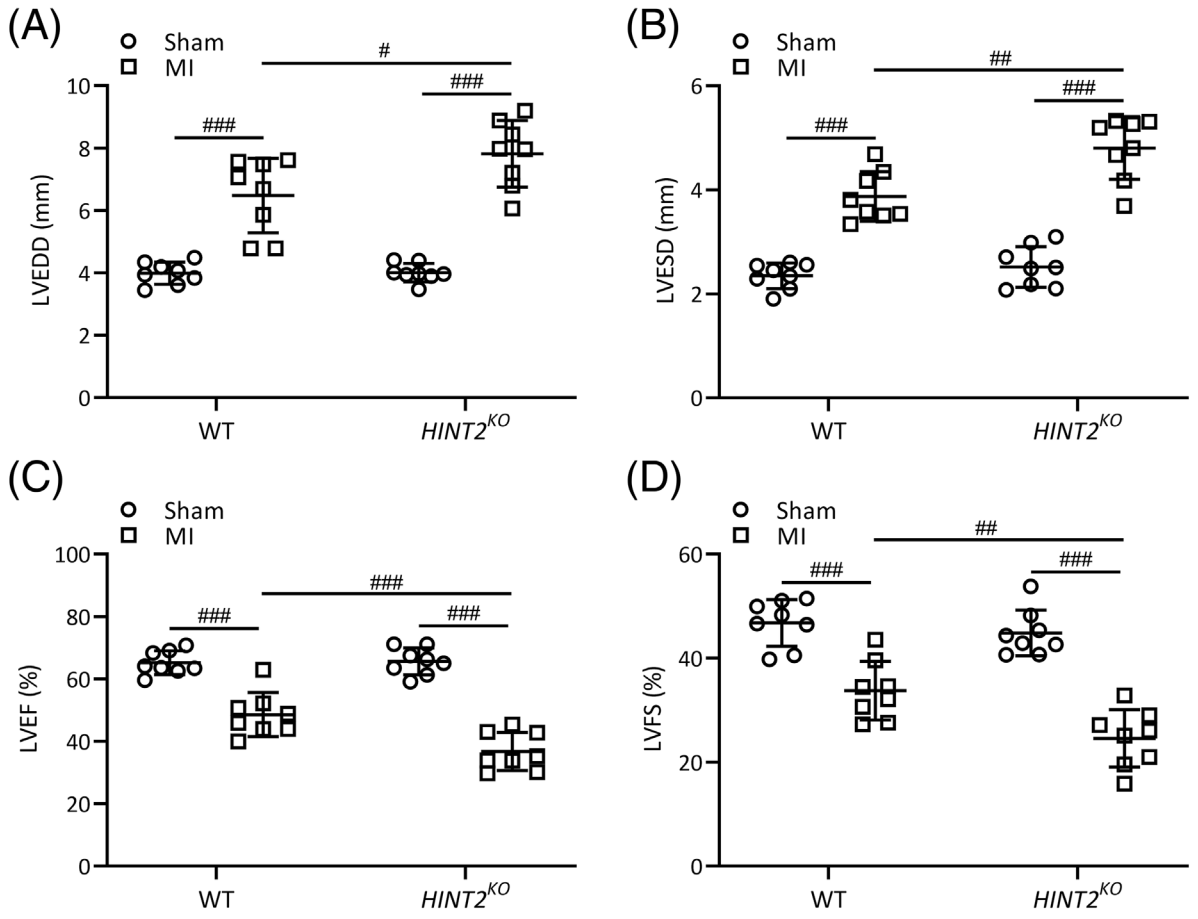
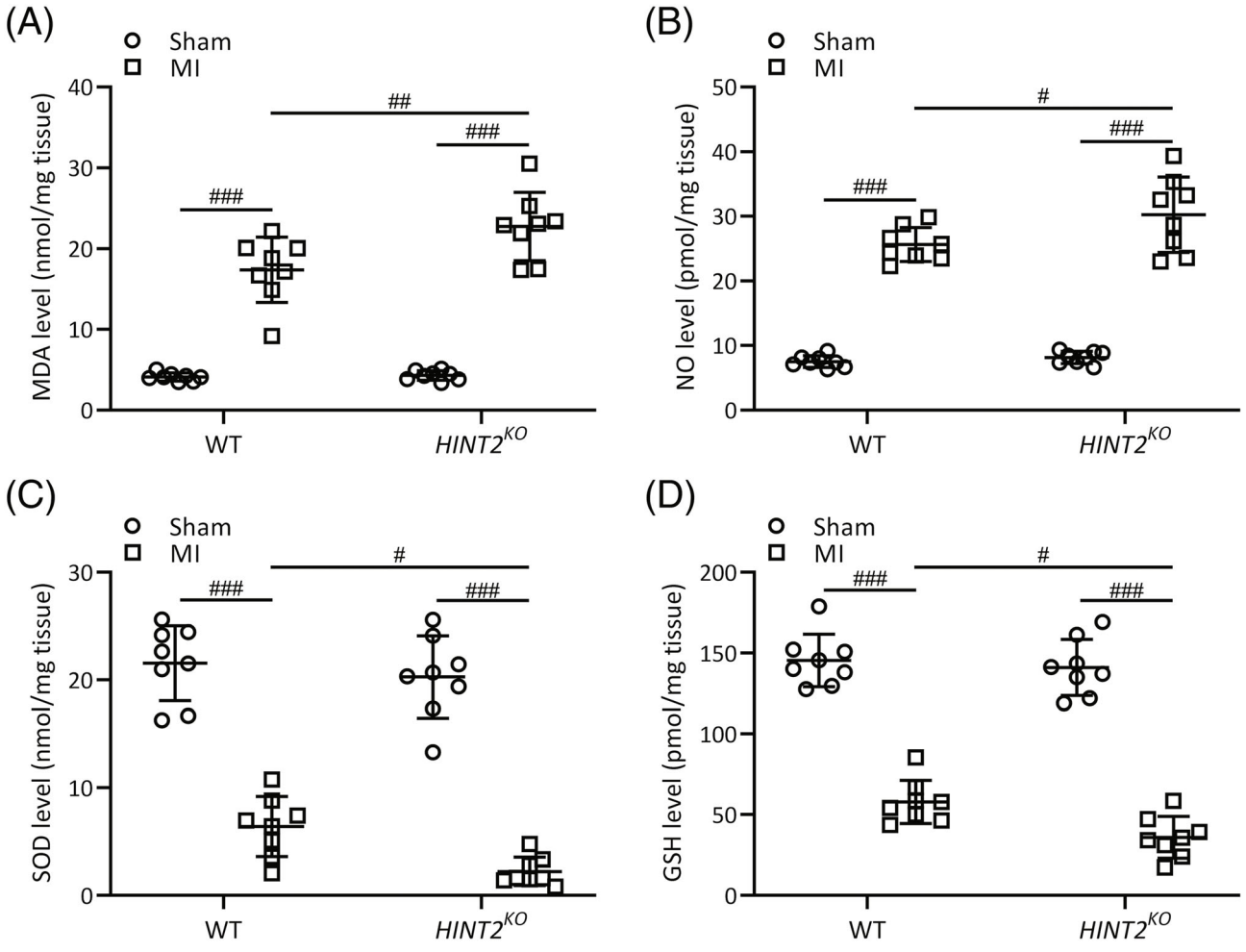


Figure 6 HINT2 deficiency exacerbated myocardial infarction-induced oxidative stress in the myocardial tissues. The levels of oxidative stress indicators malondialdehyde (MDA) (A), nitric oxide (NO) (B), superoxide dismutase (SOD) (C), and glutathione (GSH) (D) in left ventricle tissues were examined 7 days post-myocardial infarction (MI). Eight mice were used in each group. WT, wild-type. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.



NAD. NAD is a coenzyme central to metabolism that exists in both oxidized (NAD^+) and reduced (NADH) forms. The balance between the oxidized and reduced forms is essential for the normal function and cell survival. Interestingly, NAD^+ has been reported to attenuate oxidative DNA damages in primary rat cortical neurons[19] and to protect cardiac myoblasts and neurons against death induced by oxidative stress.[20,21] The regulation of NAD homeostasis by HINT2 could contribute to its effects on oxidative stress during MI. Interestingly, we found that HINT2 deficiency enhanced the expression of pro-apoptotic protein Bax, indicating more cell apoptosis in HINT2-deficient MI mice. This result was also correlated to NAD homeostasis.

In the present study, we utilized coronary artery ligation to induce mild MI, but not heart failure in mice, which was reflected by the LVEF (around 40%) of MI mice. It should be interesting to induce severe MI and compare the phenotypes between WT and HINT2-deficient mice. There are other limitations in this study. We only analysed the oxidative stress in HINT2-deficient MI mice. It should be interesting to explore the potential effects of HINT2 deficiency on inflammation during MI. We only measured the myocardial function of MI mice but did not evaluate the infarction. Experiments need to be designed to address these questions. Although previous studies and our study elucidated the effects of HINT2 on MI in mice and suggested that HINT2 could be a therapeutic target of MI, the description of HINT2 in human MI is limited. More studies need to be performed on human patients. If similar functions of HINT2 can be confirmed in human, gene therapy targeting this protein should have promising prospect to treat the disease.

Conclusion

Deficiency of HINT2 exacerbates oxidative stresses in MI mice, which may lead to deteriorative myocardial function.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study is supported by the departmental funding.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. HINT2 deficiency exacerbated myocardial infarction induced cell apoptosis in the myocardial tissues. Western blotting was used to measure the protein expressions of Bax and Bcl2 (a). GAPDH was used as a loading control and the expressions were normalized to sham-WT (b and c). Left ventricle tissues from 8 mice in each group were mixed and the experiments were repeats for 3 times. #p $<$ 0.05, ##p $<$ 0.01, ###p $<$ 0.001.

Table S1. Echocardiographic outcomes.

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