

TIPE2 protects cardiomyocytes from ischemia-reperfusion-induced apoptosis by decreasing cell autophagy via the mTORC1 signaling pathway

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Abstract. In cardiac ischemia-reperfusion (I/R), autophagy of hyperactivated cardiomyocytes degrades normal proteins and organelles, destroys cells and causes irreversible cell death. The present study aimed to determine the molecular mechanism through which TNF- α -induced protein 8-like protein 2 (TIPE2) regulates cardiomyocyte apoptosis via autophagy in I/R. The results revealed that the number of apoptotic cells and the protein expression levels of TIPE2 in the heart tissue of I/R model mice were significantly increased. *In vitro*, the overexpression of TIPE2 decreased oxygen glucose deprivation (OGD)-induced autophagy, apoptosis and activation of the mTOR complex 1 (mTORC1) signaling pathway in H9c2 cells. Treatment with the mTORC1 inhibitor not only inhibited the TIPE2-activated mTORC1 signaling pathway, but also increased OGD-induced autophagy and apoptosis of H9c2 cells. In conclusion, the results of the present study revealed that TIPE2 may protect cardiomyocytes from I/R-induced apoptosis by decreasing cell autophagy via the mTORC1 signaling pathway.

Introduction

Coronary artery disease has one of the highest morbidity and mortality rates worldwide, and myocardial infarction is the most common coronary artery disease. Percutaneous coronary

intervention (PCI) is the most commonly used and most effective treatment for myocardial infarction in the clinic due to its ability to clear a narrow or occluded coronary lumen; however, ischemia-reperfusion (I/R) injury remains the biggest obstacle for the successful treatment of PCI (1,2). I/R injury has been established as one of the main causes of arrhythmia (3,4), myocardial contractile dysfunction (5,6) and causes irreversible damage of cardiomyocytes (7), while an excessive autophagy response caused by myocardial I/R was found to be one of the main causes of myocardial cell apoptosis (8,9).

Autophagy is an adaptive response of cells against metabolic stress and environmental changes. It degrades or removes cytoplasmic components, including damaged proteins, lipids, carbohydrates and dysfunctional organelles through lysosomal degradation pathways (10,11). However, cardiomyocyte autophagy was discovered to be a double-edged sword in cardiac I/R (12,13). For example, low levels of autophagy during the myocardial ischemic phase compensated for the cells by providing nutrients and energy to decrease the damage (14), while during the reperfusion phase, excessive activation of autophagy was demonstrated to degrade normal proteins and organelles, destroying cells and causing irreversible cell death (13,15). Therefore, identifying targets that can selectively regulate autophagy may represent novel targets for the prevention and treatment of myocardial I/R injury.

TNF- α -induced protein 8 (TIPE)-like protein 2 (TIPE2) is an important member of the TIPE protein family, and has been discovered to improve cell survival and inhibit apoptosis (16). TIPE2 was shown to be a negative regulator of the maintenance of inflammation and immune homeostasis, and downregulated expression of TIPE2 was reported to promote the progression of numerous severe inflammatory diseases (17,18). Previous studies have reported that autophagy was closely associated with inflammation. For example, numerous cytokines, reactive oxygen species and inflammation-related transcription factors in inflammatory mediators were discovered to regulate autophagy, whereas autophagy was found to regulate inflammatory responses through the toll-like receptor and NLR signaling pathways (19). In addition, TIPE2 was found to exert an inflammatory regulatory effect in I/R injury (20). However,

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it remains unclear whether TIPE2 can alleviate myocardial I/R injury by regulating autophagy. The results of the present study revealed that the expression levels of TIPE2 were upregulated in myocardial I/R injury *in vivo*. *In vitro*, TIPE2 was discovered to attenuate oxygen glucose deprivation (OGD)-induced autophagy by activating the mTOR complex 1 (mTORC1) signaling pathway, thereby attenuating the OGD-induced apoptosis of H9c2 cells. In conclusion, the findings of the current study may provide a novel target for attenuating I/R-induced myocardial injury by inhibiting autophagy.

Materials and methods

Animal studies and establishment of myocardial I/R model. Male C57BL/6J mice (age, 5–6 weeks; weight, 25–30 g) were acclimatized at room temperature (20–24°C), with a 12-h light/dark cycle and 60% humidity for a week prior to use in subsequent experiments. The left anterior descending coronary artery (LAD) of the mice was ligated to establish a myocardial I/R model. Briefly, the mice were injected peritoneally with 350 mg/kg chloral hydrate (4%; Sinopharm Chemical Reagent Co., Ltd.) for anesthesia and fixed in a supine position fixed; breathing was monitored to ensure the airways were unobstructed. A 1-cm incision was subsequently made between the 3rd and 4th ribs on the left sternal border, and the skin was gently cut using tissue scissors, separating the tissue layer by layer to open the thoracic cavity. The LAD was located and then ligated. After 30 min, the ligature was opened and the incision was sutured. The surgical procedure in the sham group was conducted in the same manner, but the LAD was not ligated. Postoperatively, the animals were housed at room temperature (25–28°C) and were allowed free access to food and water. At the end of the experiment, the animals were sacrificed via cervical dislocation and the lack of heartbeat for a long time was used to confirm death. The present study was approved by Ethics Committee of Shaanxi Provincial People's Hospital (Xi'an, China).

Cell culture and OGD establishment. H9c2 cells (crl-1446; American Type Culture Collection) were cultured in DMEM (cat. no. 12491-15; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. 10100-147; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. 15640055; Gibco; Thermo Fisher Scientific, Inc.).

OGD cell models were subsequently established. Briefly, 1×10^7 H9c2 cells were seeded into a 100-mm dish, and the culture medium was replaced with HEPES medium (cat. no. 15630106; Gibco; Thermo Fisher Scientific, Inc.). H9c2 cells were sequentially exposed to hypoxia for 12 h (CO_2/N_2 at a 95:5 ratio) and then reoxygenated (O_2/CO_2 at a 95:5 ratio) for 2 h.

Myocardial infarction area measurements. Following 24 or 48 h of reperfusion, 1% Evans blue was injected into the aorta of each mouse and the hearts were harvested to prepare heart sections. Then, 1% 2, 3, 5-triphenyl tetrazolium chloride (TTC) was used to stain the heart slices, which were visualized using a photomicroscope. ImageJ software 6.0 (National Institutes of Health) were used to quantify the size of the left ventricle (LV), area at risk (AAR) and infarct area (IA).

Cardiac function analysis. An animal electrocardiogram (ECG) recording analyzer (LS20; B&E TEKSYSTEMS)

was used to perform the ECG of mice at 0 days (baseline), 7 and 14 days following myocardial I/R injury. The fractional shortening of the LV [FS (%)], left ventricular end-diastolic pressure (LVEDP) and left ventricular diameter of systolic (LVDs) was measured using an ECG.

TUNEL staining. Heart tissues from each mouse were fixed with paraformaldehyde and embedded in paraffin. TUNEL staining was subsequently used to detect the apoptosis of cardiomyocytes in each group using a TUNEL Cell Apoptosis Detection kit (cat. no. TA201-02; Beijing Transgen Biotech Co., Ltd.) or a One-step TUNEL cell apoptosis detection kit (green fluorescence) (cat. no. C1086; Beyotime Institute of Biotechnology). Briefly, the paraffin-embedded sections were deparaffinized and dehydrated, then incubated with proteinase K for 30 min (room temperature for 15 min and 37°C for 15 min). The sections were subsequently incubated with TUNEL reaction mixture for 1 h at 37°C, incubated with transformant-POD for 30 min at 37°C, incubated with DAB for 30 min at room temperature and counterstained with hematoxylin. Sections were mounted and visualized using a light microscope.

Cell transfection. The pRK5-TIPE2 recombinant plasmid (Over-TIPE2) was transfected into H9c2 cells using Lipofectamine® 2000 reagent (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) to overexpress the TIPE2 protein, while an empty pRK5 plasmid was transfected into cells as the control.

Tandem sensor for autophagy. A total of 0.5×10^6 H9c2 cells were seeded into a 6-well plates, and pRK5 and pRK5-TIPE2 plasmids were transfected into H9c2 cells. Following 48 h of transfection, an adenovirus containing GFP-RFP-LC3 plasmid (cat. no. HB-LP2100001; Hanbio Biotechnology Co., Ltd.) was added to the cell culture and incubated for 24 h. Following the incubation, cells were fixed with 4% polyaldehyde, rinsed with PBS and sealed.

Western blotting. Total protein was extracted from cells and tissues using a One Step Animal Tissue Active Protein Extraction kit (cat. no. C500006-0020; Sangon Biotech Co., Ltd.). Tissue or cell lysates were separated using SDS-PAGE and subsequently transferred onto PVDF membranes, which were blocked with 5% skimmed milk at room temperature for 1 h. The membranes were then incubated with the following primary antibodies overnight at 4°C: Anti-TIPE2 (cat. no. ab110389; Abcam; 1:500), anti-LC3B (cat. no. ab51520; Abcam; 1:3,000), anti-autophagy related 15 (ATG14; cat. no. ab227849; Abcam; 1:1,000), anti-regulatory associated protein of mTOR complex 1 (Raptor; cat. no. ab5454; Abcam; 1:3,000), anti-mTOR (cat. no. ab134903; Abcam; 1:1,500), anti-mTOR phospho S2448 (p-mTOR; cat. no. ab84400; Abcam; 1:1,000), anti-eukaryotic translation initiation factor 4E (4EBP1; cat. no. ab32042; Abcam; 1:2,000), anti-4EBP1 phospho T37 (p-4EBP1; cat. no. ab75767; Abcam; 1:1,000) and anti-GAPDH (cat. no. ab9484; Abcam; 1:3,000). Following the primary antibody incubation, the membranes were incubated with goat anti-rabbit (cat. no. ab150077; Abcam; 1:1,000) or goat anti-mouse (cat. no. ab150117; Abcam; 1:1,000) secondary antibodies for 1 h at room temperature. Protein bands were visualized and densitometric analysis was performed.

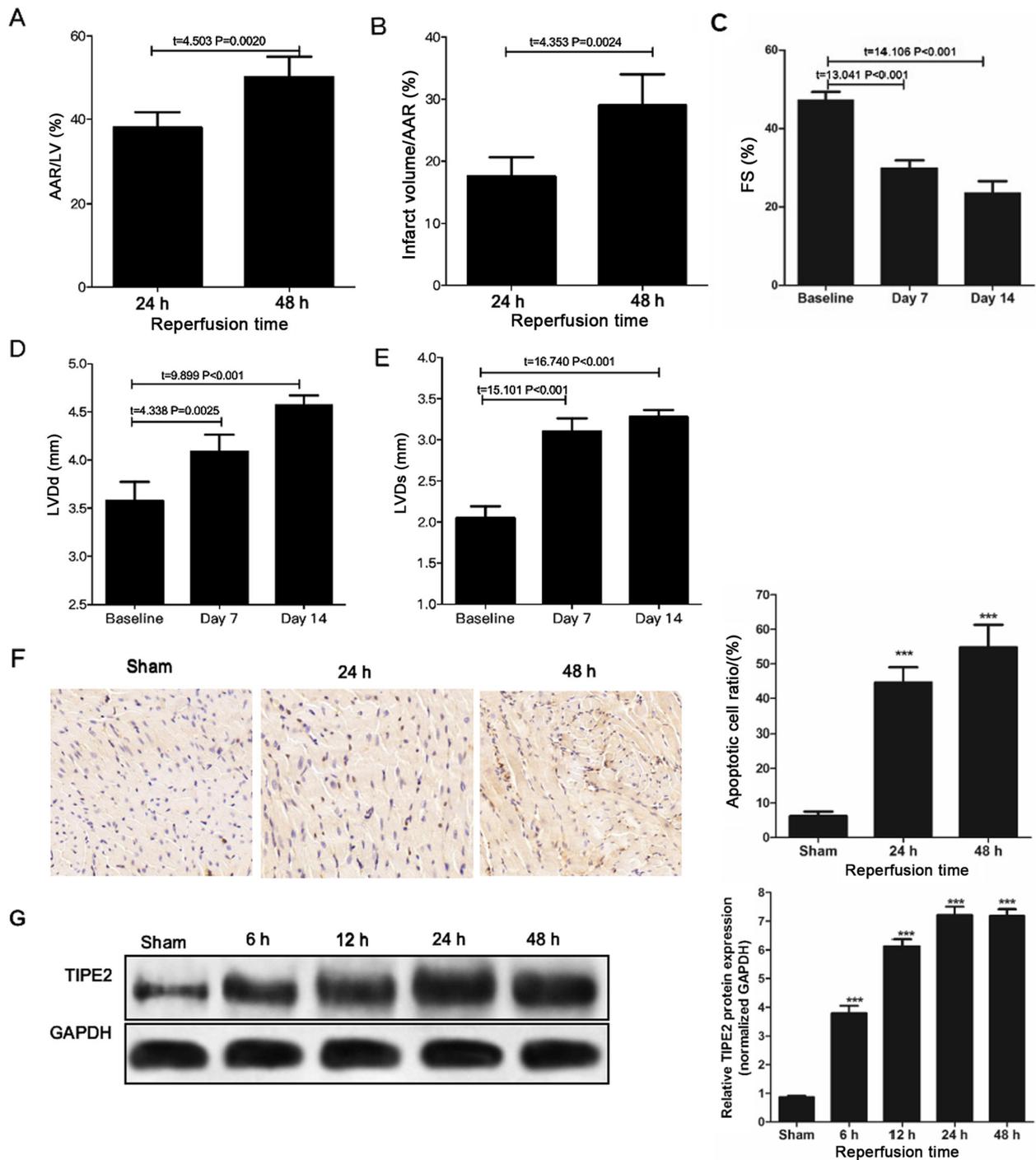


Figure 1. Establishment of an I/R model and analyzing the protein expression levels of TIPE2. (A) AAR/LV and (B) IA/AAR were quantitatively measured in the heart of mice from different groups. (C) FS (%), (D) LVDd and (E) LVDs were measured using echocardiography in the different groups of mice at baseline and 7/14 days after myocardial I/R injury. (F) TUNEL assay was used to detect the levels of myocardial apoptosis. (G) TIPE2 protein expression levels were analyzed in cardiac injury tissues following I/R at different reperfusion times. n=5 mice/group. Data are representative of three independent repeats per experiment. ***P<0.001 vs. sham. I/R, ischemia-reperfusion; TIPE2, TNF- α -induced protein 8-like protein 2; AAR, area at risk; LV, left ventricle; IA, infarct area; FS (%), fractional shortening of the left ventricle (%); LVDd, LVDs, left ventricular diameter of systolic.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp.) and the data are presented as the mean \pm SD. Statistical differences between two groups were determined using a Student's t-test, while a one-way ANOVA followed a Duncan's or Tukey's post hoc test was used to determine statistical differences between three groups or more than three groups, respectively. P<0.05 was considered to indicate a significantly difference.

Results

TIPE2 expression levels are upregulated in myocardial I/R injury. The LAD of each mouse was ligated through surgery for 30 min, and then blood flow reperfusion was resumed at different times (6, 12, 24 or 48 h) by relieving the surgical ligation. The AAR/LV and IA/AAR in the ischemic group was significantly increased (Fig. 1A and B). An ECG was

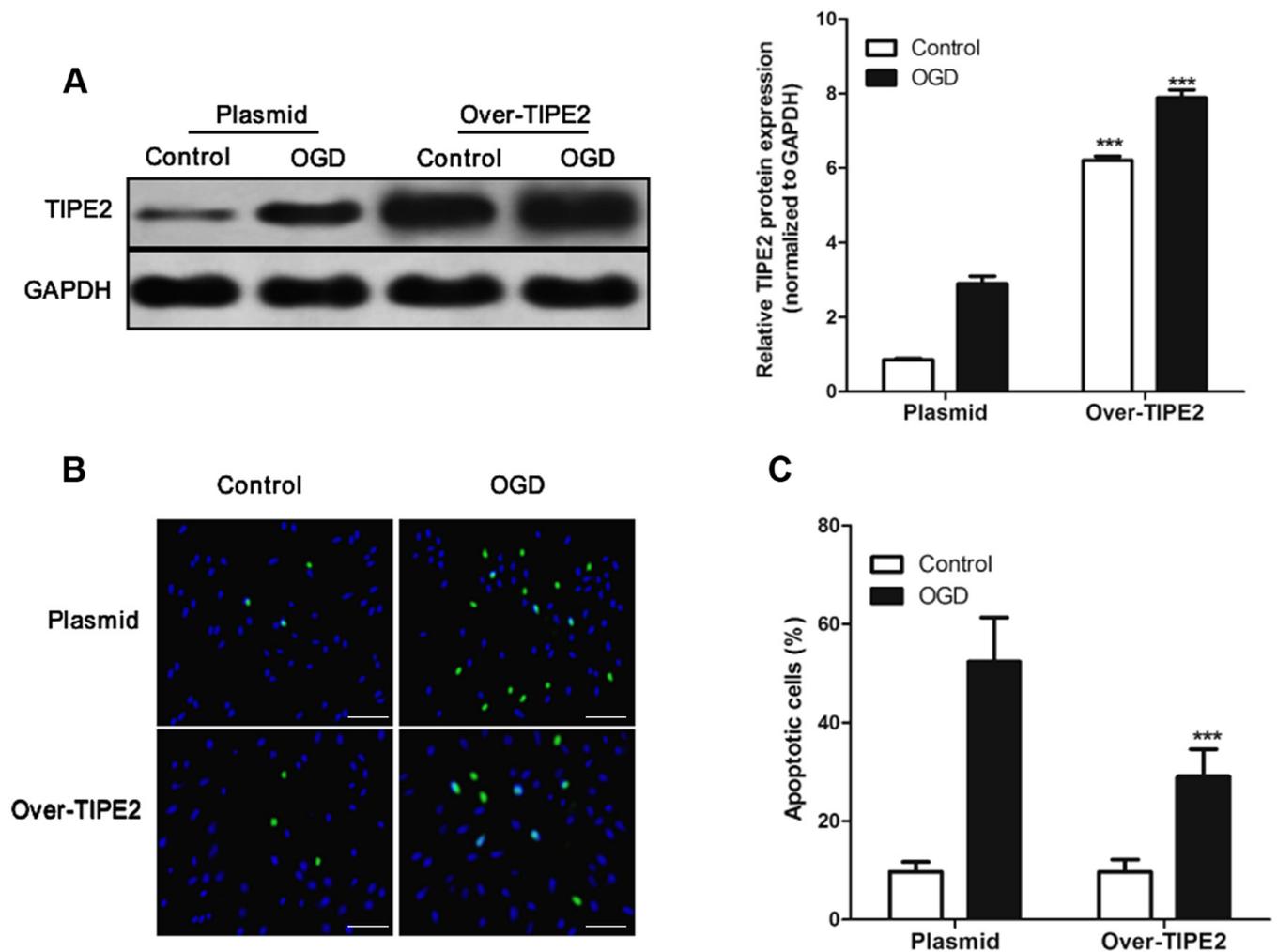


Figure 2. Overexpression of TIPE2 reduces OGD-induced H9c2 cell apoptosis. (A) Western blotting was used to analyze the expression levels of TIPE2 in H9c2 cells. (B and C) TUNEL staining was used to detect OGD-induced apoptosis in H9c2 cells. Data are representative of three independent repeats per experiment. *** $P < 0.001$ vs. plasmid. TIPE2, TNF- α -induced protein 8-like protein 2; OGD, oxygen glucose deprivation; over-, overexpression.

performed to assess the baseline left ventricular function of the mice and then at 7 and 14 days following I/R injury. As shown in Fig. 1C-E, the FS (%) gradually decreased, while the LVDd and LVDs gradually increased from baseline to day 14. These results indicated that the I/R mouse model was successfully established.

In addition, the number of apoptotic cells in the heart tissue of I/R model mice was significantly increased at 24 and 48 h compared with the sham group (Fig. 1F), and the protein expression levels of TIPE2 in the heart tissue of I/R model mice were significantly upregulated compared with the sham group in a time-dependent manner following reperfusion, reaching a peak value after 24 h (Fig. 1G).

Overexpression of TIPE2 decreases OGD-induced H9c2 cell apoptosis. TIPE2-overexpressing H9c2 cells were successfully established (Fig. 2A), and then *in vivo* I/R was simulated in an *in vitro* setting through OGD. Following 30 min of hypoxia/reoxygenation *in vitro*, apoptosis was detected using TUNEL staining. As shown in Fig. 2B and C, the overexpression of TIPE2 significantly decreased the levels of OGD-induced apoptosis of H9c2 cells.

Overexpression of TIPE2 decreases OGD-induced autophagy in H9c2 cells. H9c2 cells were used to perform Tandem sensor experiments *in vitro* to detect the autophagic flow. The results revealed that there was an increase in autophagy levels in H9c2 cells following OGD, whereas the overexpression of TIPE2 significantly inhibited the autophagy induced by OGD (Fig. 3A). Moreover, the expression levels of autophagy-related proteins, LC3B and ATG14, were analyzed, and the results demonstrated that OGD significantly upregulated the protein expression levels of ATG14 and LC3B in H9c2 cells compared with the plasmid group; however, the overexpression of TIPE2 significantly downregulated the expression levels of these proteins (Fig. 3B). These results suggested that the overexpression of TIPE2 may decrease OGD-induced autophagy in H9c2 cells.

Overexpression of TIPE2 activates the mTORC1 signaling pathway in H9c2 cells. The mTORC1 signaling pathway is one of the key pathways that regulates cell autophagy. The present results found that the overexpression of TIPE2 not only upregulated the protein expression levels of TIPE2, but also upregulated the protein expression levels of Raptor, p-mTOR and 4EBP1 in H9c2 cells (Fig. 4A and B). However,

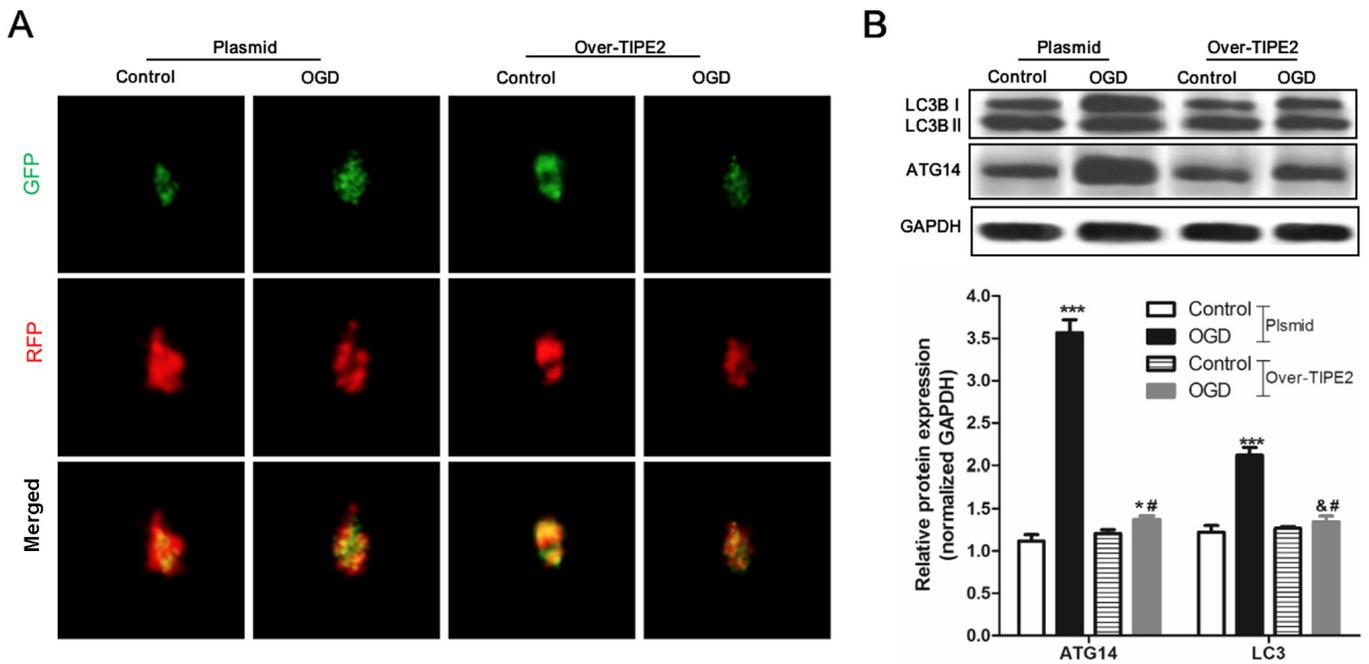


Figure 3. TIPE2 inhibits autophagy levels in H9c2 cells. (A) Levels of autophagy were measured using a tandem sensor. OGD increased autophagy levels in H9c2 cells, while the overexpression of TIPE2 reduced autophagy levels in H9c2 cells with/without OGD. (B) Western blotting was used to analyze the proteins expression levels of autophagy-related LC3B and ATG14 in H9c2 cells. Data are representative of three independent repeats per experiment. $&P>0.05$ vs. plasmid; $*P<0.05$, $***P<0.001$ vs. plasmid; $#P<0.001$ vs. plasmid + OGD. TIPE2, TNF- α -induced protein 8-like protein 2; OGD, oxygen glucose deprivation; over-, overexpression; ATG14, autophagy related 14.

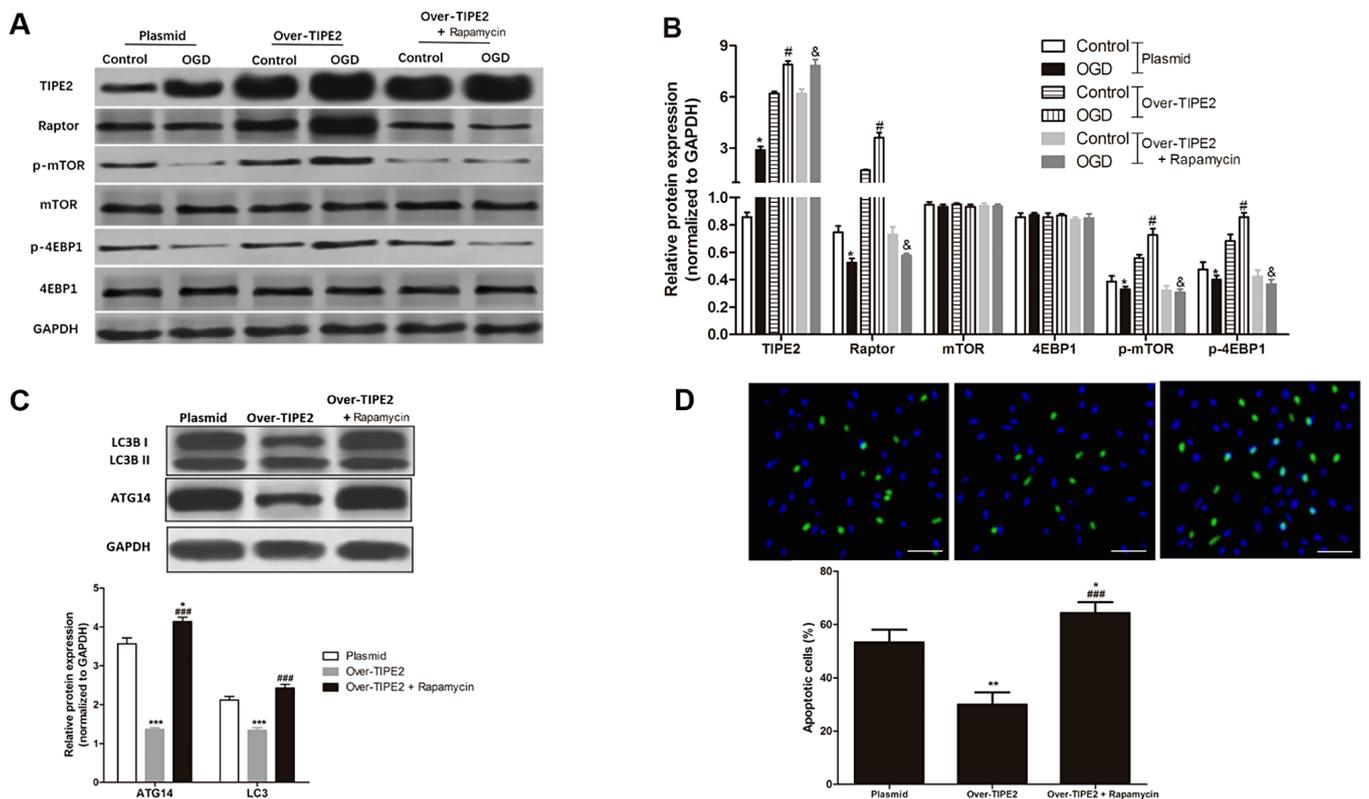


Figure 4. TIPE2 reduces autophagy and OGD-induced apoptosis in H9c2 cells by activating the mTORC1 signaling pathway. (A) Western blotting was used to analyze the protein expression levels of TIPE2, Raptor, p-mTOR/mTOR and p-4EBP1/4EBP1 in H9c2 cells following different treatments. (B) Semi-quantification of the expression levels presented in part (A). (C) Expression levels of autophagy-related proteins, LC3B and ATG14, in H9c2 cells following different treatments. (D) TUNEL staining was used to detect OGD-induced apoptosis in H9c2 cells. Data are representative of three independent repeats per experiment. $*P<0.05$, $**P<0.01$, $***P<0.001$ vs. plasmid; $#P<0.05$ and $###P<0.001$ vs. over-TIPE2; $&P<0.05$ vs. over-TIPE2 + OGD. TIPE2, TNF- α -induced protein 8-like protein 2; OGD, oxygen glucose deprivation; over-, overexpression; ATG14, autophagy related 14; p-, phosphorylated; Raptor, regulatory associated protein of mTOR complex 1; 4EBP1, eukaryotic translation initiation factor 4E.

treatment with rapamycin, an inhibitor of the mTORC1 signaling pathway, downregulated Raptor, p-mTOR and 4EBP1 protein expression levels in H9c2 cells overexpressing TIPE2, without altering TIPE2 protein expression levels (Fig. 4A and B). Therefore, inhibit and promote synergy may cause insignificant changes in protein expression. It is important to note that a suitable concentration of rapamycin was chosen to ensure that the expression of the Raptor, p-mTOR and 4EBP1 proteins in H9c2 cells were similar between the plasmid and Over-TIPE2 + rapamycin groups. Subsequently, the autophagy levels of H9c2 cells under different treatment conditions were compared by detecting the expression levels of autophagy-related proteins, LC3B and ATG14. The results revealed that the overexpression of TIPE2 significantly downregulated the protein expression levels of ATG14 and LC3B compared with the plasmid group, while subsequent treatment with rapamycin could ameliorate this effect (Fig. 4C). In addition, following the overexpression of TIPE2 in H9c2 cells, rapamycin treatment increased the levels of OGD-induced apoptosis in H9c2 cells (Fig. 4D). The aforementioned findings suggested that TIPE2 may decrease OGD-induced apoptosis levels in H9c2 cells by activating the mTORC1 signaling pathway to decrease autophagy.

Discussion

Following the societal advances in the economy and lifestyle changes, especially the increased aging population and the acceleration of urbanization, the prevalence of cardiovascular risk factors in China has significantly increased, which has led to an increase in the number of patients diagnosed with cardiovascular disease. In fact, myocardial infarction has become one of the most common causes of mortality in the Chinese population (21,22). Treatment methods, including thrombolysis, coronary angioplasty and coronary artery bypass grafting, can restore blood perfusion to the ischemic myocardium. However, reperfusion is a double-edged sword, because reperfusion itself does not protect the heart muscle, and it can accelerate and cause new myocardial damage, which is known as myocardial I/R damage (1,2).

In the present study, the LAD of mice was ligated followed by a period of reperfusion to establish a myocardial I/R model, which has been widely used to study I/R injury, due to its simplicity to construct and the resulting high survival rate. The results of the present study found that not only did the number of apoptotic cardiomyocytes significantly increase, but the protein expression levels of TIPE2 also significantly increased in the injured heart tissue of I/R model mice. The human TIPE2 gene is located on chromosome 1q21.2-1q21.3, and it encodes a protein that can bind to a protein containing a death effect domain to exert an inhibitory effect on apoptosis (16,23). Previous studies have identified TIPE2 served a role as an immuno-negative regulator and was essential for inflammation and immune homeostasis, as TIPE2 deficiency was found to promote the pathogenesis of severe inflammatory diseases (17,18). Sun *et al* (17) reported that TIPE2-knockout mice gradually showed signs of weight loss, spleen enlargement, leukocytosis and increase levels of multiple organ spontaneous

inflammatory mediators. In addition, TIPE2 was also found to decrease myocardial I/R injury by inhibiting nucleotide binding oligomerization domain containing 2-mediated inflammatory responses (20).

Autophagy is an important self-protection mechanism used by cells to maintain homeostasis (10,11). Inflammation is a protective response that activates the immune system to promote the secretion of multiple cytokines in response to injury or invasion by pathogens (24,25). Abnormal inflammatory reactions can disrupt the stability of the intracellular environment and autophagy has been discovered to suppress these inflammatory responses. Autophagy was reported to act as a protective mechanism for cells against excessive inflammation or persistent inflammatory responses through two pathways (19,26). The first is the indirect pathway, in which cells effectively remove strong stimulating factors, such as intracellular damaged organelles, including mitochondria, or intracellular pathogenic microorganisms, which trigger inflammatory responses through autophagy (27,28). The second is a direct pathway, in which cells can protect themselves by inhibiting the formation of inflammatory complexes (29). Previous studies have found that a regulatory network that controls autophagy monitored output signals associated with various inflammatory mediators, which appropriately regulated autophagy depending on the state of the inflammatory response (19,26). Since TIPE2 was found to attenuate I/R injury by inhibiting inflammation (20), it was hypothesized that TIPE2 may be able to decrease the levels of I/R-induced cardiomyocyte apoptosis by regulating autophagy.

In the present study, to determine the relationship between TIPE2, autophagy and I/R injury, H9c2 cells overexpressing the TIPE2 protein were established and I/R injury was simulated *in vitro* through OGD. The results revealed that the overexpression of TIPE2 could not only decrease the levels of OGD-induced H9c2 cell apoptosis, but it could also decrease OGD-induced autophagy in H9c2 cells. The effect of autophagy on I/R injury is determined by the extent by which it is activated. On the one hand, appropriate autophagy can help cardiomyocytes to clear damaged mitochondria to decrease the levels of reactive oxygen species production, which damage intracellular functional proteins and DNA (30,31), and it also helps to clear ubiquitinated proteins to maintain the homeostasis of intracellular proteins (32). On the other hand, while excessively-activated autophagy can degrade harmful proteins in cells, it can also degrade normal proteins and organelles, thereby destroying cells and causing irreversible cell death (13,15). The aforementioned results suggested that TIPE2 may not only be an inflammation/immuno-negative regulator, but also a negative regulator of autophagy in I/R injury. Moreover, a phenomenon not shown in the article should be mentioned, that is, that TIPE2 knockout could affect the proliferation and activity of H2c9 cells, and this may also relate to autophagy, because autophagy is required for the normal functioning of cells.

Further analysis of the downstream signaling pathway involved in the regulatory effects of TIPE2 in OGD-induced autophagy found that the overexpression of the TIPE2 upregulated the protein expression levels of Raptor, p-mTOR/mTOR

and p-4EBP1/4EBP1 in H9c2 cells. However, the treatment with the mTORC1 signaling pathway inhibitor, rapamycin, subsequently downregulated Raptor, p-mTOR/mTOR and p-4EBP1/4EBP1 proteins without altering the TIPE2 protein expression levels in H9c2 cells. Moreover, the overexpression of TIPE2 decreased the levels of OGD-induced apoptosis and autophagy in H9c2 cells, while the treatment with the mTORC1 signaling pathway inhibitor, rapamycin, partially reversed these effects. mTORC1 is a complex composed of mTOR, Raptor, DEP domain containing mTOR interacting protein, AKT1 substrate 1 and mTOR associated protein, LST8 homolog, which has been found to be involved in the regulation of cell proliferation, apoptosis and autophagy (33,34). In numerous types of malignant tumor cells, the activation of the mTORC1 signaling pathway was discovered to inhibit autophagy and promote the apoptosis of cancer cells; however, mTORC1 signaling pathway activation has been found to be inhibited in numerous types of tumor tissue (35,36). Although the association between mTORC1-mediated autophagy and apoptosis remains uncertain, due to the interaction between autophagy and apoptosis being different under different conditions, it has been reported that the mTORC1 signaling pathway inhibits autophagy (37,38).

In conclusion, the results of the present study suggested that TIPE2 may protect cardiomyocytes from I/R-induced apoptosis by decreasing cellular autophagy via the mTORC1 signaling pathway. However, the present study did not overexpress or knockdown TIPE2 expression *in vivo*, thus the effects of TIPE2 on autophagy and apoptosis *in vivo* were not investigated and should be the focus of future investigations. In addition, future research should also use primary cardiomyocytes to further investigate the effects of TIPE2.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FC conceived the study, wrote the manuscript, designed and supervised the study and edited the manuscript. GC, XH, PY, PF, SJ, JZ and HY performed the data analysis, participated in the experiments and data collection. FC and GC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Ethics Committee of Shaanxi Provincial People's Hospital (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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