EXPERIMENTAL STUDY

HIF-1/AKT Signaling-Activated PFKFB2 Alleviates Cardiac Dysfunction and Cardiomyocyte Apoptosis in Response to Hypoxia

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Summary

Myocardial infarction (MI) is the most prevalent disease with severe mortality, and hypoxia-induced cardiac injury and cardiomyocyte apoptosis are the significant and harmful consequences of this disease. The cross talk between hypoxia signaling and glycolysis energy flux plays a critical role in modulating MI-related heart disorder. However, the underlying mechanism remains unclear. Here, we aimed to explore the effect of a key glycolytic enzyme of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 (PFKFB2) on cardiac dysfunction and apoptosis in response to hypoxia. Our data demonstrated that the mRNA and protein expression of PFKFB2 were significantly elevated in the MI mice. The MI treatment promoted the activation of PFKFB2 in vivo, as presented by the remarkably increased phosphorylation levels of PFKFB2. PFKFB2 depletion enhanced MI-induced cardiac dysfunction and cardiomyocyte apoptosis in the MI mouse model. Moreover, hypoxia treatment dramatically upregulated the expression and activation of PFKFB2 in a time-dependent manner in cardiomyocytes. Hypoxia-stimulated PFKFB2 relieved hypoxia-induced cardiomyocyte apoptosis in vitro. PFKFB2 activated the fructose-2,6-bisphosphate (Fru-2,6-p2)/PFK/anaerobic adenosine triphosphate (ATP) glycolysis energy flux in response to hypoxia in cardiomyocytes. Mechanically, hypoxia-activated PFKFB2 by stimulating the hypoxia-inducible factor 1 (HIF-1)/AKT signaling. Thus, we conclude that HIF-1/AKT axis-activated PFKFB2 alleviates cardiac dysfunction and cardiomyocyte apoptosis in response to hypoxia. Our finding presents a new insight into the mechanism by which HIF-1/AKT/PFKFB2 signaling modulates MI-related heart disorder under the hypoxia condition, providing potential therapeutic targets and strategy for hypoxia-related myocardial injury.

Key words: MI, Heart disorder, Myocardial injury

Myocardial infarction (MI) is a sort of disease of the coronary artery that has high morbidity and mortality globally.1,2 The occurrence of MI is usually correlated with severe constant hypoxia-induced oxidative stress and cardiomyocyte apoptosis, leading to cardiac dysfunction.3 Suspension of the blood supply of the heart may emerge from the blockage of the coronary artery, a status called atherosclerosis, as a consequence of an increase of plaque, a matter mostly made of cellular waste products, cholesterol, and fat.4 Thus, hypoxia is a significant outcome of myocardial ischemia, which drives to cell apoptosis and worsens cardiac tissue injury.5 Understanding the molecular mechanisms that underlie hypoxia-mediated cardiomyocyte apoptosis and cardiac injury will benefit to the development of effective treatments for MI.6,7 However, the advancement in this research field is still limited.

Glycolysis is an essential source of anaerobic adenosine triphosphate (ATP) during both acute ischemia and reperfusion of a restricted-span coronary occlusion, preserving the myocardium from cell damage and consequent cardiac dysfunction for the maintenance of contractility of the heart.8 Hypoxic signaling is a central portion of the reply to MI-induced ischemia, stimulating the adjustment processes, including enhanced glycolysis.9 The primary operator of this signaling is hypoxia-inducible factor 1 (HIF-1), a transcription factor maintained at low oxygen conditions to organize many alterations in gene expression.10 The cross talk of hypoxia/HIF-1 signaling and glycolysis pathway plays a critical role in modulating MI-related heart injury and cardiomyocyte apoptosis.11 A previous study revealed that HIF-1 modulated the activity of...
hexokinase II and pyruvate dehydrogenase kinase-1 to attenuate ischemia/reperfusion injury and apoptosis in the heart by reprogramming cell metabolism of glycolysis. Although some clues are presented, the profound understanding of the interplay of hypoxia/HIF-1 signaling and glycolytic energy flux in the MI-induced cardiac injury is still obscure.

Furthermore, 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 2 (PFKFB2), as a critical regulator of glycolysis, controls both the synthesizing and degrading of fructose-2, 6-bisphosphate (Fru-2, 6-p2), which serves as a signal metabolite and activator for phosphofructokinase (PFK), playing an important role in balancing energy homeostasis. It has been reported that the heart-specific isoform of PFKFB2 is an important contributor to glycolytic flux in cardiomyocytes. Further studies showed that PFKFB2 was highly sensitive to hypoxia mimics such as iron chelators, transition metals, and HIF hydroxylase inhibitors in the heart. Besides, as an essential cellular regulating pathway, AKT signaling is involved in the modulation of cardiac function, in which AKT-mediated PFKFB2 phosphorylation and activation increase glycolysis flux in the heart. However, the effect of PFKFB2 and AKT signaling on MI-induced cardiac injury in response to hypoxia remains elusive.

In this study, we aimed to explore the role of PFKFB2 in hypoxia-induced cardiomyocyte apoptosis and cardiac injury. We identified a novel function of HIF-1/AKT signaling-activated PFKFB2 in protecting MI-induced cardiac dysfunction and attenuating cardiomyocyte apoptosis in response to hypoxia.

**Methods**

**MI mouse model:** To establish the MI mouse model, the C57BL/6 mice (4-6 months old, male, 25-30 g) were intraperitoneally injected with xylazine (5 mg/kg body weight) for the anesthetization. The left anterior descending (LAD) coronary artery occlusion/reperfusion (LAD/reperfusion) was performed. Briefly, we placed the LAD on the heart surface using an anatomy microscope and ligated the LAD for 30 minutes, followed by the restoration of blood flow. The surgery was conducted on sham mice without LAD occlusion. The untreated and sham mice were used for control. In addition to the untreated mice (control), we randomly separated the mice into the following groups: sham, MI, MI + control shRNA, and MI + PFKFB2 shRNA. The targeted sequence of PFKFB2 shRNA was as follows: 5'-AGGAAAUAACAGACCUCAA-3'. We injected the lentiviral vectors comprising shRNA of PFKFB2 (Genechem, China) (1 × 10⁷ TU/mice) or the lentivirus comprising control shRNA, or the corresponding control at a dosage of 50 nmol/L by X-treme GENE (Roche, Germany) according to the manufacturer’s guidelines before hypoxia treatment. The vector carrying the complete PFKFB2 coding sequence for PFKFB2 overexpression vector, or the corresponding control at a dosage of 50 nmol/L by X-treme GENE (Roche, Germany) according to the manufacturer’s guidelines before hypoxia treatment. The HIF-1 inhibitor Bay87-2243 (Selleckchem, USA) was used at the dose of 25 and 50 nM. The PI3K inhibitor LY294002 (InvivoGen, USA) was used at the dose of 10 and 20 μM.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL):** The apoptosis was analyzed using the TUNEL Detection Kit (Roche, Germany) in the ischemic border region of the heart in the 3 days post-MI or sham mice according to the product’s instruction. After the staining of TUNEL, the ventricular samples were dyed using DAPI (Sigma, USA) to stain nuclear. Fluorescence observation was performed using a confocal microscope (Olympus Fluoview1000, Tokyo, Japan).

**Analysis of cell apoptosis:** About 2 × 10⁴ H9C2 cells were plated on 6-well dishes. Apoptosis-mediated cell death was analyzed using an Annexin V-FITC/PI Apoptosis Detection Kit (Keygen Biotech, China) according to the manufacturer’s instruction. Briefly, about 2 × 10⁴ cells were collected using a binding buffer, washed, and dyed with Annexin V-FITC and PI at 25°C; flow cytometry analysis was then performed.

**Quantitative reverse transcription-PCR (qRT-PCR):** The total RNAs were extracted by TRIzol (Invitrogen, USA). The first-strand cDNA was manufactured as per the manufacturer’s instruction (Rever-Tra Ace, Japan). The qRT-PCR was performed by applying SYBR Real-time PCR I Kit (Roche, Germany). The standard control was GAPDH. Quantitative examination of the RNA levels was conducted in triplicate independent experiments. The primer sequences are as follows: PFKFB2 forward: 5'-CC ATGAAAGATCGCAAAACAGT-3' PFKFB2 reverse: 5'-TC TTCAGTGAGATACGCCTTAACAT-3' GAPDH forward: 5'-AAGAAGGTGGTGAAGCAGGC-3' GAPDH reverse: 5'-GTCACCAAGGTTGTCCGGGC-3'

**Western blot analysis:** Total proteins were extracted from the cells or mouse heart tissues with RIPA buffer (Be-
The PFKFB2 is activated in the myocardial tissue from MI mice. To understand the potential correlation of PFKFB2 with the MI, we established the MI mouse model and detected the expression and activation of PFKFB2 in the mice. Significantly, qPCR assays revealed that the mRNA expression levels of PFKFB2 were enhanced in the peri-infarct area of myocardial tissues from the MI mice relative to that from the control and sham surgery mice (Figure 1A). It has been reported that the phosphorylation of PFKFB2 is critical for its activation. Consistently, compared with the control groups, the MI-post mice demonstrated remarkably elevated phosphorylation levels of PFKFB2 in cardiac tissues (Figure 1B). The IHC staining further revealed that the protein expression of PFKFB2 was increased in the peri-infarct region of heart tissues from the MI mice (Figure 1C), suggesting that PFKFB2 may be closely correlated with the development of MI.

The depletion of PFKFB2 enhances MI-induced cardiac dysfunction and cardiomyocyte apoptosis: We then further explored the effect of PFKFB2 on MI-induced injury in vivo. To this end, we injected the lentiviral plasmids carrying PFKFB2 shRNA and corresponding control shRNA into the mice ventricular chamber and constructed the MI mouse model for 3 days. The elevated expression of PFKFB2 induced by MI and the efficiency of PFKFB2 knockdown by shRNA were confirmed in the mice (Figure 2A). As expected, echocardiography analysis demonstrated that the percentage of EF and FS was decreased in the heart of MI mice compared with the control group (Figure 2B), indicating that the cardiac function was impaired. Significantly, the depletion of PFKFB2 by shRNA further reduced the levels of EF and FS (Figure 2B), implying the potentially protective effect of PFKFB2 on heart function after MI. Moreover, TUNEL analysis revealed that the TUNEL-positive cells were elevated in the border region of the heart from the MI mice, in which PFKFB2 knockdown enhance this elevation (Figure 2C), suggesting that PFKFB2 is critical for the protection of cardiomyocyte from MI-induced apoptosis.

Hypoxia-activated PFKFB2 alleviates hypoxia-induced cardiomyocyte apoptosis: Acute myocardial infarction (AMI) is described by pathological alterations correlated with hypoxia, and the hypoxia-induced cell apoptosis aggravates the MI progression and MI-related heart injury. Hence, we were interested in the impact of hypoxia on PFKFB2 and the function of PFKFB2 in the hypoxia-caused cardiomyocyte apoptosis. For this purpose, we constructed a hypoxia-exposure cardiomyocyte model using H9C2 cells. Our data showed that the hypoxia treatment dramatically enhanced the expression levels of PFKFB2 in a time-dependent manner in the H9C2 cells (Figure 3A), implying that PFKFB2 can respond to the hypoxia in cardiomyocytes. Notably, hypoxia treatment enhanced the phosphorylation levels of PFKFB2 in the cells (Figure 3B), indicating that hypoxia may activate PFKFB2. We then depleted and overexpressed the PFKFB2 by PFKFB2 siRNA and the vector carrying the complete PFKFB2 coding sequence in the H9C2 cells, respectively. The efficiency of PFKFB2 knockdown and overexpression was validated in the cells (Figure 3C). Significantly, the hypoxia-induced cardiomyocyte apoptosis was reinforced by PFKFB2 depletion but was impaired by PFKFB2 overexpression (Figure 3D). Similarly, PFKFB2 knockdown remarkably reduced the expression of the apoptotic Bcl-2 but enhanced the pro-apoptotic Bax and cleaved caspase-3 expression under the hypoxia condition in the cells (Figure 3E). Besides, the PFKFB2 overexpression displayed a reverse effect (Figure 3F). Together, these data suggest that hypoxia-activated PFKFB2 can alleviate hypoxia-induced cardiomyocyte apoptosis.

PFKFB2 activates glycolysis energy axis in response to hypoxia in cardiomyocytes: The glycolysis-mediated energy axis plays a critical role in response to the hypoxic-ischemia injury after MI. The glycolysis is negatively related to the apoptosis in hypoxic myocytes by balancing...
Figure 1. PFKFB2 is activated in the myocardial tissue from MI mice. MI mouse model was successfully established. A: mRNA expression levels of PFKFB2 were analyzed by qPCR in the heart tissues of control (n = 4), sham (n = 4), and MI (n = 4) mice. B: Phosphorylation of PFKFB2 was measured using the Western blot analysis in the heart tissues of control, sham, and MI mice. C: Protein expression levels of PFKFB2 were assessed by immunohistochemical staining in the heart tissues of control, sham, and MI mice. Data are presented as mean ± SD. Statistic significant differences were indicated. *P < 0.05.

the energy homeostasis.9) Because PFKFB2 is an essential mediator in the glycolysis, we hypothesized whether PFKFB2 induced the protective role in hypoxia-promoted apoptosis by modulating glycolysis. We observed that hypoxia treatment remarkably reduced the cellular ATP levels, in which PFKFB2 depletion further enhanced this reduction, but PFKFB2 overexpression was able to rescue the ATP levels in the H9C2 cells (Figure 4A). Lactate production was inhibited by PFKFB2 knockdown, while it was increased by PFKFB2 overexpression under the hypoxia condition in the cell (Figure 4B). PFKFB2 catalyzes the synthesis of Fru-2, 6-p2 to modulate the activation of its downstream glycolytic enzyme PFK, which is a powerful regulator of glycolysis flux.19) The elevation of Fru-2, 6-p2 and activation of PFK induced by PFKFB2 lead to a higher glycolysis energy rate.20) Significantly, we found that the depletion of PFKFB2 diminished the levels of Fru-2, 6-p2, and the overexpression of PFKFB2 displayed a reverse effect in the hypoxia-treated H9C2 cells (Figure 4C). Consistently, the enzyme activity of PFK was attenuated by PFKFB2 knockdown but was reactivated by the reintroduced PFKFB2 in the system under the hypoxia condition (Figure 4D). These data indicate that PFKFB2 activates glycolysis energy axis in response to hypoxia in cardiomyocytes.

Hypoxia activates PFKFB2 by HIF-1/AKT signaling in cardiomyocytes: Next, we further explored the underlying mechanism by which hypoxia enhanced the phosphorylation and thereby promoted the enzyme activity of PFKFB2. It has been identified that AKT-mediated phosphorylation and activation of PFKFB2 increase glycolysis flux in the heart.13) The transcriptional factor HIF-1 is the primary executive molecule of the function of hypoxia in multiple diseases including cardiac dysfunction.21) Besides, hypoxia-induced HIF-1 activates the PI3K/AKT pathway by phosphorylation, contributing to the glycolysis.22,23) Accordingly, we were concerned about whether hypoxia-activated PFKFB2 through HIF-1/AKT signaling. Interestingly, the phosphorylation of AKT and PFKFB2, along with the expression of HIF-1, was significantly enhanced.
Figure 2. Depletion of PFKFB2 enhances MI-induced cardiac dysfunction and cardiomyocyte apoptosis. A–C: Left ventricular chamber of 3 days post-MI and sham mice after being injected with the lentiviral vector of control shRNA or PFKFB2 shRNA. Untreated mice with no surgery were used as control. A: Protein expression levels of PFKFB2 were analyzed using Western blot and. B: mRNA expression levels of PFKFB2 were analyzed by qPCR in the heart tissues of the indicated mice. C: Representative echocardiographic tracings (timestamps, 100 ms; calibration bar, 2 mm) were shown. D: Percentage of ejection fractions was assessed by echocardiographic analysis. E: Fractional shortening was assessed by echocardiographic analysis. F: Quantification of TUNEL staining was shown. G: Apoptosis was measured by TUNEL staining in the mice. Data are presented as mean ± SD. Significant differences were indicated. *P < 0.05.

by hypoxia treatment in a time-dependent manner in the H9C2 cells (Figure 5A). The inhibitor of HIF-1 called BAY87-2243 inhibited the expression of HIF-1 and blocked the hypoxia-induced phosphorylation of AKT and PFKFB2 in a dose-dependent manner in the cells (Figure 5B). Moreover, the PI3K inhibitor called LY294002 impaired hypoxia-enhanced AKT and PFKFB2 phosphorylation in a dose-dependent manner (Figure 5C). Together, these suggest that hypoxia activates PFKFB2 by HIF-1/ATK signaling.

Discussion
AMI is one of the most severe matters to human beings, which is not only the vital disorder of unexpected cardiac mortality but also the essential contributor driving to heart failure, and it affects an expanding quantity of people worldwide. The primary pathological variation of MI is cardiomyocyte apoptosis, which leads to the irreversible damage of cardiac function and severe ischemic heart injury. Glycolytic enzymes are involved in the modulation of apoptosis in MI-induced cardiac dysfunction. It has been reported that hexokinase regulates the mitochondria metabolism and decreases ischemia/reperfusion injury, controlling cardiomyocyte apoptosis in the heart. Cardiac PFK-2 enhances glycolysis, myocyte resistance to hypoxia, and hypertrophy. The activity of phosphoglycerate kinase is increased in MI mice and modulates heart failure in a phosphotransfer-dependent manner. Phosphoglycerate mutase (PGAM) reduction inhibits necroptosis in hearts of mice following ischemia and reperfusion by the destruction of dynamin-related protein 1. Pyruvate kinase 2 (PKM2) controls the cardiac cell cycle and promotes the regeneration of cardiomyocyte. In this study, we identified that the mRNA and protein expression of PFKFB2 was elevated in the peri-infarct area of myocardial tissues from the MI mice. Moreover, the activity of cardiac PFKFB2 was signifi-
Hypoxia-activated PFKFB2 alleviates hypoxia-induced cardiomyocyte apoptosis. A: mRNA expression levels of PFKFB2 were measured by qPCR in the H9C2 cells and H9C2 cells treated with hypoxia for indicate time. B: Phosphorylation of PFKFB2 was analyzed using Western blot analysis in the H9C2 cells and H9C2 cells treated with hypoxia for indicate time. C: Protein expression levels of PFKFB2 were tested using Western blot analysis in the H9C2 cells transfected with control siRNA, PFKFB2 siRNA, pcDNA3.1 vector, and pcDNA3.1-PFKFB2 vector. D–F: H9C2 cells were treated with hypoxia and transfected with control siRNA, PFKFB2 siRNA, pcDNA3.1 vector, or pcDNA3.1-PFKFB2 vector. D: Cell apoptosis was examined by flow cytometry analysis in the indicated cells. Quantification of flow cytometry analysis was shown. E and F: Expression levels of Bcl2, Bax, and cleaved caspase-3 were tested using Western blot analysis in the indicated cells. All experiments were repeated three times, and mean ± SD of at least three experiments was shown. Statistic significant differences were indicated. *P < 0.05, **P < 0.01.

Significantly enhanced in the MI mice, as demonstrated by the increased phosphorylation levels of PFKFB. These data indicate that PFKFB2 may be closely related to the progression of MI. Furthermore, our data revealed that the depletion of PFKFB2 promoted MI-induced cardiac dysfunction and cardiomyocyte apoptosis. It suggests that PFKFB2 is required for the protection of cardiac function and preserves cardiomyocyte from MI-induced apoptosis. These observations further emphasize the critical role of glycolytic enzymes in the regulation of MI-induced cardiac disorder.

Hypoxia-induced cardiac ischemia injury and cardiomyocyte apoptosis are significant complications and harmful consequences of cardiac diseases such as MI and heart failure. Hypoxia signaling and glycolysis pathway presents the crucial mechanisms in the MI progression. Hypoxia exposures stimulate the adaptations of cardiomyocytes, such as increased carbohydrate metabolism and glycolytic capability, paving the way for the accelerated recovery of cardiac ATP for the regular function of the heart after severe anoxia. It has been found that miR-21 promotes glycolysis and cardiac protection by Per2 signaling under the hypoxia condition. Inhibition of the hypoxia-mediated miR-34a preserves cardiomyocyte by maintaining glucose metabolism. The depletion of GCN5 L1 in hypoxia-treated cardiac cells disturbs glucose metabolism and elevates apoptosis by AKT/mTORC2 pathway. Reduced expression of heart PPARα is required for adaptive metabolism remodeling under hypoxia to modulate fatty acid oxidation and glycolysis.
PGAM5 is downregulated in cardiomyocytes treated with hypoxia/reoxygenation and modulates the apoptosis of cardiomyocyte.40) Our study demonstrated that hypoxia treatment significantly enhanced the expression and phosphorylation levels of PFKFB2 in a time-dependent manner in the H9C2 cells. It suggests that hypoxia induces PFKFB2 activation in cardiomyocytes. Moreover, hypoxia-activated PFKFB2 could relieve hypoxia-induced cardiomyocyte apoptosis. Meanwhile, PFKFB2 activated glycolysis energy flux in response to hypoxia. These data suggest that PFKFB2 displays an adaptive and protective role for cardiomyocyte under hypoxia stimulation. Our investigation provides valuable information for the interplay of hypoxia and glycolytic enzyme-mediated energy regulation in the MI-related apoptosis.

Hypoxia exerts its impact usually in a HIF-dependent manner in many physiological and pathological processes such as cell proliferation, apoptosis, glycolysis, energy metabolism, cancer, and cardiac diseases.41-44) It has been reported that hypoxia-enhanced HIF-1 stimulates glycolysis as an adjustment in heart failure upon damaged energy metabolism by modulating the expression of ET-1.45) HIF-1 negotiates a switch of PKM after MI.11) The stabilization of HIF-1 guards the heart against ischemia and reperfusion injury through reducing oxidative stress and increasing glycolysis.12) In addition, hypoxia-activated HIF-1 mediates the PI3K/AKT signaling by modulating its phosphorylation, contributing to glycolysis.22,23) The delivered lipoprotein after MI improves heart glucose uptake in mice by AKT pathway.8) The mTOR/AKT signaling-enhanced glycolysis defends against ischemia/reperfusion injury by decreasing ROS generation.46) AKT-mediated phosphorylation and activation of PFKFB2 increase glycolysis flux in the heart.13) Our mechanism study displayed that hypoxia induced the activation of PFKFB2 through HIF-1/ATK signaling in cardiomyocytes. These data provide a new scenario of the crossroad involving HIF signaling, glycolytic enzyme PFKFB2, and AKT pathway in response to hypoxia in cardiomyocytes. Further explorations are needed to figure out the relationship of HIF signaling of other glycolytic enzymes in MI development.

In conclusion, we uncovered that HIF-1/AKT signaling-activated PFKFB2 relieved cardiac dysfunction and cardiomyocyte apoptosis in response to hypoxia (Figure 5D). Our finding presents a new insight into the mechanism by which HIF-1/AKT/PFKFB2 signaling modulates MI-related heart injury under the hypoxia condition, providing potential target and strategy for the therapy of hypoxia-related myocardial injury.
Conflicts of interest: The authors declare no competing financial interests.

Disclosure

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