β-Hydroxybutyrate Exacerbates Hypoxic Injury by Inhibiting HIF-1α-Dependent Glycolysis in Cardiomyocytes—Adding Fuel to the Fire?

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β-hydroxybutyrate exacerbates hypoxic injury by inhibiting HIF-1α-dependent glycolysis in cardiomyocytes—Adding fuel to the fire?

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Abstract

Purpose: Ketone body oxidation yields more ATP per mole of consumed oxygen than glucose. However, whether an increased ketone body supply in hypoxic cardiomyocytes and ischemic hearts is protective or not remains elusive. The goal of this study is to determine the effect of β-hydroxybutyrate (β-OHB), the main constituent of ketone bodies, on cardiomyocytes under hypoxic conditions and the effects of ketogenic diet (KD) on cardiac function in a myocardial infarction (MI) mouse model.

Methods: Adult mouse cardiomyocytes and MI mouse models fed a KD were used to research the effect of β-OHB on cardiac damage. qPCR, western blot analysis and immunofluorescence were used to detect the interaction between β-OHB and glycolysis. Live/dead cell staining and imaging, lactate dehydrogenase, Cell Counting Kit-8 assays, echocardiography and 2,3,5-triphenyltetrazolium chloride staining were performed to evaluate the cardiomyocyte death, cardiac function and infarct sizes.

Results: β-OHB level was significantly higher in acute MI patients and MI mice. Treatment with β-OHB exacerbated cardiomyocyte death and decreased glucose absorption and glycolysis under hypoxic conditions. These effects were partially ameliorated by inhibiting hypoxia-inducible factor 1α (HIF-1α) degradation via roxadustat administration in hypoxia-stimulated cardiomyocytes. Furthermore, β-OHB metabolisms were obscured in cardiomyocytes under hypoxic conditions. Additionally, MI mice fed a KD exhibited exacerbated cardiac dysfunction compared with control chow diet (CD)-fed MI mice.

Conclusion: Elevated β-OHB levels may be maladaptive to the heart under hypoxic/ischemic conditions. Administration of roxadustat can partially reverse these harmful effects by stabilizing HIF-1α and inducing a metabolic shift toward glycolysis for energy production.

Keywords: Beta-hydroxybutyrate; Hypoxia; Glycolysis; Hypoxia-Inducible Factor 1α

Declarations

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Conflict of interest: The authors declare no conflict of interest.

Data availability: The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions: Xiurui Ma, Zhen Dong and AiJun Sun conceived and designed the study. Jingyi Liu collected patient plasma and information. Xiurui Ma, Zhen Dong, Leilei Ma, Rifeng Gao, Lihong Pan, Jinyan Zhang, and Dilan A. performed the animal and cell culture experiments. Xiurui Ma, Aijun S, Xiaolei Sun, Jian An, Kai Hu and Junbo Ge interpreted the data. Xiurui Ma, Aijun Sun and Kai Hu. wrote the manuscript. Aijun Sun and Junbo Ge supervised the study. Aijun S and Junbo G reviewed and edited the manuscript. All authors approved the final manuscript.

Compliance with ethical standards:

Ethics approval

All animal protocols performed in this study were approved by and complying with the guides of the Animal Care and Use Committee of the Zhongshan Hospital, Fudan University.

All patients information was obtained from hospital medical records, test databases, or direct interviews with patients. The study protocol was approved by the Ethics Committee of Shanxi Cardiovascular Hospital (clinical registry number: 2018002).

Consent to participate: Informed consent was obtained from all individual participants included in the study and the written informed consent was provided by each patient at the time of registration.

Consent for publication: Not applicable.

1 Introduction

The heart is a high energy-consuming organ; therefore, cardiac energy metabolism is essential for its normal biological and physiological functions[1]. Oxygen plays a critical role in cardiac energy metabolism because mitochondrial oxidative phosphorylation provides 95% of the ATP in the healthy, adult mammalian heart[2-5]. Upon limited oxygen supply, the heart can switch from using oxygen-dependent substrates to more oxygen-efficient energy sources[6]. It has previously been shown that glucose yields 11% more ATP per consumed oxygen atom than fatty acids[7]. However, ketone body oxidation yields more ATP per mole of consumed oxygen than glucose[8]. When the heart experiences hypoxia, such as during a myocardial infarction (MI) or in response to other pathologies, including chronic intermittent hypoxia, sleep apnea, and anemia, the heart can decrease fatty acid oxidation and concomitantly increase its use of glucose and ketone bodies as energy substrates[9]. There is a growing body of evidence showing that the increased utilization of glucose during periods of ischemia is cardioprotective[1, 6, 10]. However, it is not yet clear whether an increased use of ketone bodies under hypoxic conditions is adaptive or maladaptive in the heart.

It has been previously shown that the mammalian heart is capable of avid ketone body uptake and oxidation[11]. Furthermore, the role of ketone bodies in cardiometabolic health has been increasingly recognized. In an ischemia-reperfusion rat model, fasting-induced ketosis[12] and intravenous injection of β-hydroxybutyrate (β-OHB), the main component of ketone bodies[13], have been shown to attenuate ischemic injury. In a recent clinical trial, the beneficial effect of the sodium-glucose cotransporter 2 (SGLT2) inhibitor, empagliflozin, on the cardiovascular outcomes of patients with diabetes[14] is thought to be associated with hyperketonemia[15]. It has also been suggested that the heart is better protected against MI in the fed state with a lower level of ketone bodies compared with the fasted state[16]. However, a recent study has shown that both glucose and β-OHB are positively associated with an increased risk of MI[17]. Dietary carbohydrate intake has been associated with a significant increase in cardiovascular mortality[18] and an increased risk of subsequent coronary artery calcium progression[19]. Furthermore, long-term ketogenic diet-induced β-OHB accumulation has been shown to be detrimental to heart health by promoting cardiac fibrosis[20].

Despite these controversial findings, there is currently limited research on the impact of high levels of ketone bodies on heart metabolic adaptations in response to hypoxia. Here, we aim to investigate whether high ketone body levels can modulate cardiac substrate metabolism and/or induce functional alterations in hypoxic cardiomyocytes and mice after MI surgery.

2 Materials and Methods

2.1 Human peripheral blood collection

Human peripheral blood was collected from acute MI patients at Shanxi Cardiovascular Hospital, and peripheral blood samples from healthy volunteers served as controls. The patient characteristics are shown in Supplemental Table S1. The

clinical outcomes of the acute MI patients were assessed by N-terminal proB-type natriuretic peptide (NT-proBNP) levels and left ventricular ejection fractions (LVEFs), which were measured by echocardiography 7 days after acute MI.

The use of human peripheral blood for scientific purposes was approved by the Ethics Committee of Shanxi Cardiovascular Hospital. All methods were conducted in accordance with the approved guidelines and regulations. Written informed consent was obtained from all patients.

2.2 Adult cardiomyocyte isolation and culture

Cardiomyocytes were isolated from adult, male C57BL/6 mice (6–8 weeks old, weighing 20–22 g) that were obtained from the Shanghai Jiesijie Laboratory Animal Centre, according to institutional guidelines. The isolation of adult mouse cardiomyocytes was performed as described previously[21].After euthanasia, the heart was exposed. Following injection of 7 mL EDTA buffer into the right ventricle, the aorta was clamped with forceps, and the heart was removed. Multiple rounds of injections to the apex of the heart were performed with the following: 10 mL EDTA buffer, 3 mL perfusion buffer, and 30 mL collagenase buffer containing collagen II, collagen IV, and proteins. The heart was then pulled apart using forceps and filtered through a 100-μm strainer to remove large tissue debris.

After three rounds of gravity sedimentation, the adult cardiomyocytes were resuspended in culture media. After enzymatic isolation, the cardiomyocytes were seeded onto laminin-coated coverslips (2×10^4 cardiomyocytes/coverslip) in plating media (M199 culture media [Thermo-Fisher Scientific], penicillin-streptomycin [Gibco], and 5% fetal bovine serum [Gibco]) and cultured at 37°C for 2 h to facilitate attachment. Then, the cardiomyocytes were cultured in M199 culture media containing 0, 0.1, 0.5, 1, 5, 10, 20, 50, or 100 mM Na-β-OHB (Sigma-Aldrich) (Na-β-OHB media). The cardiomyocytes were cultured in these media for 12 h before hypoxia was induced. The cardiomyocytes used in the roxadustat experiments were cultured in Na-β-OHB media before hypoxia as well as treatment with 50 μM roxadustat (Dalian Meilun Biotechnology). To simulate hypoxia, cardiomyocytes were placed in a hypoxia incubator maintained at 37°C, 1% O_2, and 5% CO_2 for 12 h.

Human AC16 cardiomyocytes were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco).

2.3 Animals and surgical procedures

Male C57BL/6 mice (6–8 weeks old) were randomly assigned to be fed a normal chow diet (CD; 11 kcal% fat, 20 kcal% protein, 69 kcal% carbohydrates) or a low-carbohydrate, low-protein ketogenic diet (KD; 93.5 kcal% fat, 4.7 kcal% protein, 1.8 kcal% carbohydrates; Dyets #HF93.5) for 4 weeks. After the 4 weeks, permanent ligation of the left anterior descending artery (LAD) was performed to induce MI in the mice. Echocardiographic analysis was performed 1 day after MI surgery. Mice with LVEFs between 30–45% were used for further experiments and were continued

on their respective CD or KD. Echocardiographic analysis was repeated 4 weeks later. Sham mice underwent similar surgical procedures without LAD ligation.

All experimental procedures were conducted in accordance with the animal welfare guidelines. All animal protocols were approved by the Animal Care and Use Committee of the Zhongshan Hospital, Fudan University.

2.4 Statistics

The Student's t-test was used to analyze parametric variables between two groups, and one-way analysis of variance (ANOVA) with a post-hoc test was used to compare parametric variables among three or more groups. Linear regression and Pearson correlation analyses were used to analyze the relationship between β-OHB levels and patient clinical outcomes. All values were presented as the mean ± standard deviation (SD), and n was used to refer to the sample size. A P-value <0.05 was considered statistically significant.

2.5 Experimental setup

Detailed descriptions of the experimental setup and chemicals, including quantitative PCR (qPCR) analysis, western blot analysis, immunofluorescence, ketone body assay, intracellular ATP, live/dead cell staining and imaging, lactate dehydrogenase (LDH) and Cell Counting Kit-8 (CCK-8) assays, echocardiography, 2,3,5-triphenyltetrazolium chloride (TTC) staining, plasmid construction and transfection and RNA interference, are given in the Supplementary Methods.

3 Results

3.1 β-OHB levels were increased in response to MI in humans and mice

Baseline characteristics, including age and the proportion of men to women, were not significantly different between the healthy volunteers and acute MI patients (Supplemental Table S1). The serum β-OHB levels were significantly higher in the acute MI patients (Figure 1a), indicating the *in vivo* formation of β-OHB during acute MI. The serum β-OHB levels were negatively correlated with LVEFs (Figure 1b) and were positively correlated with NT-proBNP levels (Figure 1c) in acute MI patients, indicating that β-OHB may be related to severe myocardial injury.

The serum β-OHB levels were also significantly higher in the MI mice compared with the sham mice (Figure 1d) and were negatively correlated with LVEFs (Figure 1e). Additionally, the myocardial β-OHB levels were significantly increased in the mice 3 days after MI surgery (Figure 1f).

3.2 β-OHB enhanced cardiomyocyte death under hypoxic conditions

The live/dead cell assay was used to determine whether β-OHB influenced the death of cardiomyocytes in response to 12 h of hypoxia. Cardiomyocytes were cultured with media containing different doses of β-OHB. Under normoxic conditions, treatment

with 1 mM, 10 mM, 20 mM, or 50 mM β-OHB did not affect the percent of live cells. However, after 12 h of hypoxia, β-OHB decreased the percent of live cells in a dose-dependent manner (Figure 2a, b). Cell survival was determined using the CCK-8 assay, and results similar to those of the live/dead cell assay were observed (Figure 2c). As expected, β-OHB treatment increased necrotic cell death, as demonstrated by increased LDH release into the culture media (Figure 2d).

3.3 β-OHB altered glycolysis in cardiomyocytes under hypoxic conditions

Our results confirmed that the expression of a glycolytic glucose transporter, GLUT1, was upregulated in cardiomyocytes after 12 h of hypoxia. Accordingly, both the mRNA and protein expression levels of an important glycolytic regulator, PFKFB3, as well as other glycolytic rate-limiting enzymes, including HK2, PKM1, and LDHA, were all upregulated after 12 h of hypoxia compared with the control normoxia group (Figure 3a–f, h–k).

To further demonstrate the influence of β-OHB on glycolysis in cardiomyocytes, the cells were treated with different doses of β-OHB before undergoing 12 h of hypoxia. Western blot analysis showed that the protein expression levels of GLUT1, HK2, PFKFB3, PKM1, and LDHA decreased in response to β-OHB treatment in a dose-dependent manner compared with the vehicle control group (Figure 3a–f), suggesting decreased glucose absorption and glycolysis in the β-OHB-treated cardiomyocytes under hypoxic conditions. Furthermore, immunofluorescence revealed low levels of GLUT1 expression in the nuclei and cytoplasm of the β-OHB-treated cardiomyocytes (Figure 3g). Similar to the Western blot results, the mRNA expression levels of the glycolytic enzymes were also decreased in a dose-dependent manner in response to β-OHB treatment under hypoxic conditions (Figure 3h–k).

Because GLUT1 expression was decreased in response to β-OHB treatment, we measured the glucose concentrations in the culture media as an indicator of cellular glucose uptake. The media glucose concentrations proportionally increased with the dose of β-OHB, indicating that β-OHB decreased cellular glucose transportation (Figure 3l). Altogether, these results demonstrated that β-OHB inhibited cellular glucose uptake and glycolysis under hypoxic conditions.

3.4 β-OHB downregulated HIF-1α in cardiomyocytes in a dose-dependent manner under hypoxic conditions

Next, the expression of glycolysis regulator HIF-1α was analyzed. Consistent with our observation of decreased glycolysis in the β-OHB-treated cardiomyocytes, HIF-1α expression was also significantly downregulated in these cells. Cardiomyocytes exhibited significantly higher levels of HIF-1α in response to hypoxia compared with normoxia. However, high-dose β-OHB treatment resulted in decreased HIF-1α expression under hypoxic conditions (Figure 4a–d). High levels of HIF-1α were detected in the nuclei of cardiomyocytes after 12 h of hypoxia, thus indicating HIF-1α pathway activation. However, these levels were significantly reduced with β-OHB

treatment (Figure 5a, b). HIF-1α mRNA levels were also elevated in the cardiomyocytes in response to hypoxia, but these levels remained unchanged after β-OHB treatment (Figure 5e), indicating that β-OHB decreased HIF-1α at the posttranslational level.

Additionally, AC16 human cardiomyocyte cells were transfected with HIF-1α siRNA, which resulted in reduced HIF-1α and GLUT1 expression. This result was similar to what was observed in response to β-OHB treatment (Figure 4f, h). Furthermore, HIF-1α overexpression in AC16 cells suppressed the effects of β-OHB treatment (Figure 4g, i). These results indicated that β-OHB reduced cellular glucose uptake via the HIF-1α signaling pathway.

3.5 Roxadustat partially reversed the effects of β-OHB in cardiomyocytes under hypoxic conditions

A HIF prolyl hydroxylase inhibitor, roxadustat (Roxa), was used in further experiments to determine if it could reverse the effects of β-OHB by increasing HIF-1α levels. Western blot and immunofluorescence analyses were used to examine the expression of both HIF-1α and GLUT1 and confirmed that roxadustat administration partially reversed the effects of β-OHB. Immunofluorescence results showed that roxadustat administration partially reversed the effects of β-OHB (Figure 5a, b). Additionally, Western blot confirmed elevated HIF-1α levels after roxadustat administration in cardiomyocytes under normoxic conditions. Roxadustat also resulted in elevated HIF-1α expression in cardiomyocytes under hypoxic conditions after β-OHB treatment (Figure 5c, d).

Next, GLUT1 expression was evaluated after roxadustat administration as a downstream indicator of HIF-1α activity. Western blot and immunofluorescence analyses both revealed that roxadustat administration increased GLUT1 expression in cardiomyocytes treated with 10 mM β-OHB under hypoxic conditions (Figure 5c, e, f, h).

Furthermore, the live/dead cell assay showed that roxadustat administration substantially diminished cardiomyocyte death in response to β-OHB treatment under hypoxic conditions (Figure 5g, i).

Then VHL expression was evaluated after roxadustat administration to explore the mechanism of HIF-1α degradation. Western blot analyses revealed that β-OHB upregulated VHL in cardiomyocytes in a dose-dependent manner under hypoxic conditions and roxadustat administration decreased VHL expression in cardiomyocytes treated with 10 mM β-OHB under hypoxic conditions (Figure 5j, k).

3.6 β-OHB metabolisms were obscured under hypoxic conditions in cardiac myocytes.

Upon entering the cell, ketone bodies rapidly form acetyl-CoA via a series of reactions catalyzed by BDH1, OXCT1, and mitochondrial acetyl-CoA acetyltransferase 1 (ACAT1), as shown in Figure 6a. The levels of β-OHB were first

detected to observe its metabolism under hypoxic conditions in cardiomyocytes. Under normoxic conditions, β-OHB treatment did not result in elevated β-OHB levels in cardiomyocytes. However, under hypoxic conditions, β-OHB treatment resulted in increased intracellular β-OHB levels in cardiomyocytes in a dose-dependent manner (Figure 6b). Furthermore, the decreased expression of transporter SLC16A1 and the enzyme BDH1 were observed after β-OHB treatment, suggesting a low ketone body metabolic capacity in the cardiomyocytes under hypoxic conditions (Figure 6c, d).

Although β-OHB is an energy substrate, there was no change in ATP production in response to β-OHB treatment (Figure 6e). In contrast to the increased ketone utilization previously reported in end-stage heart failure[22] and HFpEF (heart failure with preserved ejection fraction)[23], the β-OHB treatment did not contribute to cardiomyocyte ATP production under hypoxic conditions, suggesting that the cardiomyocytes did not use ketone bodies as an alternative energy source.

3.7 KD exacerbated cardiac dysfunction in mice after MI surgery

Mice were fed either a KD or control CD for 4 weeks prior to MI surgery (Supplemental Table S2). The KD-fed mice exhibited higher plasma β-OHB levels compared with the CD-fed mice (Figure 7a). Infarct sizes and cardiac function were evaluated 4 weeks after MI surgery. Compared with the CD-fed mice, infarct size was larger in the KD-fed mice (Figure 7b, c) and cardiac function was worse in the KD-fed mice (Figure 7d–f) at 4 weeks post MI surgery. These data suggested that KD enhanced MI-induced cardiac injury.

4 Discussion

In this study, we demonstrated that plasma β-OHB levels were elevated in acute MI patients compared with healthy control volunteers, and increased β-OHB levels were also correlated with disease progression. Furthermore, β-OHB treatment resulted in the increased death of adult mouse cardiomyocytes in response to hypoxia as well as larger infarct sizes and deteriorated cardiac function in mice after MI surgery. Metabolic characteristics can influence the function and fate of cardiomyocytes[24]. Under hypoxic conditions, cardiomyocytes have been shown to utilize anaerobic glycolysis instead of oxidative phosphorylation to meet their energy demands and to reduce damage[25]. Here, we showed that β-OHB treatment decreased glycolysis in cardiomyocytes under hypoxic conditions and that downregulated HIF-1α was a key cause of this effect. The HIF prolyl hydroxylase inhibitor, roxadustat, had a therapeutic effect in the β-OHB-treated cardiomyocytes under hypoxic conditions, which was mostly due to increased levels of HIF-1α and GLUT1. In contrast to the alterative ketone utilization observed in advanced-stage heart failure, increased β-OHB utilization was not observed in cardiomyocytes under hypoxic conditions. However, intracellular β-OHB accumulation occurred in these cardiomyocytes and resulted in HIF-1α destabilization (Figure 8).

Elevated β-OHB concentrations have been found to be significantly higher in the hearts of patients with arrhythmogenic cardiomyopathy than in non-diseased donor

hearts[26]. Furthermore, increased concentrations of serum ketone bodies, but decreased concentrations of myocardial ketone bodies, have been detected in patients with dilated myocardial disease[27]. In our study, we demonstrated that β-OHB levels were increased in both the serum of acute MI patients as well as the cardiomyocytes of mice after MI surgery; however, β-OHB metabolism was not increased in cardiomyocytes under hypoxic conditions. This observation is obscure because it is contradictory to the alterative ketone utilization observed in advanced-stage heart failure. Previous research has shown that hypertrophied and failing hearts undergo regulatory gene reprogramming to increase the uptake and oxidation of ketone bodies[23, 28]. Specifically, the expression of BDH1 and the transporter SLC16A1 have been shown to be increased during heart failure[22]. However, we found that cardiomyocytes exhibited decreased expression levels of BDH1 and SLC16A1 in response to hypoxia with β-OHB treated environment.

It is known that enhanced myocardial cell glucose metabolism increases cardiac tolerance to ischemic injury[29]. In this study, glycolysis increased in cardiomyocytes in response to hypoxia; however, elevated levels of β-OHB decreased glycolysis. Therefore, alterations in this metabolic mode due to elevated β-OHB levels may contribute to the decreased adaptability of cardiomyocytes in response to hypoxia. These observations support the hypothesis that ketone body metabolism can regulate the energy source selection of cardiomyocytes under hypoxic conditions.

It is known that HIF-1α regulates the expression of key glycolytic genes, including glucose transporters *GLUT1* and *GLUT4*, *LDH*, phosphoglycerate kinase (*PGK1*), glucose-6-phosphate isomerase (*GPI*), and *PFK1[30]*. Our data showed that β-OHB decreased the expression of HIF-1α in cardiomyocytes under hypoxic conditions and also downregulated the expression of the glycolysis-associated proteins, GLUT1, PKM1, and LDHA. Furthermore, the expression of HIF-1α decreased after β-OHB treatment, and this effect was partially reversed by roxadustat. Therefore, our data collectively indicate that normal glycolysis in cardiomyocytes may be partially regulated by β-OHB via its regulation of HIF-1α. Because HIF-1α is essential for cellular and systemic responses to low oxygen availability, reduced HIF-1α levels may therefore be responsible for the increased death of cardiomyocytes under hypoxic conditions[31].

HIF-1 plays a dominant role during cellular adaptation in response to changes in oxygen availability. HIF-1 comprises two subunits: the hypoxia-regulated α subunit, HIF-1α, and the oxygen-insensitive β subunit, HIF-1β[32]. Under normoxic conditions, HIF-1α is rapidly degraded via the von Hippel-Lindau tumor suppressor (pVHL)-mediated ubiquitin-proteasome pathway[33-35]. The association of pVHL and HIF-1α under normoxic conditions is triggered by the posttranslational hydroxylation of prolines (Pro402 and Pro564) by specific HIF prolyl hydroxylases[36]. In our study, β-OHB decreased the protein level of HIF-1α in the cardiomyocytes after hypoxia, but the mRNA levels remained unchanged and stabilization of HIF by roxadustat through inhibiting the prolyl hydroxylases can partially reversed the effects of β-OHB. So the mechanism of declining HIF-1α may

be partly prolyl hydroxylases-VHL dependent. Furthermore our data collectively indicate that β-OHB metabolism was obscured and β-OHB accumulation in cardiomyocytes under hypoxic, so the stability of HIF-1α may be modulated by β-OHB itself not the productions of its metabolism.

In the current study, we revealed that the β-OHB/HIF-1α/glycolysis pathway was associated with cardiac injury under hypoxic conditions. However, several questions remain. First, roxadustat only partially reversed the decreased expression of GLUT1 in response to β-OHB treatment under hypoxic conditions, suggesting that it did not completely reverse the effects of the β-OHB-mediated HIF-1α protein degradation. Further studies are required to reveal if β-OHB independently regulates HIF-1α stability. Second, in contrast to what we observed under hypoxic conditions, we observed increased levels of the glycolytic metabolites, PFKFB3, HK2, and PKM1, in β-OHB-treated cardiomyocytes under normoxic conditions. Nonetheless, our goal of this study was to confirm the contribution of the β-OHB/HIF-1α/glycolysis pathway to hypoxic injury. Therefore, we did not investigate the mechanism of these β-OHB-mediated effects under normoxic conditions. Third, besides glycolysis, HIF-1α has many downstream effects. Further studies are required to reveal the regulatory effects of β-OHB on myocardial injury in response to hypoxia. Last, we validated that β-OHB metabolism inhibited HIF-1α-dependent glycolysis in cardiomyocytes under hypoxic conditions. However, the mechanism of this β-OHB-mediated regulation of HIF-1α remains unclear and warrants further investigation.

In conclusion, our results demonstrated that increased β-OHB levels may be maladaptive to cardiomyocytes under hypoxic conditions. Therefore, patients with high levels of β-OHB may experience extensive injury due to ischemic heart disease, and a KD should not be recommended for individuals who have an increased risk of MI.

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Figure legends

Fig. 1 β-OHB levels were increased in response to MI in humans and mice. **a** Plasma β-OHB levels in healthy volunteers (n = 32) or AMI patients (n = 45), **b** Linear regression and Pearson correlation analysis of β-OHB level and LVEF, **c** Linear regression and Pearson correlation analysis of β-OHB level and NTproBNP, **d** Mice with similar body weight were randomly assigned to the sham-treated group (n = 13) or MI group (n = 11), plasma β-OHB level was significantly increased, **e** Linear regression and Pearson correlation analysis of plasma β-OHB level and LVEF in mice of MI group, **f** Myocardial β-OHB was significantly increased in mouse 4 weeks after MI. ***P<0.001, ****P<0.0001

Fig. 2 β-OHB enhanced cardiomyocyte death under hypoxic conditions. **a, b** Live (green) or dead (red) CMs under normoxia or 12 h of hypoxia in the different dose of β-OHB treated environment, **c** CCK-8 cell survival detection of CMs under normoxia or 12 h of hypoxia in the different dose of β-OHB treated environment, **d** LDH release in the culture media under normoxia or 12 h of hypoxia in the different dose of β-OHB treated environment **P<0.01, ****P<0.0001 vs. hypoxia 0 h. Scale bars, 50μm.

Fig. 3 β-OHB altered glycolysis in cardiomyocytes under hypoxic conditions. **a-f** Representative western blot of enzymes involved in myocardial glycolysis in CMs cultured with β-OHB at 0 mM, 10 mM, 20 mM, or 50 mM under normoxia or hypoxia, **g** Immunofluorescence imaging showing GLUT1 expression in cardiomyocytes cultured with β-OHB under normoxia or hypoxia, **h-k** qPCR of enzymes involved in myocardial glycolysis in CMs cultured with β-OHB under normoxia or hypoxia, **l** Glucose in the culture media which cardiac myocytes cultured with β-OHB at different concentrations under normoxia or hypoxia. *P<0.05,

**P<0.01, ***P<0.001, ****P<0.0001.

Fig. 4 β-OHB downregulated HIF-1α in cardiomyocytes in a dose-dependent manner under hypoxic conditions. **a-d** Western blot of HIF-1α in CMs cultured with β-OHB at different concentrations under normoxia or hypoxia, **e** PCR of HIF-1α in CMs cultured with β-OHB at different concentrations under normoxia or hypoxia, f and h, Western blot of GLUT1 and HIF-1α in HIF-1α siRNA transfection AC16 cells, **g and i** Western blot of GLUT1 and HIF-1α in HIF-1α overexpress (HIF-1α OE) AC16 cells.

Fig. 5 Roxadustat partially reversed the effects of β-OHB in cardiomyocytes under hypoxic conditions. **a** Immunofluorescence imaging showing HIF-1α expression in cardiomyocytes cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12 h, **b** Quantitation of HIF-1α in the nuclei of CMs cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12 h, Western blot of HIF-1α in CMs cultured with 50 μM roxadustat and β-OHB at different concentrations under normoxia or hypoxia, **c, d** and **e** Western blot of HIF-1α and GLUT1 expression in cardiomyocytes cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12 h, **f** Immunofluorescence imaging of GLUT1 in CMs cultured with 50 μM roxadustat and β-OHB at different concentrations under normoxia or hypoxia, **g** Live (green) or dead (red) CMs cultured with 50 μM roxadustat and β-OHB at different concentrations under normoxia or hypoxia, **h** Quantitation of GLUT1 in CMs cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12h, **i** Quantitation of live cell percent in CMs cultured with 50 μM roxadustat and β-OHB at different concentrations under normoxia or hypoxia, **j, k** Western blot of VHL, PHD2 and HIF-1α expression in cardiomyocytes cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12 h.* P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Fig. 6 β-OHB metabolisms were obscured under hypoxic conditions in cardiac myocytes. **a** Diagrammatic drawing of ketone metabolism in the mitochondria. BDH1: β-OHB dehydrogenase; OXCT: succinyl-CoA:3-oxoacid-CoA transferase; ACAT1: acetyl-CoA acetyltransferase 1; TCA: tricarboxylic acid cycle, **b** Detected concentration of β-OHB in CMs cultured with β-OHB at different concentrations under normoxia or hypoxia, **c** and **d** Western blot of SLC16A1, BDH1, OXCT1, OXCT2 and ACTA1 which are β-OHB transporters and the key enzymes for ketone body metabolism in CMs cultured with β-OHB at different concentrations under normoxia or hypoxia, **e** ATP in CMs cultured with β-OHB at different concentrations under normoxia or hypoxia. * P<0.05, **P<0.01, ***P<0.001

Fig. 7 KD exacerbated cardiac dysfunction in mice after MI surgery. **a** The level of β-OHB in plasma of model mice, **b** Triphenyl tetrazolium chloride staining and Masson's trichrome staining 4weeks post-myocardial infarction, n=8 per group, **c** The infarct area was quantified to the left ventricular area, **d, e** and **f** left ventricular ejection fraction (LVEF) and fractional shorting (FS) were measured by echocardiography, n= 9–10 per group. **P<0.01

Fig.8 Schematic diagram of how β-OHB exacerbates hypoxic/ischemic myocardial injury. Under hypoxia, β-OHB accumulation occurred in the cardiomyocytes and resulted in HIF-1α destabilization through regulated PHD/VHL. Then β-OHB induced more cardiomyocyte death by decreasing HIF-1α and the downstream GLUT1 and the expression of key glycolytic genes.

Figures

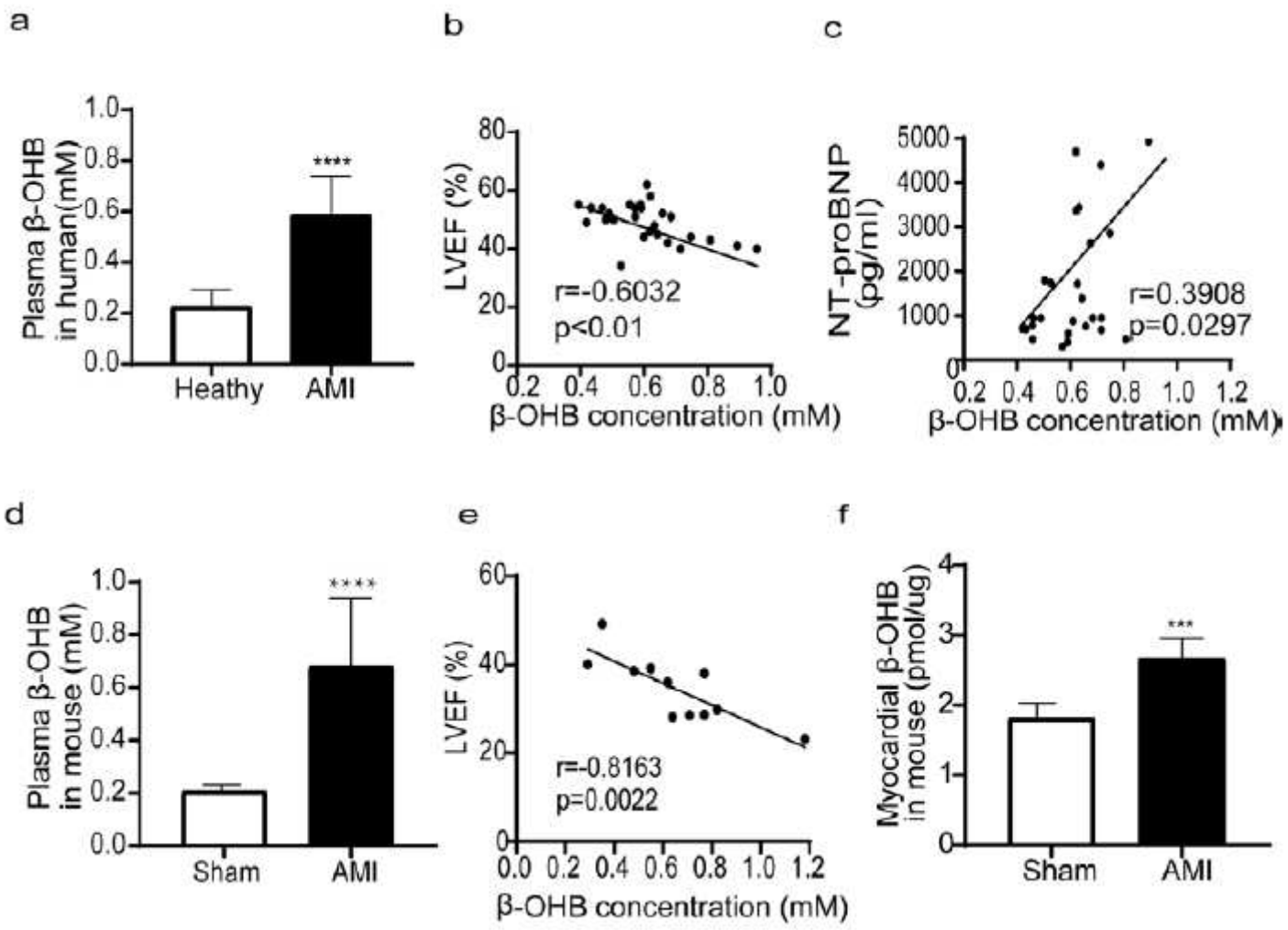

Figure 1

β-OHB levels were increased in response to MI in humans and mice. a Plasma 19 β-OHB levels in healthy volunteers (n = 32) or AMI patients (n = 45), b Linear 20 regression and Pearson correlation analysis of β-OHB level and LVEF, c Linear 21 regression and Pearson correlation analysis of β-OHB level and NTproBNP, d Mice 22 with similar body weight were randomly assigned to the sham-treated group (n = 13) 23 or MI group (n = 11), plasma β-OHB level was significantly increased, e Linear 24 regression and Pearson correlation analysis of plasma β-OHB level and LVEF in mice 25 of MI group, f Myocardial β-OHB was significantly increased in mouse 4 weeks after 26 MI. ***P<0.001, ****P<0.0001

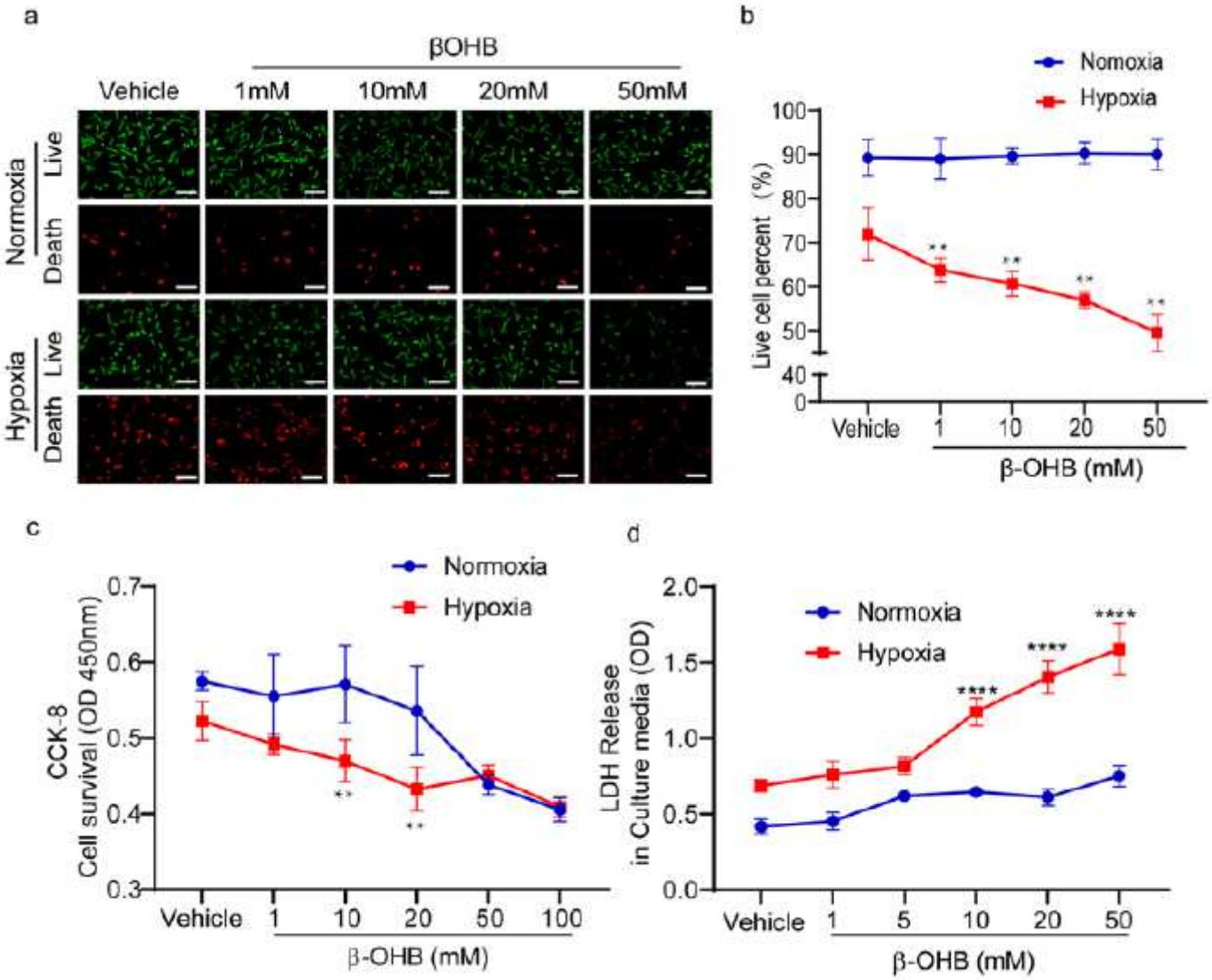

Figure 2

β-OHB enhanced cardiomyocyte death under hypoxic conditions. a, b Live 28 (green) or dead (red) CMs under normoxia or 12 h of hypoxia in the different dose of 29 β-OHB treated environment, c CCK-8 cell survival detection of CMs under normoxia 30 or 12 h of hypoxia in the different dose of β-OHB treated environment, d LDH release 31 in the culture media under normoxia or 12 h of hypoxia in the different dose of 32 β-OHB treated environment **P<0.01, ****P<0.0001 vs. hypoxia 0 h. Scale bars, 33 50μm.

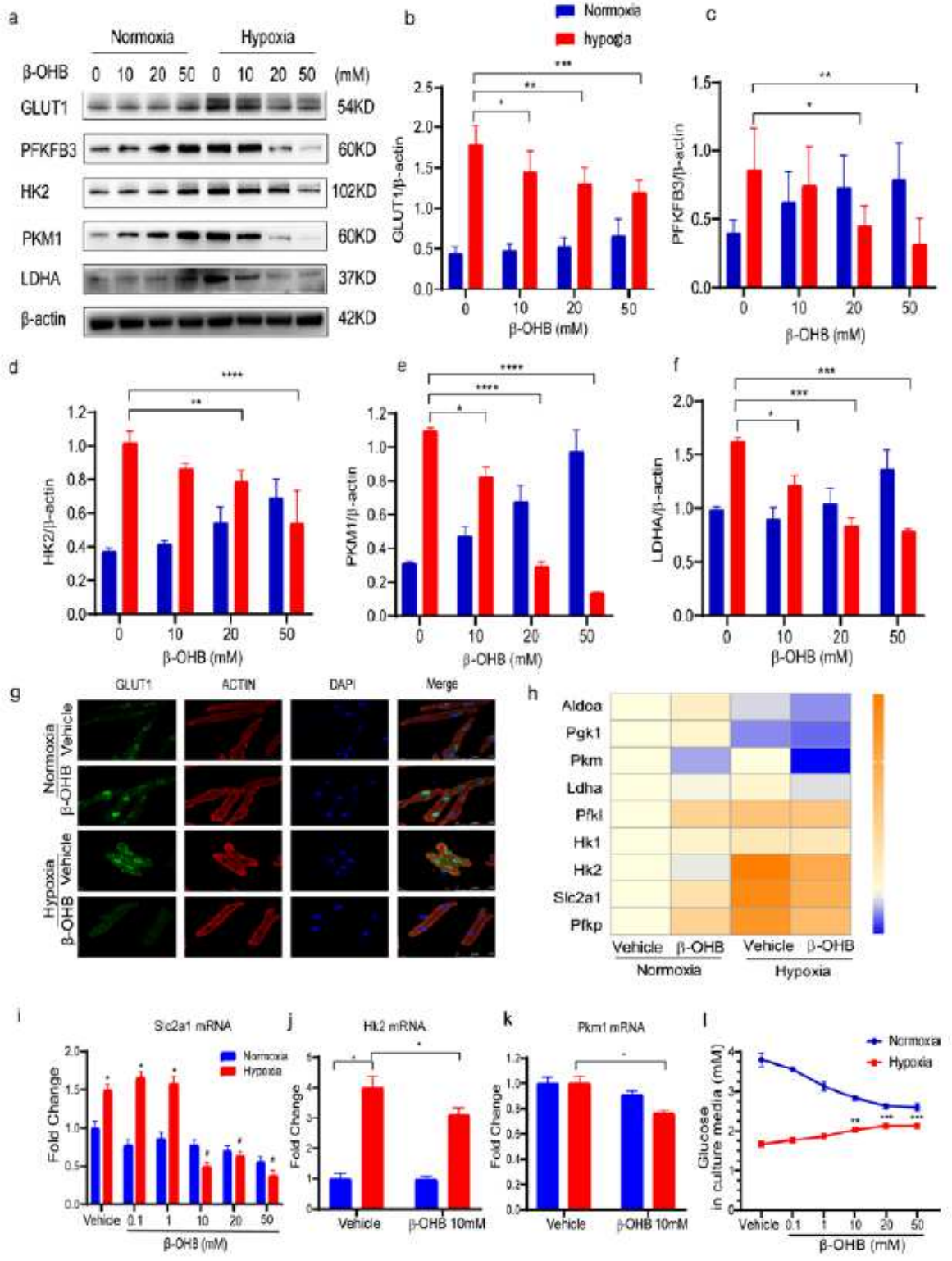

Figure 3

β-OHB altered glycolysis in cardiomyocytes under hypoxic conditions. a-f 35 Representative western blot of enzymes involved in myocardial glycolysis in CMs 36 cultured with β-OHB at 0 mM, 10 mM, 20 mM, or 50 mM under normoxia or 37 hypoxia, g Immunofluorescence imaging showing GLUT1 expression in 38 cardiomyocytes cultured with β-OHB under normoxia or hypoxia, h-k qPCR of 39 enzymes involved in myocardial glycolysis in CMs cultured with β-OHB under 40 normoxia or hypoxia, l Glucose in the culture

media which cardiac myocytes cultured 41 with β-OHB at different concentrations under normoxia or hypoxia. *P<0.05,**P<0.01, ***P<0.001, ****P<0.0001.

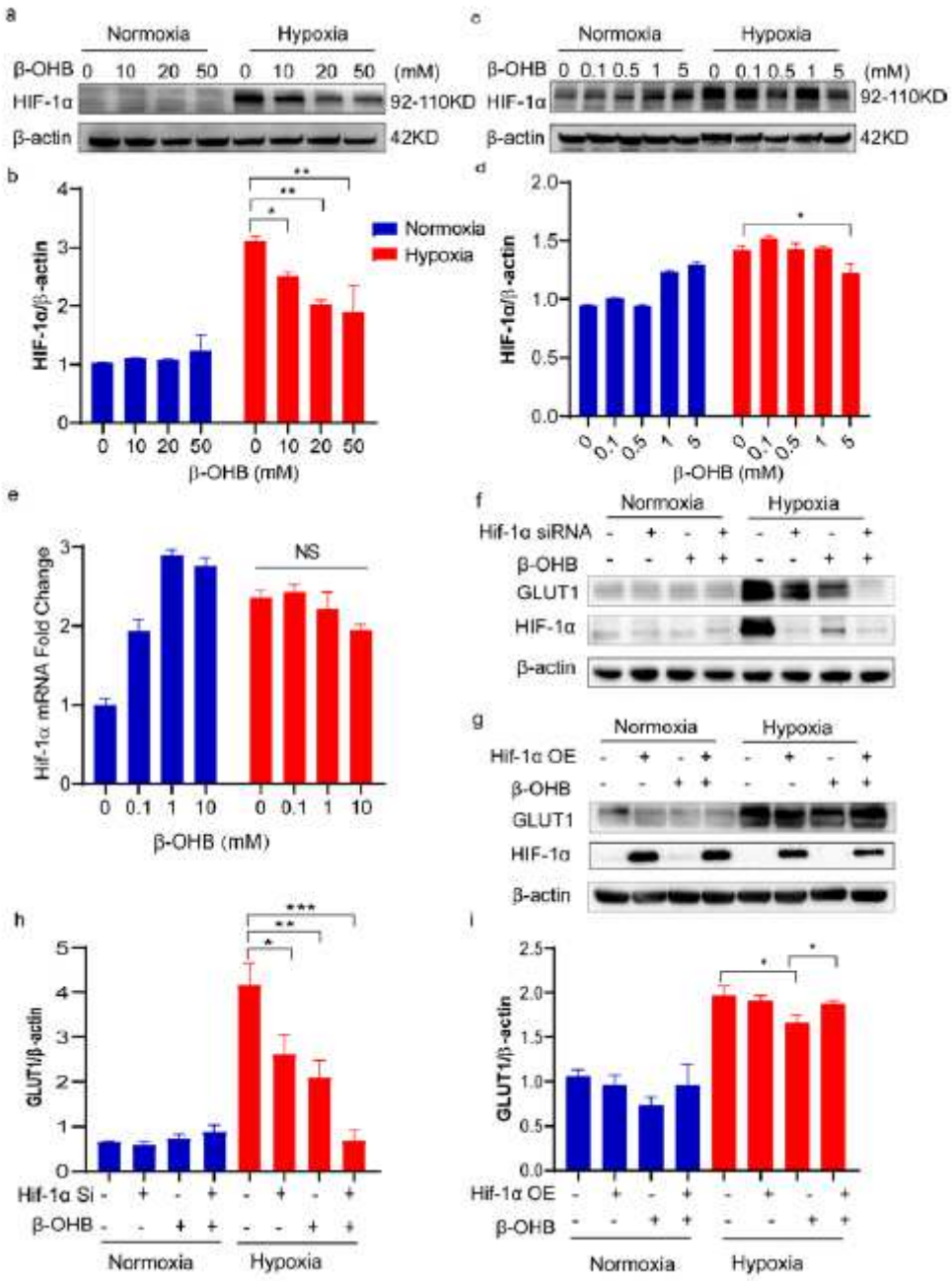

Figure 4

β-OHB downregulated HIF-1α in cardiomyocytes in a dose-dependent manner 2 under hypoxic conditions. a-d Western blot of HIF-1α in CMs cultured with β-OHB at 3 different concentrations under normoxia or hypoxia, e PCR of HIF-1α in CMs 4 cultured with β-OHB at different concentrations under normoxia or hypoxia, f and h, 5 Western blot of GLUT1 and HIF-1α in HIF-1α siRNA transfection AC16 cells, g and 6 i Western blot of GLUT1 and HIF-1α in HIF-1α overexpress (HIF-1α OE) AC16 7 cells.

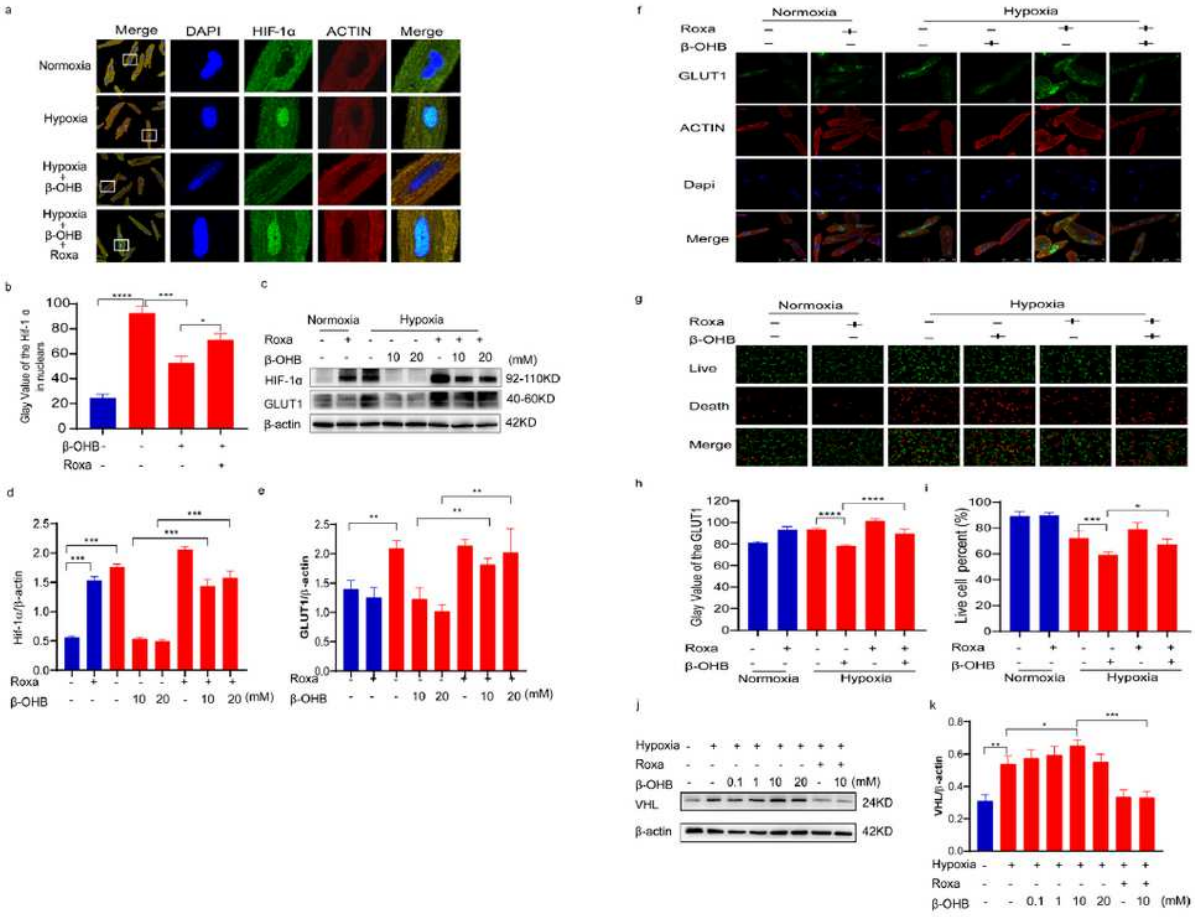

Figure 5

Roxadustat partially reversed the effects of β-OHB in cardiomyocytes under 9 hypoxic conditions. a Immunofluorescence imaging showing HIF-1α expression in 10 cardiomyocytes cultured with 50 μM roxadustat and β-OHB under normoxia or 11 hypoxia for 12 h, b Quantitation of HIF-1α in the nuclei of CMs cultured with 50 μM 12 roxadustat and β-OHB under normoxia or hypoxia for 12 h, Western blot of HIF-1α in 13 CMs cultured with 50 μM roxadustat and β-OHB at different concentrations under 14 normoxia or hypoxia, c, d and e Western blot of HIF-1α and GLUT1 expression in 15 cardiomyocytes cultured with 50 μM roxadustat and β-OHB under normoxia or 16 hypoxia for 12 h, f Immunofluorescence imaging of GLUT1 in CMs cultured with 50 17 μM roxadustat and β-OHB at different concentrations under normoxia or hypoxia, g 18 Live (green) or dead (red) CMs cultured with 50 μM roxadustat and β-OHB at 19 different concentrations under normoxia or hypoxia, h Quantitation of GLUT1 in 20 CMs cultured with 50 μM roxadustat and β-OHB under normoxia or hypoxia for 12h, 21 i Quantitation of live cell percent in CMs cultured with 50 μM roxadustat and β-OHB 22 at different concentrations under normoxia or hypoxia, j, k Western blot of VHL, 23 PHD2 and HIF-1α expression in cardiomyocytes cultured with 50 μM roxadustat and 24 β-OHB under normoxia or hypoxia for 12 h.* P<0.05, **P<0.01, ***P<0.001, 25 ****P<0.0001

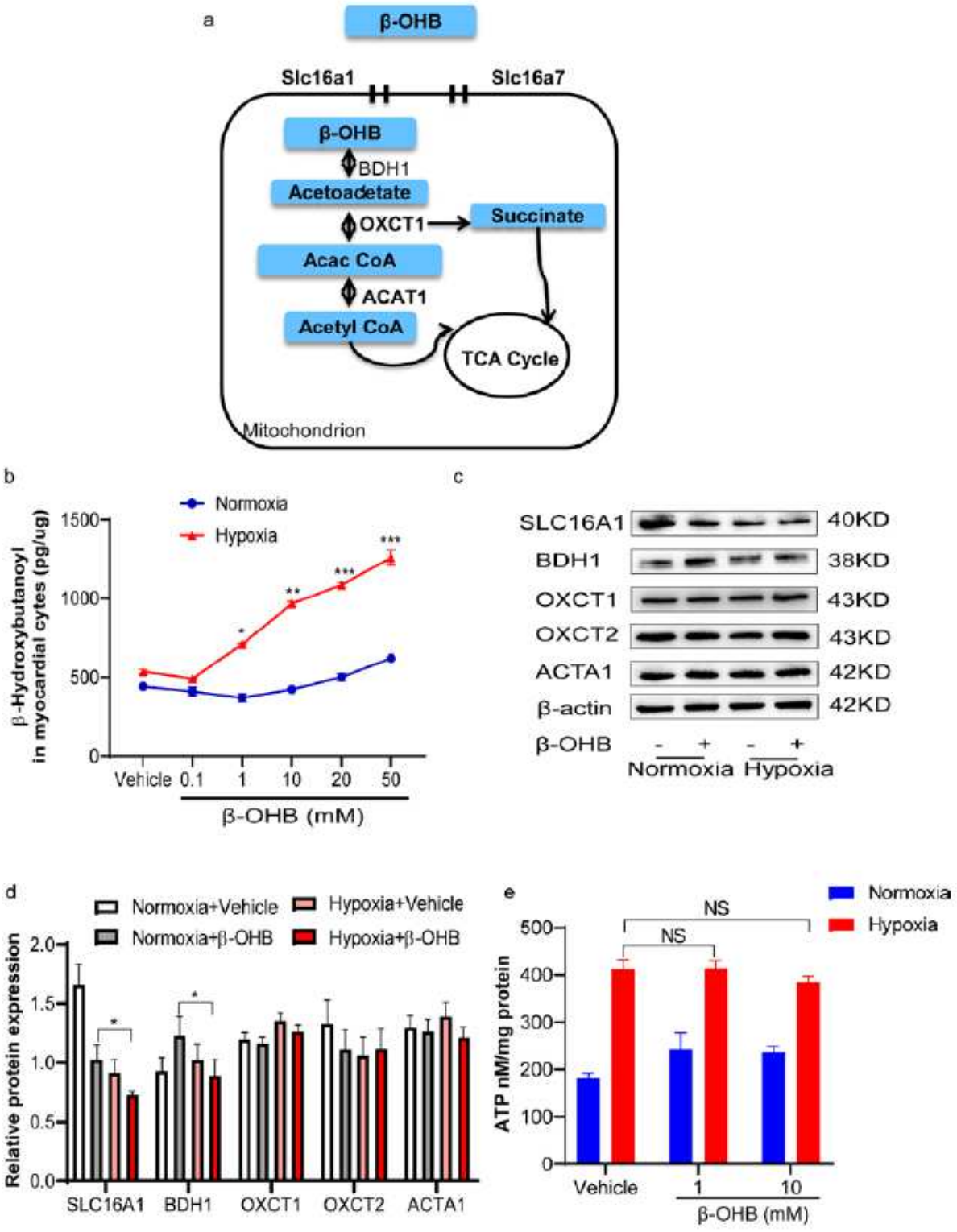

Figure 6

β-OHB metabolisms were obscured under hypoxic conditions in cardiac 27 myocytes. a Diagrammatic drawing of ketone metabolism in the mitochondria. BDH1: 28 β-OHB dehydrogenase; OXCT: succinyl-CoA:3-oxoacid-CoA transferase; ACAT1: 29 acetyl-CoA acetyltransferase 1; TCA: tricarboxylic acid cycle, b Detected 30 concentration of β-OHB in CMs cultured with β-OHB at different concentrations 31 under normoxia or hypoxia, c and d Western blot of SLC16A1, BDH1, OXCT1, 32 OXCT2 and ACTA1 which are β-

OHB transporters and the key enzymes for ketone 33 body metabolism in CMs cultured with β-OHB at different concentrations under 34 normoxia or hypoxia, e ATP in CMs cultured with β-OHB at different concentrations 35 under normoxia or hypoxia. * P<0.05, **P<0.01, ***P<0.001

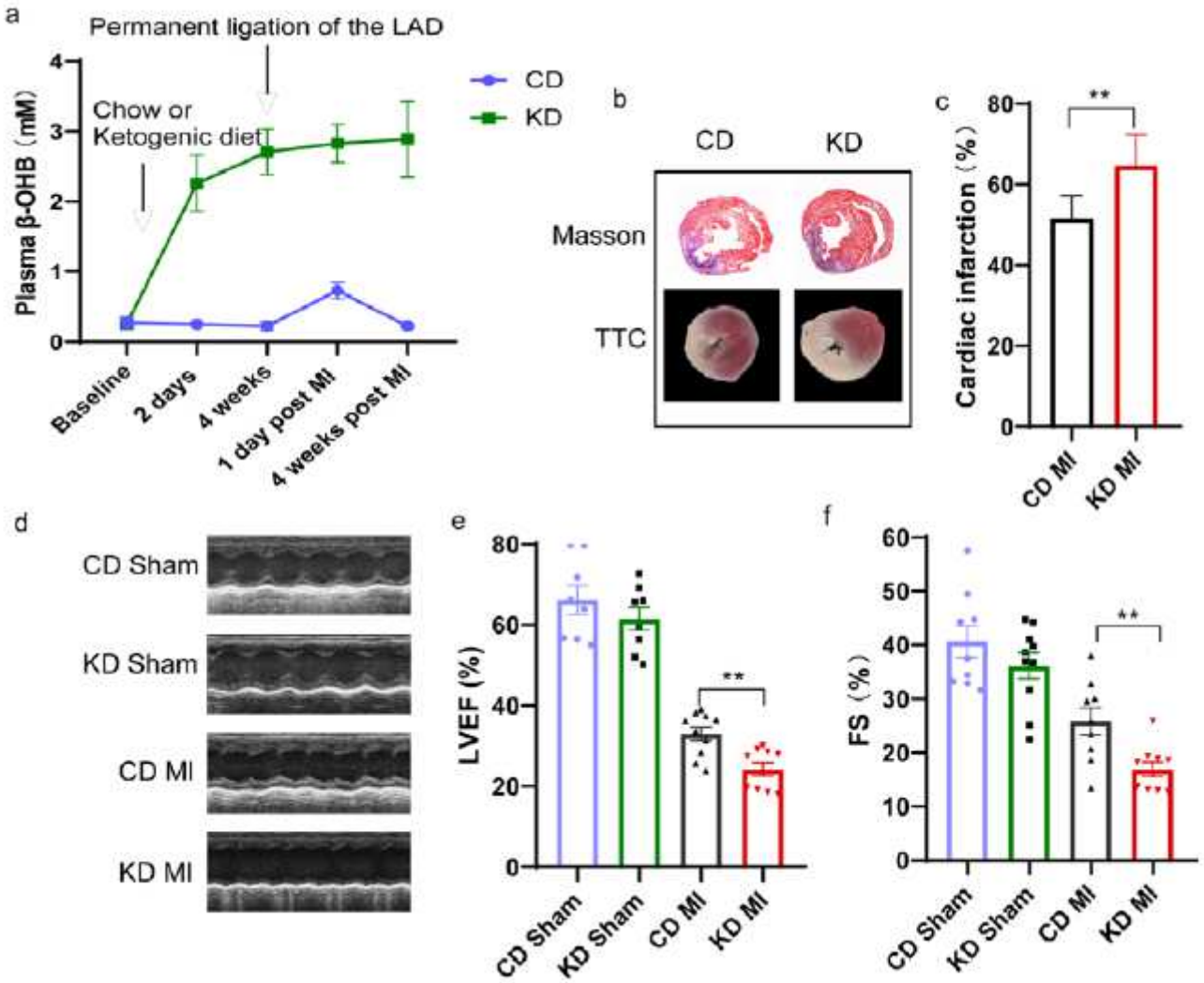

Figure 7

KD exacerbated cardiac dysfunction in mice after MI surgery. a The level of 37 β-OHB in plasma of model mice, b Triphenyl tetrazolium chloride staining and 38 Masson's trichrome staining 4weeks post-myocardial infarction, n=8 per group, c The 39 infarct area was quantified to the left ventricular area, d, e and f left ventricular 40 ejection fraction (LVEF) and fractional shorting (FS) were measured by 41 echocardiography, n= 9–10 per group. **P<0.01

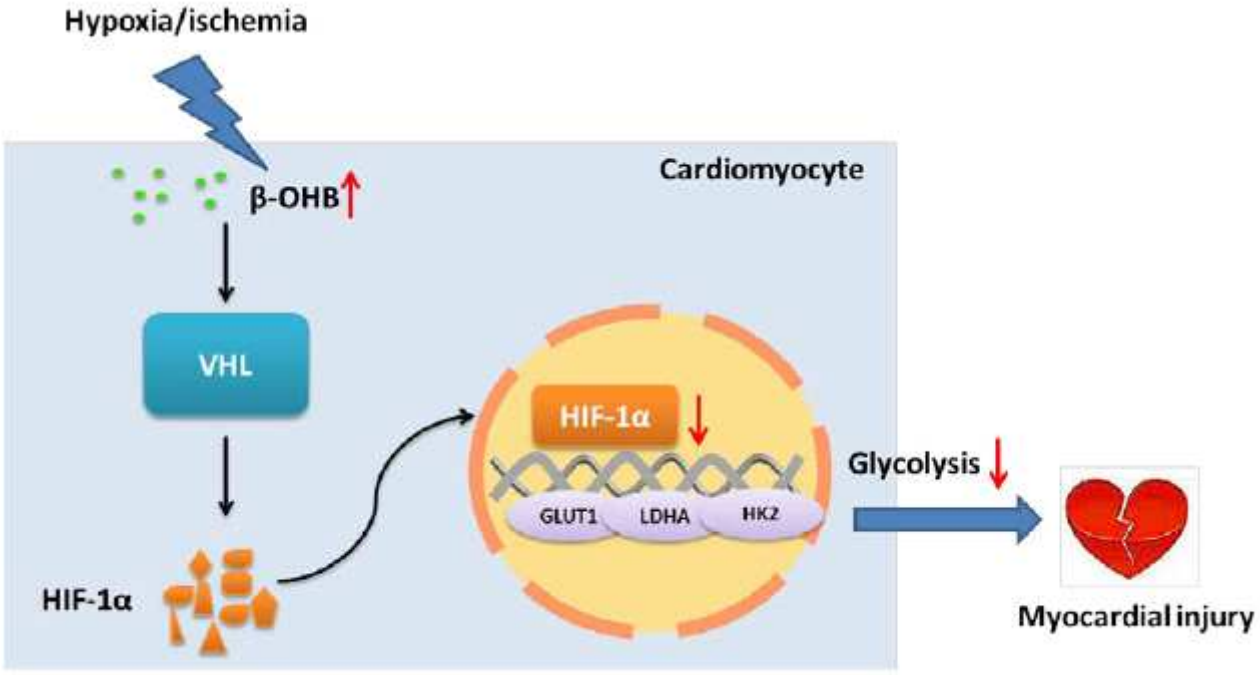

Figure 8

Schematic diagram of how β-OHB exacerbates hypoxic/ischemic myocardial 1 injury. Under hypoxia, β-OHB accumulation occurred in the cardiomyocytes and 2 resulted in HIF-1α destabilization through regulated PHD/VHL. Then β-OHB induced 3 more cardiomyocyte death by decreasing HIF-1α and the downstream GLUT1 and the 4 expression of key glycolytic genes.

Supplementary Files

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