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Research Article
Exploring the Cardioprotective Effects of Pharmacological Inhibitors of PFKFB3 in Ischemia-Reperfusion-Subjected Rats

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Abstract

Background and Objective: PFKFB3 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase-3) is an enzyme that regulates glycolysis and is activated under hypoxic conditions. However, the role of this enzyme and its pharmacological inhibitors in ischemia-reperfusion injury is not explored yet. The present study was aimed to explore the effects of two pharmacological inhibitors of PFKFB3, 3-PO and AZ PFKFB3 in ischemia-reperfusion-induced myocardial injury along with possible mechanisms. **Materials and Methods:** Wistar albino rats were subjected to *ex vivo* ischemia-reperfusion injury on the Langendorff apparatus. The degree of myocardial injury was assessed by measuring the release of cTnT and CK-MB in the coronary effluent. The Left Ventricular Developed Pressure (LVDP) was measured to assess the heart contractility. The hearts were perfused with 3-PO and AZ PFKFB3 before subjecting them to ischemia and reperfusion injury. The hearts were homogenized to measure the levels of H_2S, p-Akt and p-GSK-3β/GSK-3β ratio to explore the mechanism of PFKFB3 inhibitors. **Results:** Perfusion of hearts with 3-PO and AZ PFKFB3 alleviated ischemia-reperfusion-induced increase in CK-MB, cTnT release and restored the LVDP values suggesting their cardioprotective actions. These drugs restored the levels of H_2S and p-Akt in the heart along with the increase in the p-GSK-3β/GSK-3β ratio. **Conclusion:** Pharmacological inhibitors of PFKFB3 may be potentially employed as cardioprotective agents to attenuate ischemia-reperfusion-induced injury and these effects may be mediated involving H_2S, Akt and GSK-3β signalling.

Key words: PFKFB3, Akt, GSK-3β signalling, cardioprotective, ischemia-reperfusion-induced injury

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The development of ischemia is one of the most common causes of myocardial injury and restoration of blood in the form of reperfusion is essential to salvage the ischemic heart. However, reperfusion per se also induced myocardial injury and therefore, the term ischemia-reperfusion is collectively used to described injury resulting during the process of ischemia and reperfusion[1]. Despite the widespread prevalence of ischemia-reperfusion-induced myocardial injury, there are not appropriate drugs to attenuate this form of injury. Thus, there is a need to explore and identify new pharmacological agents and targets for the effective management of ischemia-reperfusion-induced heart injury.

6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) is an enzyme that plays a significant role in regulating glycolysis and it is generally activated under hypoxic conditions[2]. In recent years, the importance of this pharmacological target has increased due to an understanding of its role in the pathophysiology of different diseases. Therefore, PFKFB3 has emerged as a major potential therapeutic target in the management of different diseases[3]. Most of the research on this target has been related to the proliferation of cancer cells and its role in different types of cancer has been explored[4,5]. Apart from it, the role of PFKFB3 on the vascular system has also been explored and it is found to be involved in the development of pulmonary hypertension[6], endothelial injury[7]. Its role in acute lung injury[8] and endotoxemia-induced myocardial injury[9] has also been described. Moreover, it has been shown that inhibition of PFKFB3 attenuates ischemia-reperfusion-induced cerebral injury[10]. However, the role of pharmacological inhibitors of PFKB3 in ischemia-reperfusion-induced myocardial injury has not been explored. Therefore, the present study was designed to explore the therapeutic potential of pharmacological inhibitors of PFKB3 i.e., 3-PO and AZ PFKFB3 in ischemia-reperfusion-induced heart injury. Moreover, the present study also explored the potential signalling pathways involved in PFKB3 inhibitors-mediated cardioprotection, particularly H_2S, Akt and GSK-3β.

MATERIALS AND METHODS

Study area: The experimental study was carried out in the Cardiovascular Division, Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine and supported by the National Natural Science Foundation of China, (Grant No. 81960849), China from December, 2019 to April, 2021.

Animals and drugs: Male Wistar albino rats (200-250 g) were used for the experiments in this study. The animals were kept in the Departmental Animal House, where they were provided with standard housing facilities including 12 hrs dark-light cycles, 25±1°C temperature and 55±5% relative humidity. The experimental protocol was approved by the ethical committee of Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine all experiments were conducted as per ethical guidelines provided by the Institutional Ethical committee.

***Ex vivo* ischemia-reperfusion injury:** The rats were sacrificed and hearts were isolated. The isolated hearts were immediately mounted on the Langendorff System and were perfused with physiological solution (Kreb's Solution, 37°C) in a retrograde manner. After stabilization of 15 min, the inflow of physiological solution was stopped to induce global ischemia for 30 min. Thereafter, the inflow of physiological solution was instituted for 120 min to induce reperfusion. A catheter with a fluid-filled latex balloon, connected to the pressure transducer, was inserted in the left ventricle to measure Left Ventricular Developed Pressure (LVDP). The physiological solution after passing through the heart was collected in the form of coronary effluent for the estimation of heart-specific biochemicals. The effluent was collected before subjecting to ischemia (basal) and immediately after instituting reperfusion (during reperfusion)[11,12]. The heart-specific biochemicals including CK-MB and cTnT were quantified in the coronary effluent using commercially available kits. The isolated rat hearts were treated with 3-PO (10 and 20 μM) and AZ PFKFB3 (10 and 20 μM) by their addition in the physiological solution and hearts were perfused with drug mixed physiological solution for 15 min before subjecting to ischemia and reperfusion.

Measurement of biochemical parameters in the heart homogenate: After 120 min of reperfusion, the hearts were isolated and were homogenized in physiological buffer solution (PBS, pH: 7.4). The homogenate was centrifuged at 3000 g for 15 min to obtain the homogenate, in which biochemical parameters including p-Akt, H_2S, GSK-3β. The p-Akt and the ratio of p-GSK-3β/GSK-3β were quantified using commercially available ELISA kits. The levels of H_2S were quantified in the heart homogenates using the reverse HPLC method[13,14].

Experimental design

Normal: Hearts were perfused with the physiological solution without any ischemia and reperfusion. After 180 min of perfusion (15 min of stabilization+15 min corresponding to drug perfusion periods in group III onwards+30 min corresponding to ischemia period in group II+120 min corresponding to reperfusion period in group II), the hearts were removed and subjected to biochemical estimations.

IR control: Hearts were perfused with the physiological solution for 30 min (15 min of stabilization+15 min corresponding to drug perfusion period in groups III onwards) followed by 30 min of ischemia and 120 min of reperfusion.

3-PO (10 μM) in IR: Hearts were perfused with the physiological solution for 15 min (stabilization) followed by perfusion with 3PO (10 μM) mixed physiological solution for 15 min. It was followed by 30 min of ischemia and 120 min of reperfusion.

3-PO (20 μM) in IR: Hearts were perfused with the physiological solution for 15 min (stabilization) followed by perfusion with 3PO (20 μM) mixed physiological solution for 15 min. It was followed by 30 min of ischemia and 120 min of reperfusion.

AZ PFKFB3 (10 μM) in IR: Hearts were perfused with the physiological solution for 15 min (stabilization) followed by perfusion with AZ PFKFB3 (10 μM) mixed physiological solution for 15 min. It was followed by 30 min of ischemia and 120 min of reperfusion.

AZ PFKFB3 (20 μM) in IR: Hearts were perfused with the physiological solution for 15 min (stabilization) followed by perfusion with AZ PFKFB3 (20 μM) mixed physiological solution for 15 min. It was followed by 30 min of ischemia and 120 min of reperfusion.

Statistical analysis: The results were represented as Mean±SD. The results of heart injury-specific parameters were analyzed using Two Way ANOVA, while the results of biochemical parameters were analyzed using One Way ANOVA. The *post hoc* analysis was done using Tukey's test. $p<0.05$ was considered as statistically significant.

RESULTS

Ischemia reperfusion-induced myocardial injury in *ex vivo* heart preparation: It was found the isolated rat hearts were treated with 3-PO (10 and 20 μM), The CK-MB released from 17.53-106.3 and 75.38, while treated with AZ PFKFB3 (10 and 20 μM), the CK-MB released from 17.53-100.03 and 67.73. It means the Institution of 30 min of global ischemia and then after, 120 min of reperfusion resulted in significant myocardial injury assessed in terms of an increase in CK-MB release during the reperfusion phase in comparison to basal (before ischemia) state, shown in Fig. 1 ($p<0.05$). Similarly, there was also a significant rise in the release of another heart injury-specific biomarker i.e., cTnT during the reperfusion phase. The cTnT level increased from 10.21-108.23 and 10.02-68.33 treated separately with 3-PO (10 and 20 μM). And when treated with AZ PFKFB3(10 and 20 μM), the cTnT increased from 10.01-105.22 and 10.22-65.37 in comparison to the basal state are shown in Fig. 2 ($p<0.05$).

There was also a significant decrease in the functional parameters in ischemia-reperfusion-subjected rat hearts assessed in terms of decrease in LVDP (heart contractility parameter) during the reperfusion phase in comparison to the basal state, when treated with 3-PO (10 μM) in IR, the functional parameters in ischemia-reperfusion-subjected rat hearts decrease from 76.1-40.4, while increased the 3-PO (20 μM) in IR, the value decrease from 73.9-60.1. While treated with AZ PFKFB3 (10 μM) in IR and AZ PFKFB3 (20 μM) in IR, the value decreased from 75.0-42.6 and from 76.9-62.1, which is a significant change are listed in Table 1 ($p<0.05$).

Ischemia reperfusion-induced biochemical changes in the *ex vivo* heart preparation: Exposure of 30 min of ischemia and 120 min of reperfusion produced significant changes in the biochemical milieu in the rat hearts. There was a significant decrease in the H_2S levels in the heart homogenates. It was found the Hydrogen Sulfide lever changed from 11.5-23.0 and 37.3 pg mg^{-1} when non-treated

Table 1: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in LVDP

Groups	Basal (before ischemia)	During reperfusion
Normal	75.5±5.7	70.8±4.5
IR control	73.1±4.7	24.7±4.1[a,b]
3-PO (10 μM) in IR	76.1±3.1	40.4±3.9[a,c]
3-PO (20 μM) in IR	73.9±5.9	60.1±5.1[a,b]
AZ PFKFB3 (10 μM) in IR	75.0±5.3	42.6±4.3[a,b]
AZ PFKFB3 (20 μM) in IR	76.9±5.2	62.1±3.0[a,b]

a: $p<0.05$ vs. basal of corresponding groups, b: $p<0.05$ vs. normal during reperfusion and c: $p<0.05$ vs. IR control during reperfusion

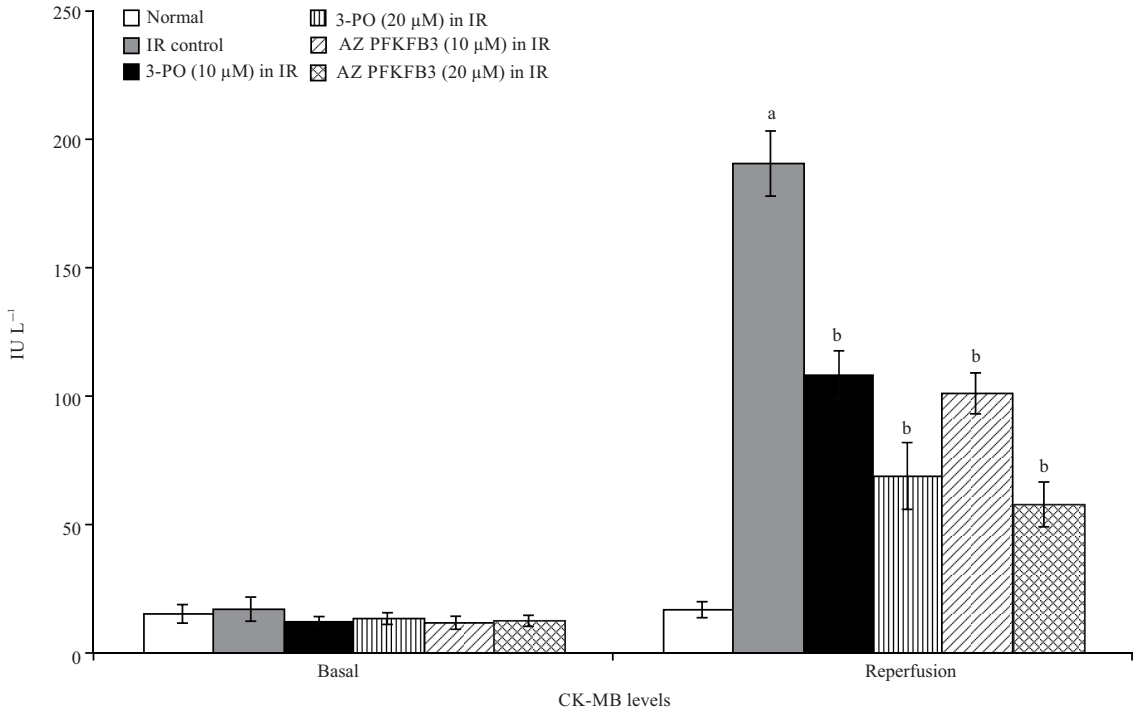

Fig. 1: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in CK-MB levels
Values are given in Mean±SD. a: p<0.05 vs. normal during reperfusion and b: p<0.05 vs. IR control during reperfusion

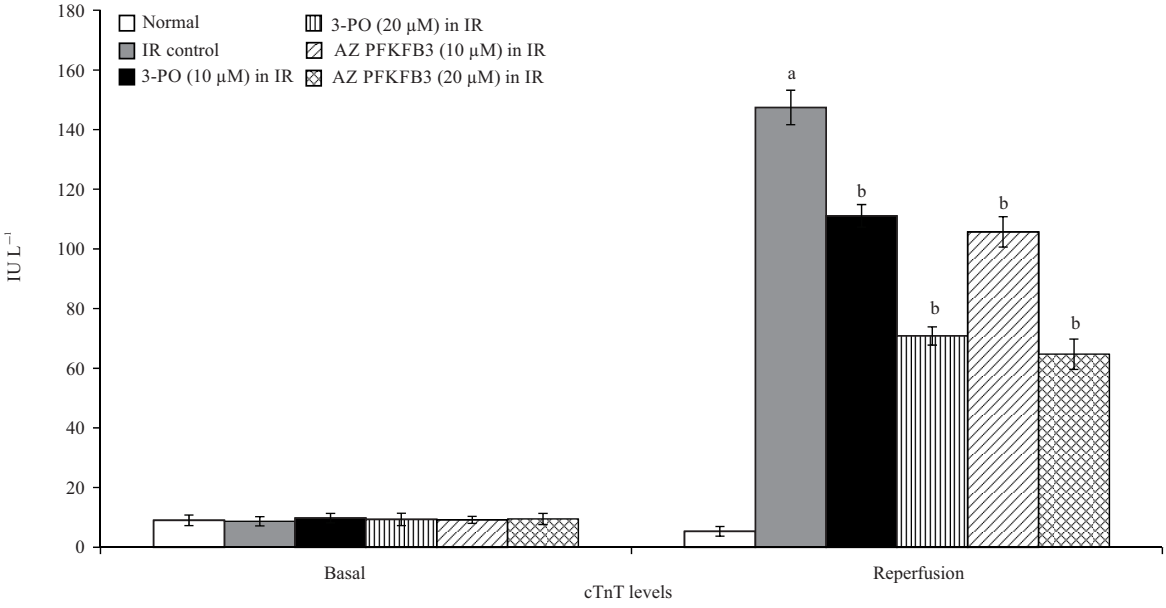

Fig. 2: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in cTnT levels
Values are given in Mean±SD. a: p<0.05 vs. normal during reperfusion and b: p<0.05 vs. IR control during reperfusion

and treated with 3-PO (10 and 20 μM) to the IR model. While treated with AZ PFKFB3 (10 and 20 μM), The IR control showed 11.5 pg mg^{-1} but the experiment group showed the Hydrogen Sulfide lever was 25.2 and 48.5 pg mg^{-1} in Fig. 3 ($p<0.05$). The result along with a significant decrease in the p-Akt levels in ischemia-reperfusion-subjected hearts. It was found that, when treated with 3-PO (10 and 20 μM) and AZ PFKFB3 (10 and 20 μM) to the isolated rat hearts, the p-Akt levels increased from 50-70 and 83% compared with and used AZ PFKFB3 (10 and 20 μM), the result changed from 50-68 and

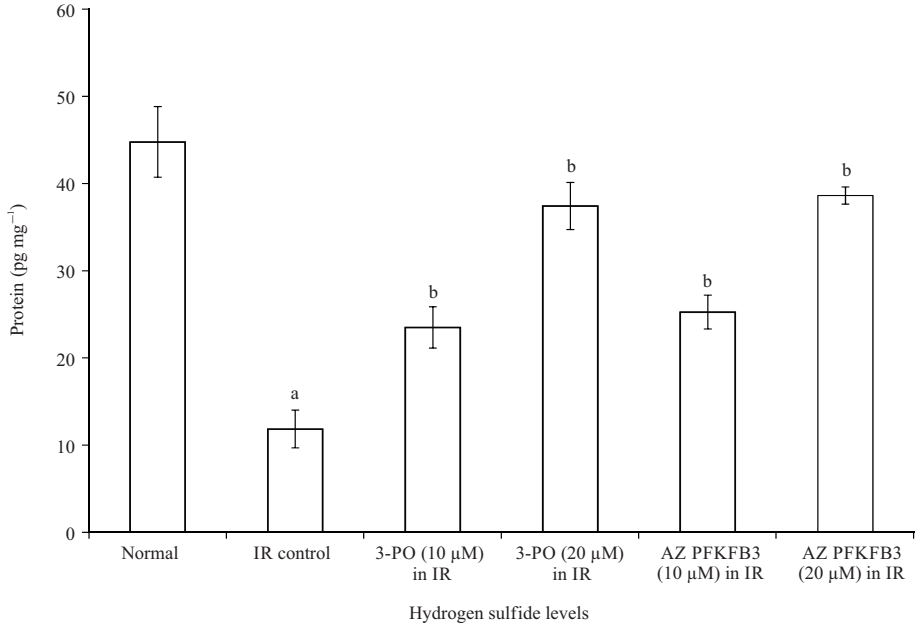

Fig. 3: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in H_2S levels in the heart homogenates
Values are given in Mean±SD. a: p<0.05 vs. normal during reperfusion and b: p<0.05 vs. IR control during reperfusion

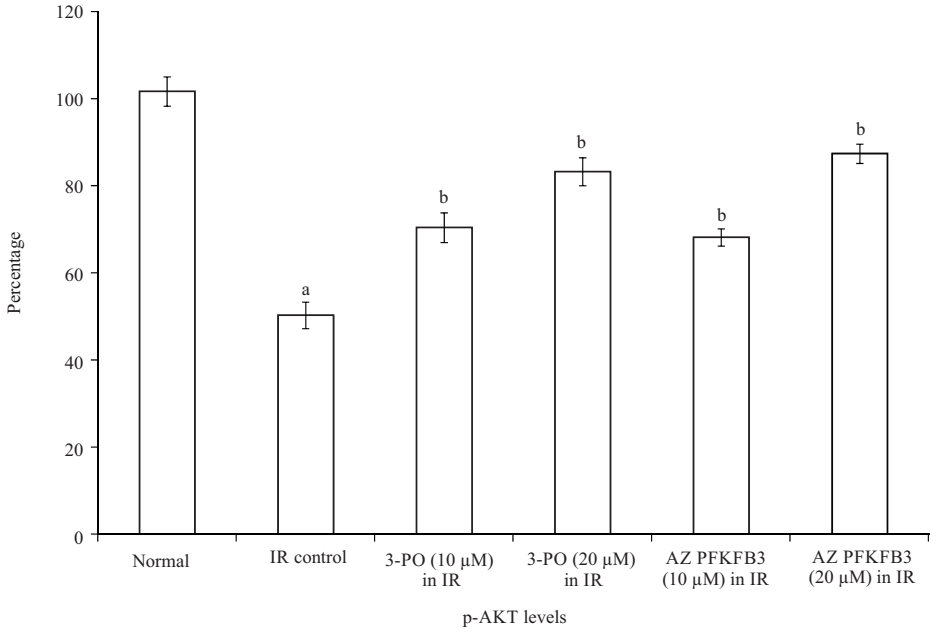

Fig. 4: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in p-Akt levels in the heart homogenates
Values are given in Mean±SD. a: p<0.05 vs. normal during reperfusion and b: p<0.05 vs. IR control during reperfusion

87% compared with ischemia-reperfusion-controlled hearts in rats in Fig. 4, (p<0.05). Furthermore, there was a significant decrease in the ratio of p-GSK-3β/GSK-3β in ischemia-reperfusion-subjected hearts. GSK-3β is a unique enzyme, whose phosphorylation leads to a decrease in its enzyme activity and therefore, a decrease in the levels of p-GSK-3β in response to ischemia-reperfusion signifies the increase in the GSK-3β activity.

Pharmacological inhibitors of PFKFB3 restored ischemia-reperfusion-induced heart injury and biochemical alterations: As found in the former experiment that Perfusion of isolated rat hearts with 3-PO (10 and 20 μM) and AZ PFKFB3 (10 and 20 μM) before ischemia and reperfusion significantly decreased the release of heart injury-specific biomarkers viz. CK-MB and cTnT in the coronary effluent during the reperfusion phase. Moreover, these drugs also prevented the

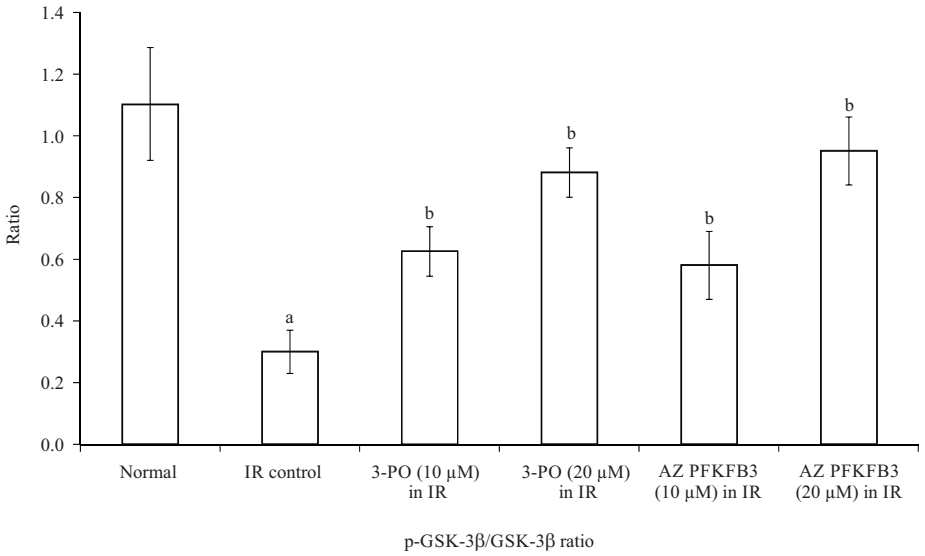

Fig. 5: Effect of 3-PO and AZ PFKFB3 on ischemia-reperfusion-induced changes in the p-GSK-3β/GSK-3β ratio in the heart homogenates

Values are given in Mean±SD. a: p<0.05 vs. normal during reperfusion and b: p<0.05 vs. IR control during reperfusion

decline in heart contractility in response to ischemia-reperfusion injury and there was significant preservation of LVDP in 3-PO and AZ PFKFB3-perfused rat hearts. It suggests that pharmacological inhibition of PFKFB3 exerted protective effects on ischemia reperfusion-subjected hearts. Along with it, these drugs also prevented ischemia-reperfusion-induced biochemical alterations on the heart homogenates. There was significant preservation of H_2S and p-AKT levels in 3-PO and AZ PFKFB3-perfused rat hearts as we found before. Besides, have found when treated isolated rat hearts with the 3-PO (10 and 20 μM), the p-GSK-3β/GSK-3β ratio increased from 0.32-0.62 and 0.88 compared with ischemia reperfusion-subjected hearts and when treated with AZ PFKFB3 (10 and 20 μM), the result changed from 0.32-0.58 and 1.75 in compared with ischemia reperfusion-controlled hearts as shown in Fig. 5. Hence, All findings showed there was also a decrease in the p-GSK-3β/GSK-3β ratio in response to treatment with PFKFB3 inhibitors.

DISCUSSION

In this study, the institution of 30 min of global ischemia and 120 min of reperfusion produced significant myocardial injury assessed in terms of an increase in the release in heart injury-specific biomarkers viz. CK-MB and cTnT in the coronary effluent during the reperfusion phase. The release of CK-MB and cTnT in coronary effluent is considered as a specific indication of myocardial injury[12]. Moreover, there was a functional impairment in ischemia-reperfusion-subjected rat hearts and there was a significant decrease in the LVDP (heart contractility parameter) in these rat hearts. The present study findings showing the development of myocardial injury are in consonance with earlier studies[15,16]. In this study, *ex vivo* heart preparation was employed as it overcomes the systemic influences encountered *in vivo* preparations[17].

In the present study, perfusion of isolated rat hearts with a pharmacological inhibitor of PFKFB3 i.e. 3-PO (10 and 20 μM) significantly ameliorated ischemia-reperfusion injury in a dose-dependent manner. In 3-PO treated rat hearts, there was a significant decrease in the release of CK-MB and cTnT in the coronary effluent. Moreover, there was a significant preservation of myocardial contractility and the values of LVDP were significantly higher in 3-PO-treated rat hearts. PFKFB3 is an enzyme that is activated during hypoxic conditions and it has a significant role in regulating glycolysis[18]. Apart from its physiological processes, it has also been shown to be involved in pathological processes. There have been studies showing the important role of this enzyme in the pathogens of cancer[4], pulmonary hypertension[6] and endotoxemia[8]. Moreover, selective inhibition of PFKFB3 has been shown to prevent ischemia-reperfusion-induced cerebral injury[10]. Moreover, PFKFB3 has been shown to promote endotoxemia-induced myocardial dysfunction through inflammatory signalling and apoptotic induction[9]. However, it is the first study showing the protective role of a PFKFB3 inhibitor in ischemia-reperfusion-

induced myocardial injury. The significant role of PFKFB3 activation in ischemia-reperfusion-injury was further supported by the results of this study showing that perfusion of hearts with another PFKFB3 inhibitor viz. AZ PFKFB3 (10 and 20 μM) significantly attenuated ischemia-reperfusion-induced myocardial injury in a dose-dependent manner. Accordingly, it may be proposed that there is a critical role of PFKFB3 activation in inducing ischemia-reperfusion-induced myocardial injury and thus, pharmacological inhibitors of PFKFB3 may be potentially employed to overcome heart injury in response to ischemia-reperfusion.

In this study, there was also a significant decrease in the H_2S levels in the heart homogenates following ischemia-reperfusion injury. H_2S is a gaseous neurotransmitter and recent studies have shown that the decrease in the H_2S levels may contribute to inducing ischemia-reperfusion injury. Moreover, there was a decrease in the p-Akt levels and p-GSK-3β/GSK-3β ratio in the heart homogenate in ischemia-reperfusion-subjected rats. Akt and GSK-3β constitute an important link in the intracellular signalling cascade in different cells including cardiomyocytes[19]. The activation of Akt occurs as a consequence of its phosphorylation and its dephosphorylation has been associated with inhibition of Akt activity[20]. There have been studies showing that dephosphorylation of Akt is a critical event in the pathogenesis of ischemia-reperfusion-induced heart injury[21]. Amongst the different functions, the activation of Akt leads to phosphorylation of GSK-3β, which is manifested in the form of inhibition of GSK-3β activity[22,23]. Alternatively, decreased Akt activation leads to decreased phosphorylation of GSK-3β and an increase in GSK-3β activity. It has also been reported that a decrease in phosphorylation of GSK-3β and an increase in GSK-3β activity is involved in inducing ischemia reperfusion-induced heart injury[24,25]. In this study, perfusion with 3-PO and AZ PFKFB3 normalized ischemia-reperfusion-induced biochemical alterations in the heart homogenate. There was the restoration of the levels of H_2S and p-Akt along with the normalization of the ratio of p-GSK-3β/GSK-3β. Since there is a significant role of H_2S, Akt and GSK-3β in ischemia-reperfusion-induced heart injury and pharmacological inhibition of PFKFB3 normalized these parameters, therefore, it may be proposed that PFKFB3 inhibitors trigger cardioprotection involving H_2S, Akt and GSK-3β signalling. Nevertheless, future studies are required to fully elucidate the role of these signalling pathways in mediating the cardioprotective effects of PFKFB3 inhibitors.

CONCLUSION

Pharmacological inhibitors of PFKFB3 are potentially employed as cardioprotective agents to attenuate ischemia-reperfusion-induced injury and these effects may be mediated involving H_2S, Akt and GSK-3β signalling.

SIGNIFICANCE STATEMENT

This study discovered the Pharmacological inhibitors of PFKFB3 may be potentially employed as cardioprotective agents to attenuate ischemia-reperfusion-induced injury that can be beneficial for heart disease treatment. This study will help the researchers to uncover the critical areas of drug therapy for myocardial ischemia that many researchers were not able to explore. Thus a new theory on drug and cardiovascular treatment may be arrived at.

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