Quercetin: A Phytoestrogen Attenuate GSK-3β Inhibitors Induced Delayed Cardioprotection in Diabetic Rat Heart

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ABSTRACT

Background and Objective: Glycogen synthase kinase (GSK-3β) play central role in Ischemic Preconditioning (IPC) mediated early as well as late effect of cardioprotection. The signaling pathway of IPC gets diminished during diabetes mellitus. The present study was designed to investigate whether GSK-3β inhibitors, administered 24 h before the ischemia, would exert the cardioprotection in diabetic rat. Methods: Diabetes Mellitus (DM) was induced by single administration of streptozotocin (STZ) (50 mg kg⁻¹, i.p.). Rat heart was isolated and mounted on Langendroff's apparatus and subjected to 30 min of ischaemia followed by 120 min of reperfusion. Myocardial infarct size was estimated by triphenyltetrazolium chloride (TTC) staining, lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were measured for the extent of myocardial damage. Results: Administration of GSK-3β inhibitors, SB 216763 (SB, 0.6 mg kg⁻¹, i.p.) and indirubin-3 monooxime (IND, 0.4 mg kg⁻¹, i.p.), 24 h before, not 12 h before the isolation of heart, significantly attenuated I/R-induced infarct size, release of LDH and CK-MB, as compared to vehicle treated group. However, this observed cardioprotection was significantly attenuated by administration of quercetin (4 mg kg⁻¹, i.p) an inhibitor of heat shock protein 72 (HSP-72) 1 h before the treatment of either drugs. Conclusion: Administration of GSK-3β inhibitors 24 h before the ischemic insult produces the cardioprotection in diabetic rat heart which may be mediated through HSP-72, since it was significantly attenuated by pretreatment with quercetin.

Key words: Glycogen synthase kinase-3β, HSP-72, ischemic preconditioning, diabetes mellitus, cardioprotection

INTRODUCTION

Ischemic Pre-Conditioning (IPC) is a well recognized cardioprotective process that protects the heart against Ischemia-Reperfusion (I/R) induced injury. This biphasic phenomenon appears immediately and 24 h after the stimuli of IPC, termed as classical and Second Window of Protection (SWOP), respectively. The cardioprotective effect of both phases gets attenuated during the metabolic disorder such as hyperlipidemia and early effect get diluted during diabetes mellitus. However, IPC mediated late phase of cardioprotection during the diabetes mellitus is not well elucidated.

Glycogen synthesizes kinase 3β (GSK-3β) is a glucose metabolizing enzyme play a key role in IPC mediated cardioprotection. During IPC, the signaling pathway which phosphorylate and inhibit the GSK-3β get impaired in diabetic and hyperlipidemic rat heart. Moreover, perfusion of selective GSK-3β inhibitor restores early cardioprotective effect of IPC during hyperlipidemia and diabetes mellitus. Heat shock protein 72 (HSP-72) is a chaperon protein is noted to exert IPC mediated late phase cardioprotection. The hearts having low cardiac content of HSP-72 are more susceptible to ischemic injury. Quercetin is a phytoestrogen which inhibits HSP-72 and attenuates the GSK-3β inhibitors induced cardioprotection during hyperlipidemia. However, the expression and activity of HSP-72 got diminished during diabetes mellitus.

This study has been designed to investigate whether or not the administration of selective GSK-3β inhibitors i.e., SB 216763 and indirubin-3 monooxime, 24 h before the ischemic insult produces the cardioprotection in diabetic rat heart if yes then what will be the effect of pretreatment with quercetin.
MATERIALS AND METHODS

Wistar rats (180-250 g), of either sex, were employed in present study. Experimental protocol was approved by Animal Ethical Committee, ISF College of Pharmacy Moga. All experiments were carried out according to guidelines of National Science Academy for care and use of animals in scientific research. All of the animals were fed to standard chow diet and were exposed to normal cycle of light and dark.

Drugs and chemicals: GSK-β inhibitors i.e., SB 216763 (0.6 mg kg$^{-1}$, i.p.)$^{16,17}$ and Indirubin-3 monooxime (0.4 mg kg$^{-1}$, i.p.)$^{18,19}$ were administered 24 h before the isolation of heart. Quercetin (4 mg kg$^{-1}$, i.p.)$^{20,21}$ was given 1 h before administration of either drugs. All of above mentioned agents were purchased from Sigma Chemicals, St. Louis, MO, USA. All the other chemicals were of analytical grade obtained from Loba chemicals (P) Ltd. Mumbai India.

Induction of experimental diabetes mellitus: Diabetes Mellitus (DM) in rat was induced by single dose of streptozotocin (STZ, 50 mg kg$^{-1}$, i.p.) and level of serum glucose was documented by commercially available kits (Vital Diagnostics (P) Ltd., Mumbai, India).

Isolated rat heart preparation: Hearts from heparinized rats 500U, i.p., were rapidly excised and immediately mounted on Langendorff’s apparatus, (Radnoti LLC, Monrovia, USA)$^{19}$. Temperature was maintained by circulating water heated to 37.8°C. The preparation was retrogradely perfused at constant pressure of 80 mm Hg with Kreb’s-Henseleit buffer (NaCl 118 mM; KCl 4.7 mM; CaCl$_2$ 2.5 mM; MgSO$_4$ 7H$_2$O 1.2 mM; NaHCO$_3$ 25 mM; KH$_2$PO$_4$ 1.2 mM; C$_6$H$_{12}$O$_6$ 11mM), pH 7.4, bubbled with 95% O$_2$ and 5% CO$_2$. Global ischaemia was produced by closing the inflow of K-H solution for 30 min followed by 120 min of reperfusion. Coronary effluent was collected after stabilization, immediately 5 and 30 min after reperfusion for estimation of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB).

Assessment of myocardial injury: The myocardial injury was determined by extent of release of LDH and CK-MB in coronary effluents using commercially available kit (Vital Diagnostics (P) Ltd., Mumbai, India). Values were expressed in international units IU per liter.

Myocardial infarct size: At the end of experiment, heart was removed from the Langendorff apparatus. Both the atria and root of aorta were separated and ventricles were kept overnight at -4°C. Frozen ventricles were sliced in uniform manner of 1-2 mm thickness. The slices were incubated in 1% TTC at 37°C in 0.2 M Tris buffer of pH 7.4 for 30 min. The non infarcted myocardium was stained brick red while the infarcted portion remained unstained. Infarct size was measured by the volume method$^{21}$.

Experimental protocol: A diagrammatic representation of experimental protocol is shown in Fig. 1. Total nine groups have been used, each group consist of 6 rats (n = 6). In all groups, the isolated rat heart was perfused with K-H solution and allowed for 10 min of stabilization. Group 1 (Sham control): isolated rat heart was perfused continuously for 200 min, without subjecting any ischemic insult. Group 2 (Ischaemia-reperfusion control): Isolated heart from normal rat after stabilization was subjected to 30 min global ischemia followed by 120 min of reperfusion. Group 3 (Vehicle control): DMSO (0.02%, w/v) was administered 24 h before the isolation of heart in diabetic rat. Heart was subjected to 30 min. of global ischemia followed by 120 min of reperfusion. Group 4 and 5: Indirubin-3 monooxime (0.4 mg kg$^{-1}$, i.p.) or SB 216763 (0.6 mg kg$^{-1}$, i.p.) was given in diabetic rat, 12 h before the isolation of heart. Isolated heart was perfused as group 2. Group 6 and 7: Diabetic rat was treated by Indirubin-3 monooxime (0.4 mg kg$^{-1}$, i.p.) and SB 216763 (0.6 mg kg$^{-1}$, i.p.) 24 h before the isolation of heart followed by as group 2. Group 8 and 9: Quercetin (4 mg kg$^{-1}$, i.p.) was administered 1 h before the administration of either of Indirubin-3 monooxime (0.4 mg kg$^{-1}$, i.p.) or SB 216763 (0.6 mg kg$^{-1}$, i.p.) in diabetic rat, 24 h before the isolation of heart. Isolated heart was followed by as group 2.

Statistical analysis: Results were expressed as Mean±SEM (N = 6). The data were statistically analyzed using one-way ANOVA followed by Tukey’s multiple comparisons test. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Effect of streptozotocin on serum glucose: Administration of single dose of streptozotocin significantly increased the serum glucose level as compared to control group (Fig. 2).

Effect of ischemia-reperfusion on myocardial injury in normal and diabetic rat heart: Global ischemia and 120 min of reperfusion significantly
increased the infarct size and the release of LDH and CK-MB in coronary effluent in normal and diabetic rat heart, as compared to sham control group. Administration of vehicle 24 h before did not have any significant effect on I/R-induced infarct size and release of LDH and CK-MB in diabetic rat heart (Fig. 3, 4 and 5).

**Effect of administration of indirubin-3 monooxime and SB 216763 on myocardial injury in diabetic rat heart:** Treatment of diabetic rats with either of GSK-3β inhibitors, indirubin-3 monooxime (0.4 mg kg⁻¹, i.p.) and SB 216763 (0.6 mg kg⁻¹, i.p.), 24 h before the isolation of heart, significantly decreased the I/R induced myocardial infarct size and the release of LDH and CK-MB as compared to I/R group. However, administration of either of above drug in same dose, 12 h before the isolation of heart did not exert significant effect on I/R-induced myocardial injury (Fig. 3, 4 and 5).

**Effect of quercetin on GSK-3β inhibitors induced cardioprotection in diabetic rat heart:** Administration of quercetin (4 mg kg⁻¹, i.p.), 1 h before the administration either of GSK-3β inhibitors i.e., indirubin-3 monooxime (0.4 mg kg⁻¹, i.p.) and...
SB 216763 (0.6 mg kg\(^{-1}\), i.p.), significantly attenuated the observed cardioprotection induced by either of the GSK-3β inhibitors in diabetic rat, noted in terms of decreased myocardial infarct size and release of LDH and CK-MB (Fig. 3, 4 and 5).

**DISCUSSION**

Glycogen synthases kinase-3β play central role in IPC mediated early and late phase of cardioprotection\(^1,3,4\). It was reported that GSK-3β is common target site responsible for attenuation of early and late cardioprotective effect of IPC in hyperlipidemic rat heart\(^3,4\). Administration of either of the GSK-3β inhibitors i.e., indirubin-3 monooxime and SB 216763 in diabetic rats, 24 h before ischemic insult, produced significant cardioprotection noted in terms of decrease in infarct size and decrease in release of LDH and CK-MB, as compared to the vehicle control group. It suggests that inhibition of GSK-3β is responsible for delayed phase of cardioprotection in diabetic rat heart. These results are in agreement with previous work\(^3\). Moreover, similar to the late phase of IPC which appears after the 24 h of stimuli, the administration of either of GSK-3β inhibitors only exerts the cardioprotection after the 24 h of administration of either drugs not after 12 h. The present outcomes demonstrates that the late phase of
Fig. 5: Effect of GSK-3\(\beta\) inhibitors on I/R induced decrease in release in of creatine kinase-MB (CK-MB) in isolated normal rat heart. Effect of HSP-72 inhibitor on Indirubin-3 Monooxime (0.4 mg kg\(^{-1}\), ip) and SB 216763 (0.6 mg kg\(^{-1}\), ip), decrease in release of CK-MB in isolated heart obtained from diabetic rat. The values are expressed as Mean±SEM *p<0.05 vs. I/R control, **p<0.05 vs. Indirubin, 24 h before the isolation of heart from diabetic rat, ***p<0.05 vs. administration of SB 216763, 24 h before the isolation of heart from diabetic rat.

The cardioprotective effect of GSK-3\(\beta\) inhibitors in diabetic rat heart, closely resembled to IPC mediated late phase of cardioprotection in normal subjects\(^6,22\). The mechanism of preconditioning-induced delayed cardioprotection is dependent on survival kinase pathways i.e., PI-3K/Akt\(^23\) which are upstream of GSK-3\(\beta\)\(^{22,24}\). Further, Gross et al.\(^{11}\) reported that the administration of SB 216763, a selective GSK-3\(\beta\) inhibitor, 24 h before ischemia, produces cardioprotection through ATP sensitive potassium channels (K\(_{ATP}\)) and thereby inhibiting the opening of Mitochondrial Permeability Transition Pore (MPTP) during reperfusion phase. Moreover, opening of K\(_{ATP}\)\(^{26}\) and inhibition of the opening of MPTP\(^{26}\) is responsible for early phase of cardioprotection\(^8\) and author suggested for further assessment of components regulated by GSK-3\(\beta\) inhibition to elucidate the mechanism involved in delayed cardioprotection.

One hour pre treatment of quercetin (4 mg kg\(^{-1}\)), a HSP-72 inhibitor\(^{5,13,14}\), significantly attenuated the observed cardioprotection by either of GSK-3\(\beta\) inhibitors i.e., SB 216763 (0.6 mg kg\(^{-1}\), i.p) and indirubin-3 monooxime (0.4 mg kg\(^{-1}\), i.p). This indicates that delayed cardioprotection induced by inhibition of GSK-3\(\beta\) may be mediated at least a part through HSP-72. Quercetin is not a specific inhibitor of HSP-72; it also inhibits the activity of several protein kinases\(^{25,27}\). Moreover, these results are supported by findings of our laboratory and of other published reports\(^5,28-30\).

"Heat Shock Protein" (HSP) are the group of proteins referred to as molecular chaperones\(^3,9\) which are highly conserved proteins that utilize ATP for conformational changes to refold other proteins and also regulate the activity of other enzymes\(^{26,31,32}\). Brief ischemic episodes have been reported to trigger the synthesis of HSP-72 in the heart which increases the resistance against myocardial infarction after 24 h\(^{33,34}\). Because of prolonged duration of protection, the second window of protection is particularly relevant for its application under clinical conditions. Furthermore, it has been reported that the decreased cardiac content of HSP-72, may decrease the ischemic tolerance in normal heart\(^{6,10}\). The activity of HSP-72 is diminished during the diabetes mellitus and pharmacological inhibition of GSK-3\(\beta\) is noted to increase its activity\(^{35,36}\). It has been reported that perfusion of either of GSK-3\(\beta\) inhibitors i.e., SB 216763 (3 \(\mu\)M) and indirubin-3 monooxime (1 \(\mu\)M) decreases the ischemia reperfusion induced injury in isolated diabetic rat heart\(^4\). Therefore, it has been suggested that inhibition of GSK-3\(\beta\) 24 h before the ischemic insult produces cardioprotection against ischemia-reperfusion induced injury in diabetic rat heart.

**Limitations:** Although the current findings are obliging with some limitations. Western blot of HSP-72 could not be conducted because of limited institutional fund.

**CONCLUSION**

This study suggests that pharmacological inhibition of GSK-3\(\beta\), 24 h before the ischemic insult, exerts cardioprotection in the diabetic rat heart which get significantly abolished by the pretreatment of quercetin.

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REFERENCES