—Regular Articles—

Possible Role of JAK-2 in Attenuated Cardioprotective Effect of Ischemic Preconditioning in Hyperhomocysteinemic Rat Hearts

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The present study investigated the possible role of Janus kinase-2 (JAK-2) in hyperhomocysteinemia-induced attenuation of the cardioprotective effects of ischemic preconditioning (IPC). Rats were administered L-methionine (1.7 g/kg/day, *p.o.*) for 4 weeks to produce hyperhomocysteinemia. Isolated Langendorff's perfused normal and hyperhomocysteinemic rat hearts were subjected to global ischemia for 30 min, followed by reperfusion for 120 min. Myocardial infarct size was assessed macroscopically using triphenyltetrazolium chloride staining. Coronary effluent was analyzed for lactate dehydrogenase (LDH) and creatine kinase (CK) release to assess the extent of cardiac injury. The oxidative stress in the heart was assessed by measuring thiobarbituric acid-reactive substances (TBARS), superoxide anion generation and the reduced form of glutathione. Ischemia-reperfusion (I/R) induced oxidative stress by increasing TBARS, superoxide anion generation and decreasing reduced form of glutathione in normal and hyperhomocystenemic rat hearts. Moreover, I/R produced myocardial injury, which was assessed in terms of the increase in myocardial infarct size, LDH and CK release in coronary effluent, and decrease in coronary flow rate in normal and hyperhomocysteinemic rat hearts. The hyperhomocysteinemic rat hearts showed enhanced I/R-induced myocardial injury with a high degree of oxidative stress as compared with normal rat hearts subjected to I/R. Four episodes of IPC (5 min each) afforded cardioprotection against I/R-induced myocardial injury in normal rat hearts as assessed in terms of improvement in coronary flow rate and reduction in myocardial infarct size, levels of LDH, CK, and oxidative stress. On the other hand, IPC-mediated myocardial protection against I/R-injury was abolished in hyperhomocysteinemic rat hearts. Tyrphostin AG490 (5 μM), a selective inhibitor of JAK-2, did not affect the cardioprotective effects of IPC in normal rat hearts, but its administration markedly restored the cardioprotective potential of IPC in hyperhomocysteinemic rat hearts. Administration of diazoxide (30 μM), an ATP-sensitive potassium (K_{ATP}) channel opener, also restored the cardioprotective effects of IPC in hyperhomocysteinemic rat hearts. In conclusion, it is suggested that the high degree of oxidative stress produced in hyperhomocysteinemic rat hearts during reperfusion and consequent activation of JAK-2 and closure of K_{ATP} channels may be responsible for abolishing the cardioprotective potential of IPC against I/R-induced myocardial injury.

Key words——hyperhomocysteinemia; oxidative stress; ischemia-reperfusion injury; ischemic preconditioning; Tyrphostin AG490; Janus Kinase-2 (JAK-2); ATP-sensitive potassium (K_{ATP}) channel

INTRODUCTION

Coronary artery disease is a leading cause of morbidity and mortality and its prevalence is continuously increasing worldwide.[1] Myocardial ischemia is a condition in which heart tissue receives inadequate blood flow, followed by inadequate oxygen and nutrient supply. The restoration of coronary blood flow to an ischemic myocardium is mandatory to avoid myocardial damage. However, reperfusion of the previously ischemic myocardium is often followed by detrimental changes in myocardial tissues, known as ischemia-reperfusion (I/R) injury.[2] Brief episodes of I/R render the heart more tolerant to subsequent sustained I/R, known as ischemic preconditioning (IPC).[3] IPC has been reported to reduce I/R-induced myocardial injury by decreasing oxidative stress,[4] limiting myocardial infarct size,[3,5] decreasing neutrophil (PMN) accumulation,[6] preserving coronary endothelial function,[7,8] and inhibiting apoptosis and necrosis.[6,9,10] Various mechanisms involved in the cardioprotective potential of IPC include activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway,[11] generation of nitric oxide (NO),[12] activation of mitochondrial ATP-sensitive K^+ channels (mito K_{ATP} channels),[13] and closure of mitochondrial permeability transition pores (mPTP).[14–16] However, the cardioprotective and infarct size-limiting effects of IPC are abolished in some pathologic conditions such as diabetes,[17,18] obesity,[19] heart

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failure,[20,21] hyperlipidemia,[22,23] and hypertension.[24] Hyperhomocysteinemia (Hhcy), a condition of elevated serum levels of homocysteine, has been considered to be an independent risk factor for cardiovascular disease.[25,26] Hhcy has been shown to generate high amounts of reactive oxygen species (ROS) by activating NADPH oxidase.[27] It has recently been reported that Hhcy decreases eNOS mRNA expression,[28] followed by increased oxidative stress and decreased bioavailability of NO, which damage the vascular endothelium.[29–31] We found that the cardioprotective potential of IPC was abolished in the Hhcy rat heart although the mechanism involved is not known. The Hhcy rat hearts showed marked oxidative stress upon reperfusion when compared with normal rat hearts subjected to I/R. Thus the signaling mechanisms activated by high levels of oxidative stress may play a detrimental role in the attenuation of the cardioprotective effects of IPC in the Hhcy rat heart. Janus kinase-2 (JAK-2) is a family of intracellular nonreceptor tyrosine kinases that mediate cytokine signal transduction *via* the janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway.[32] JAK-2 has been reported to be activated by high oxidative stress.[33,34] Further, JAK-2 was shown to inactivate the PI3K/Akt pathway, and activation of the PI3K/Akt pathway was demonstrated to be involved in the cardioprotective effects of IPC.[35] Moreover, JAK-2 activation has been implicated in the pathogenesis of I/R-induced myocardial injury.[36] Tyrphostin AG490 has been shown to be a selective inhibitor of JAK-2.[36–38] Therefore the present study investigated the effects of Tyrphostin AG-490, a selective inhibitor of JAK-2, in the abrogated cardioprotective effects of IPC in Hhcy rat hearts subjected to I/R.

MATERIALS AND METHODS

The experimental protocol used in the present study was approved by the Institutional Animal Ethical Committee. Wistar albino rats of either sex weighing 180–220 g were acclimatized in the institutional animal house and then maintained on rat chow (Kisan Feeds Ltd., Chandigarh, India) and tap water *ad libitum* with a normal day-night cycle.

Isolated Rat Heart Preparation The rat was heparinized (500 I.U., *i.p.*) and sacrificed by stunning. The heart was rapidly excised and immediately mounted on Langendorff apparatus.[39] The heart was enclosed in a double-walled jacket in which the temperature was maintained at 37°C with circulating warm water. The preparation was perfused with Krebs Henseleit (K-H) solution (118 mM NaCl, 4.7 mM KCL, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, $7H_2O$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1 mM $C_6H_{12}O_6$) of pH 7.4, maintained at 37°C, and bubbled with 95% O_2 and 5% CO_2. The coronary flow rate was maintained at around 7 ml/min by maintaining the perfusion pressure at 80 mmHg. Global ischemia was produced for 30 min by blocking the inflow of physiological solution, followed by reperfusion for 120 min. The coronary flow rate was recorded at baseline (before global ischemia), 0 min (onset of reperfusion), and after 5, 30, and 120 min of reperfusion.

Assessment of Myocardial Injury I/R-induced myocardial injury was assessed by estimating the release of lactate dehydrogenase (LDH) and creatine kinase (CK) in the coronary effluent and measuring the infarct size in the heart.

Estimation of LDH and CK Myocardial injury was assessed by measuring the release of LDH and CK in the coronary effluent using commercially available enzymatic kits (Vital Diagnostics, Thane, Maharastra, India). LDH was measured in the coronary effluent using the UV-kinetic method, which is based on the principle that LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity is proportional to the increase in absorbance due to the reduction of NAD into NADH. LDH activity was measured using the following formula: LDH activity (U/L) $=\Delta$A/min$\times$3376. CK-MB was measured in the coronary effluent using the immunoinhibition method, which is based on the principle that the CK-M fraction of CK-MM in the sample is completely inhibited by CK-M antibody present in the reagent. Then, the activity of the CK-B fraction was measured and CK-MB activity was calculated using the following formula: CK-MB activity (U/L) $=\Delta$A/min$\times$6752.

Infarct Size Measurement The heart was removed from the Langendorff apparatus. Both auricles, the root of the aorta and right ventricle were excised, and the left ventricle was kept overnight at -4 °C. Frozen ventricle was sliced into sections of 2–3 mm thick. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) solution in 0.1 M Tris buffer (pH 7.8) for 20 min at 37°C. The TTC

stain reacts with dehydrogenase enzyme in the presence of cofactor NADH to form formazon pigment in viable cells, which is brick red in color. Infarcted cells that have lost dehydrogenase enzyme remain unstained. Thus the infarcted portion of the myocardium remains unstained while the normal viable myocardium is stained brick red with TTC. Infarct size was measured macroscopically using the volume method.[40,41]

Assessment of Oxidative Stress The left ventricle was minced and homogenized in ice cold phosphate buffer 0.05 M (pH 7.4) using a Teflon homogenizer. The clear supernatant of the homogenate was used to estimate the levels of thiobarbituric acid-reactive substance (TBARS) and the reduced form of glutathione (GSH). The oxidative stress was assessed in the heart at 20-min reperfusion after 30-min ischemia.

Estimation of TBARS The quantitative measurement of TBARS, an index of lipid peroxidation in the heart, was performed according to the method of Ohkawa *et al.*[42] Supernatant homogenate (0.2 ml) was pipetted into a test tube, followed by the addition of 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 30% acetic acid (pH 3.5), and 1.5 ml of 0.8 % thiobarbituric acid (TBA), and the volume was made up to 4 ml with distilled water. The test tubes were incubated for 1 h at 95°C, cooled, and then 1 ml of distilled water and 5 ml of n-butanol-pyridine mixture (15 : 1 v/v) were added. The test tubes were centrifuged at 4000 g for 10 min. The absorbance of the developed pink color was measured spectrophotometrically (Thermo Double Beam Spectrophotometer, Thermo Electron Corporation, UK) at 532 nm. A standard calibration curve was prepared using 1–10 nM of 1,1,3,3-tetramethoxy propane. The concentration of TBARS value was expressed as nanomoles per gram of wet tissue weight.[42,43]

Estimation of Superoxide Anion Generation The heart was cut into transverse sections and placed in 5 ml of K-H solution buffer containing nitroblutetrazolium (NBT) 100 μM and incubated at 37°C for 1.5 h. The NBT reduction was stopped by adding 5 ml of 0.5 N HCl. The heart was minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing di-ethylene triamine pentaacetic acid (DTPA) 40 mg/l. The mixture was centrifuged at 20000 g for 20 min, and the resultant pellets were resuspended in 1.5 ml of pyridine and kept at 80°C for 1.5 h to extract formazan. The mixture was centrifuged at 10000 g for 10 min, and the absorbance of formazan was determined spectrophotometrically at 540 nm. The amount of reduced NBT was calculated using the following formula: Amount of reduced NBT = (A.V) / (T.Wt.ε.l), where A is absorbance, V is volume of solution (1.5 ml), T is time for which the rings were incubated with NBT (90 min), Wt is the blotted wet weight of heart, ε is the extinction coefficient (0.72 l/mM/mm), and l is the length of the light path (10 mm). Results were expressed as reduced NBT picomoles per minute per milligram of wet tissue.[43,44]

Estimation of Reduced Glutathione The reduced form of glutathione (GSH) content in the heart was estimated using method of Beutler *et al.*[45] The homogenate supernatant was mixed with trichloroacetic acid (10% w/v) in a 1 : 1 ratio. The tubes were centrifuged at 1000 g for 10 min at 4°C. The supernatant obtained (0.5 ml) was mixed with 2 ml of disodium hydrogen phosphate 0.3 M. Then, 0.25 ml of freshly prepared 5,5[1]-dithiobis [2-nitrobenzoic acid] (DTNB) 0.001 M dissolved in 1% w/v citric acid was added, and the absorbance was recorded spectrophotometrically at 412 nm. A standard curve was plotted using 5–50 μM of GSH and the results were expressed as micromoles of reduced glutathione per gram of wet tissue weight.[43,45]

EXPERIMENTAL PROTOCOL

Twelve groups were used in the present study, and each group comprised 10-12 animals. A diagrammatic representation of the experimental protocol is shown in Fig. 1. In all groups, isolated Langendorff-perfused rat hearts were allowed to stabilize for 10 min by perfusing with K-H solution.

Group I (*Normal Control*): The isolated normal rat heart was perfused for 200 min using K-H solution after 10-min stabilization.

Group II (*I/R-Control*): The isolated normal rat heart after 10-min stabilization was perfused for 50 min with K-H solution. The heart was then subjected to 30 min of global ischemia followed by 120 min of reperfusion.

Group III (*Tyrphostin AG490 Per se Normal Control*): After 10-min stabilization, the isolated normal rat heart was infused with Tyrphostin AG490 (5 μM) for 10 min. Then the heart was perfused for 190 min using K-H solution.

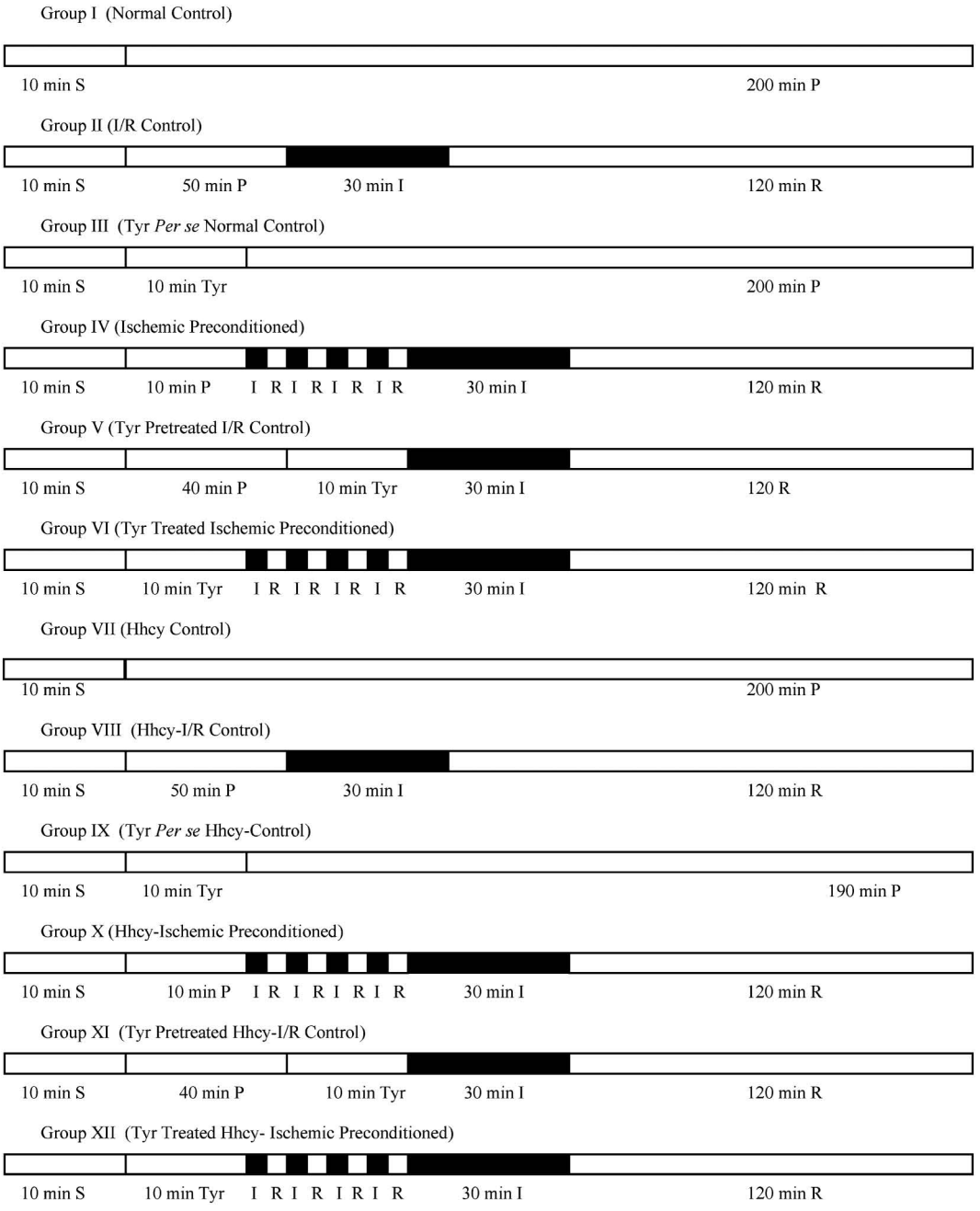

Fig. 1. Diagrammatic Representation of Experimental Protocol

S, stabilization; I, global ischemia; R, reperfusion with K-H solution; I/R, ischemia-reperfusion injury; Ischemic preconditioned, ischemic preconditioned normal rat heart; Hhcy ischemic preconditioned, ischemic preconditioned hyperhomocysteinemic rat heart; Tyr, Tyrphostin AG490 (5 μM).

Group IV (*Ischemic Preconditioned*): After 10 min of stabilization, the isolated normal rat heart was perfused for 10 min with K-H solution. The heart was then subjected to four episodes of 5-min global ischemia, followed by 5-min reperfusion to produce IPC. After four episodes of IPC, the heart was subjected to 30-min global ischemia, followed by 120-min reperfusion.

Group V (*Tyrphostin AG490-Pretreated I/R-Control*): After 10-min stabilization, the isolated normal rat heart was perfused for 40 min with K-H solution, followed by infusion with Tyrphostin AG490 (5 μM) for 10 min. The heart was then subjected to 30-min global ischemia, followed by 120-min reperfusion.

Group VI (*Tyrphostin AG490-Treated Ischemic Preconditioned*): After 10-min stabilization, the iso-

lated normal rat heart was perfused with Tyrphostin AG490 (5 μM) for 10 min. Then the heart was subjected to IPC as in group IV, followed by 30-min global ischemia and 120-min reperfusion.

Group VII (*Hhcy Control*): The isolated Hhcy rat heart was perfused for 200 min using K-H solution after 10-min stabilization.

Group VIII (*Hhcy-I/R Control*): The isolated Hhcy rat heart after 10-min stabilization was perfused for 50 min with K-H solution. The heart was then subjected to 30-min global ischemia, followed by 120-min reperfusion.

Group IX (*Tyrphostin AG490 Per se Hhcy-Control*): After 10-min stabilization, the isolated Hhcy rat heart was infused with Tyrphostin AG490 (5 μM) for 10 min. The heart was then perfused for 190 min using K-H solution.

Group X (*Hhcy-Ischemic Preconditioned*): After 10-min stabilization, the Hhcy rat heart was perfused with K-H solution for 10 min. The heart was then subjected to IPC as in Group IV. After IPC, the heart was subjected to 30-min global ischemia, followed by 120-min reperfusion.

Group XI (*Tyrphostin AG490-Pretreated Hhcy-I/R Control*): After 10-min stabilization, the Hhcy rat heart was perfused for 40 min with K-H solution, followed by infusion with Tyrphostin AG490 (5 μM) for 10 min. The heart was then subjected to 30-min global ischemia, followed by 120-min reperfusion.

Group XII (*Tyrphostin AG490-Treated Hhcy-Ischemic Preconditioned*): After 10-min stabilization, the Hhcy rat heart was perfused with Tyrphostin AG490 (5 μM) for 10 min. Then, the heart was subjected to IPC as in group IV, followed by 30-min global ischemia and 120-min reperfusion.

STATISTICAL ANALYSIS

The results were expressed as mean $\pm$ S.D. The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey's multiple-comparison test. A p value of less than 0.05 was considered to represent a statistically significant difference.

DRUGS AND CHEMICALS

The LDH and CK enzymatic estimation kits were purchased from Vital Diagnostics (Thane, Maharastra, India). L-methionine was purchased from Loba Chem (Mumbai, India). DTNB and NBT were obtained from Loba Chem. Tyrphostin AG490, 1,1,3,3-tetramethoxy propane and GSH were purchased from Sigma-Aldrich (USA). All other reagents used were of analytical grade.

RESULTS

Rats fed L-methionine (1.7 g/kg/day, *p.o.*) for 4 weeks *via* oral gavage exhibited Hhcy (25.42 ± 6.07 μM/l) when compared with normal rats (4.65 ± 0.69 μM/l). The left ventricle weight/body weight (LVW/BW) ratio (milligram/gram) was slightly increased in Hhcy rat hearts (3.26 ± 0.46) when compared with normal rat hearts (2.72 ± 0.42). L-methionine administration did not result in mortality in rats.

Effects of IPC in I/R-induced Oxidative Stress and Myocardial Injury in Normal and Hhcy Rat Hearts

The lipid peroxidation as measured in terms of TBARS and superoxide anion generation as assessed in terms of reduced NBT were increased significantly in normal and Hhcy rat hearts subjected to 30-min global ischemia and 20-min reperfusion (Figs. 2 and 3). However, the GSH was decreased in normal and Hhcy rat hearts subjected to I/R (Fig. 4). Moreover, Hhcy rat hearts showed high oxidative stress when compared with normal rat hearts subjected to I/R (Figs. 2–4). Four episodes of IPC markedly attenuated the I/R-induced oxidative stress in normal rat hearts as assessed in terms of reduction in TBARS and superoxide anion generation and consequent increase in the GSH. However, IPC-mediated reduction in oxidative stress against I/R was markedly abolished in Hhcy rat hearts (Fig. 2–4).

Global ischemia followed by reperfusion significantly increased LDH and CK levels in coronary effluent in normal and Hhcy rat hearts (Figs. 5 and 6). The maximum release of LDH was noted immediately after reperfusion, whereas peak release of CK occurred at 5th-min reperfusion. Further, I/R increased the infarct size in normal and Hhcy rat hearts (Fig. 7). Hhcy rat hearts also showed enhanced myocardial injury when compared with normal rat hearts subjected to I/R. IPC afforded cardioprotection in normal rat hearts by significantly attenuating I/R-induced myocardial injury as assessed in terms of reduction in LDH and CK levels and myocardial infarct size (Figs. 5 and 7). However, IPC-mediated cardioprotection against I/R injury was markedly abolished in Hhcy rat hearts. Global ischemia followed by reperfusion significantly decreased the

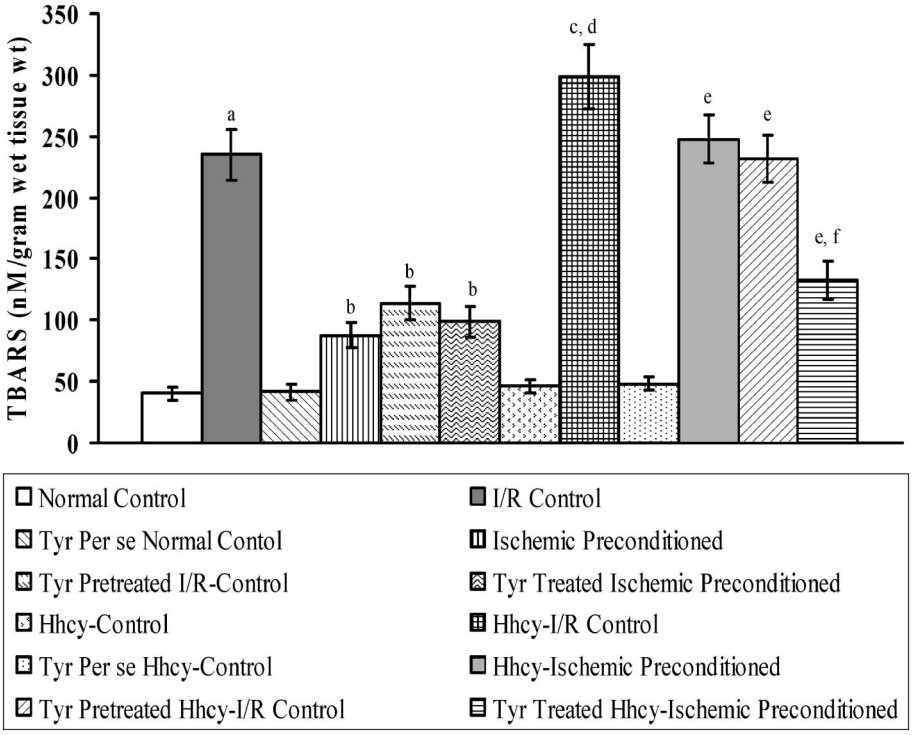

Fig. 2. Effect of Tyrphostin AG490 and IPC in I/R-induced Elevated TBARS Levels

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/RControl; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

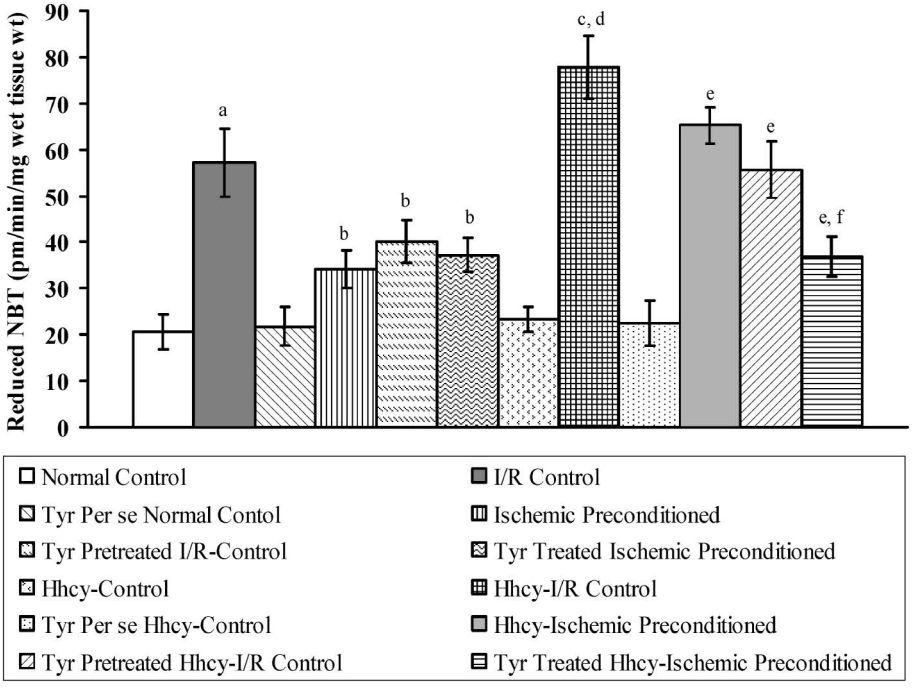

Fig. 3. Effect of Tyrphostin AG490 and IPC in I/R-induced Superoxide Anion Generation (Expressed as Reduced NBT)

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/RControl; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

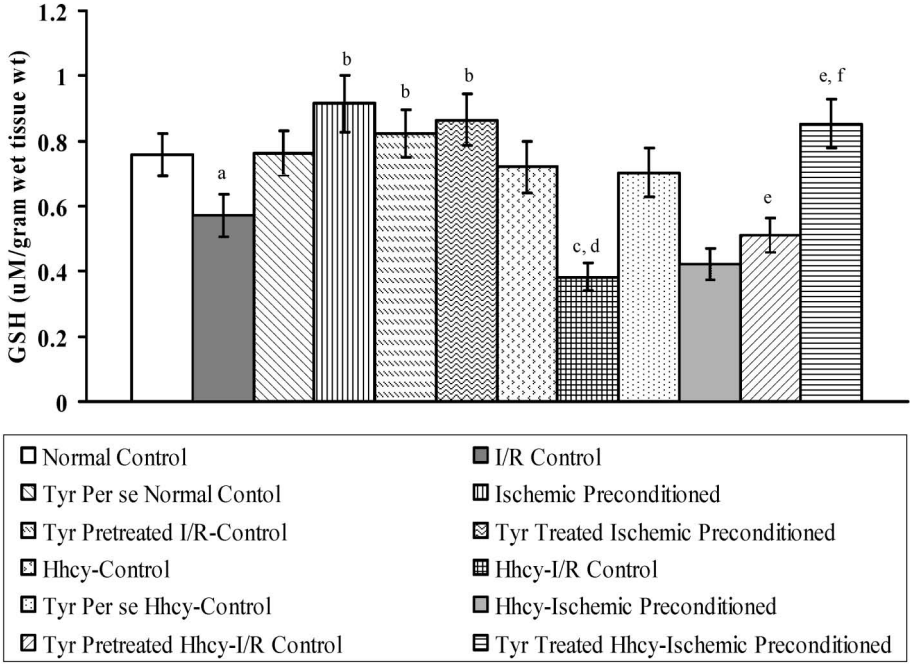

Fig. 4. Effect of Tyrphostin AG490 and IPC in I/R-induced Reduced GSH Level

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/R Control; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

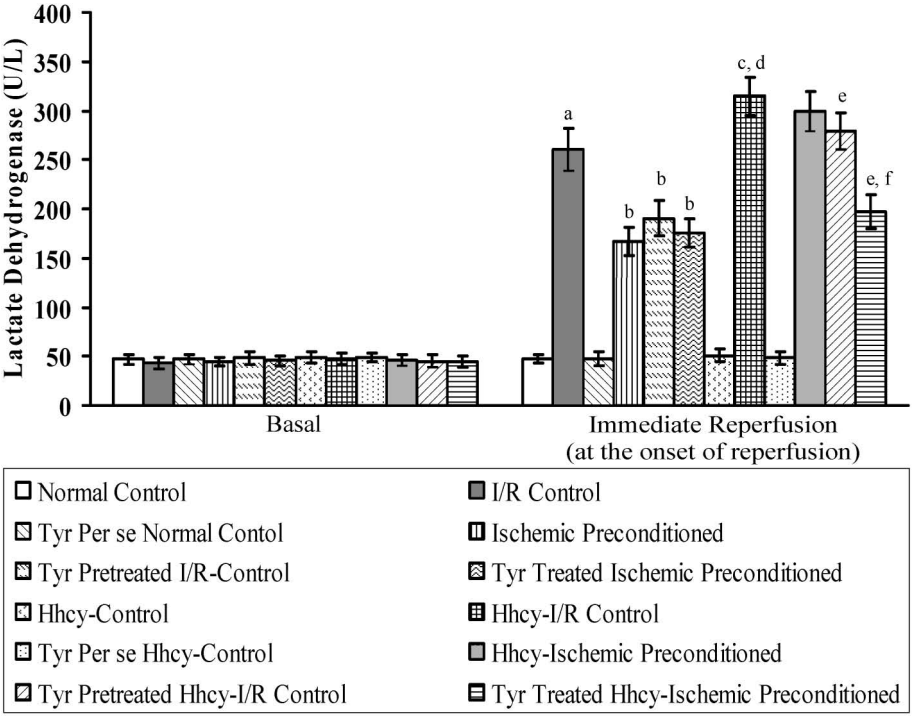

Fig. 5. Effect of Tyrphostin AG490 and IPC in I/R-induced Increase in LDH Level

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/R Control; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

amount of coronary perfuaste in normal and Hhcy rat hearts. In addition, Hhcy rat hearts showed a marked reduction in coronary perfusate when compared with normal rat hearts subjected to I/R. IPC significantly improved the coronary flow rate in normal rat hearts. On the other hand, IPC failed to im-

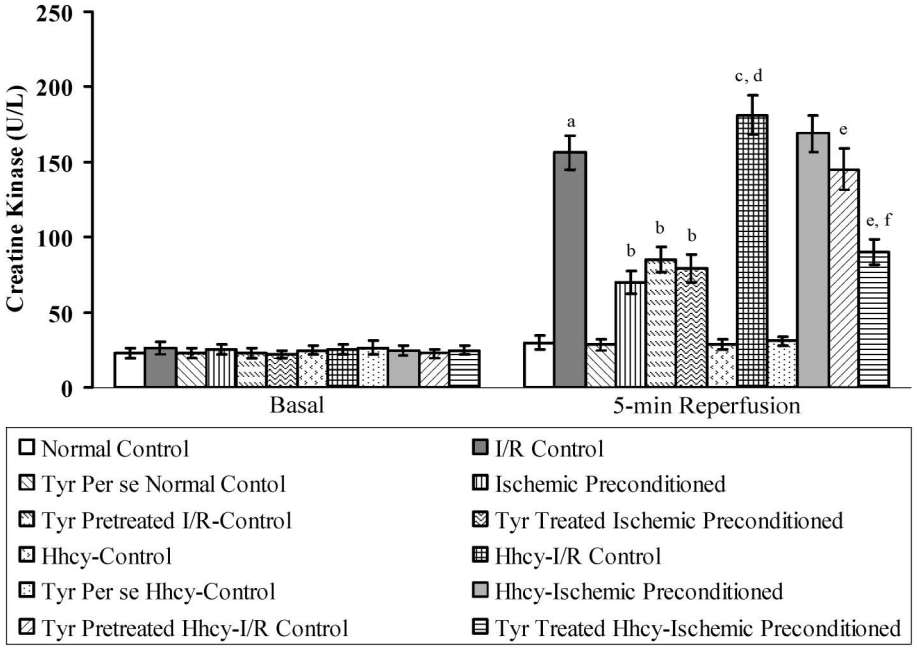

Fig. 6. Effect of Tyrphostin AG490 and IPC in I/R-induced Increase in CK Level

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/R Control; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

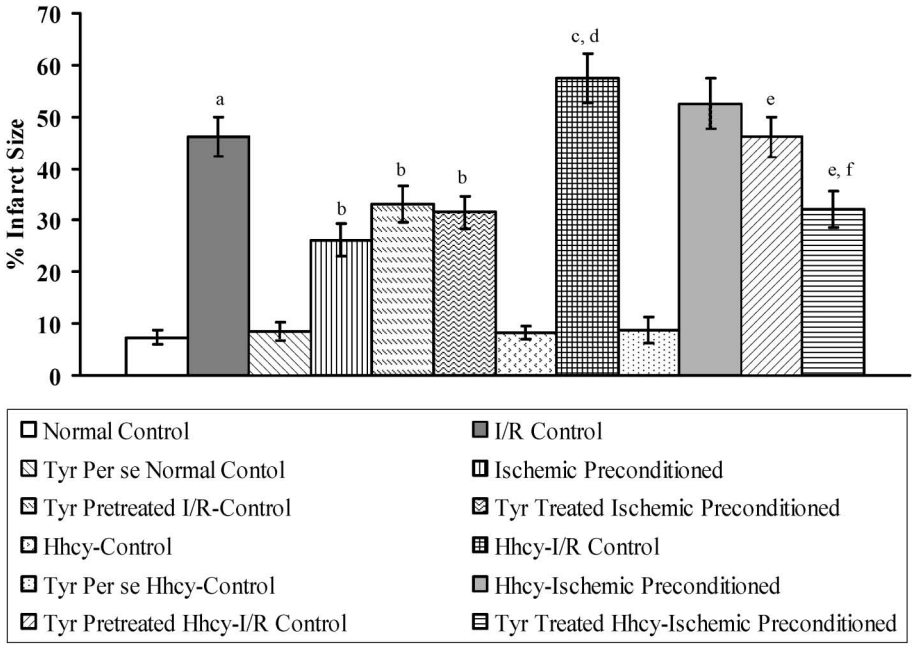

Fig. 7. Effect of Tyrphostin AG490 and IPC in I/R-induced Increase in Infarct Size

Values are expressed as mean±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/R Control; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

prove the coronary flow rate in Hhcy rat hearts subjected to I/R (Table 1).

Effects of Tyrphostin AG490 in I/R-induced Oxidative Stress and Myocardial Injury Hhcy rat hearts showed high oxidative stress and enhanced myocardial injury as compared with normal rat hearts subjected to 30-min ischemia and 20-min reperfusion. Treatment with Tyrphostin AG490 (5 μM) markedly reduced the oxidative stress produced as a result of I/R in normal rat hearts assessed in terms of reduction

in TBARS and superoxide anion generation and consequent increase in GSH, but Tyrphostin AG490 partially reduced the I/R-induced oxidative stress in Hhcy rat hearts (Fig. 2–4). Tyrphostin AG490 (5 μM) markedly reduced the I/R-induced myocardial injury in normal rat hearts assessed in terms of the reduction in myocardial infarct size, decrease in LDH and CK levels, and improvement in coronary flow rate. On the other hand, Tyrphostin AG490 (5 μM) partially reduced I/R-induced myocardial injury in Hhcy rat hearts (Figs. 5 and 7).

Effects of Tyrphostin AG490 in IPC-mediated Myocardial Protection in Normal Rat Hearts Pretreatment with Tyrphostin AG490 (5 μM) did not affect the IPC-induced attenuation of I/R-mediated oxidative stress in normal rat hearts. Moreover, its pretreatment did not modulate the IPC-induced reduction in infarct size, LDH and CK levels, and improvement in coronary flow rate (Table 1) in normal rat hearts subjected to I/R (Figs. 2 and 7).

Effects of Tyrphostin AG490 and Diazoxide in Abrogated Myocardial Protective Potential of IPC in Hhcy Rat Hearts Treatment with Tyrphostin AG490 (5 μM) did not alter the cardioprotective effects of IPC in normal rat hearts subjected to I/R. On the other hand, pretreatment with Tyrphostin AG490 markedly restored the cardioprotective potential of IPC in Hhcy rat hearts subjected to I/R as assessed in terms of improvement in coronary flow rate (Table 1) and reduction in myocardial infarct size, LDH and CK levels, and oxidative stress (Figs. 2–7). In addition, treatment with diazoxide (30 μM), an ATP-sensitive potassium (K_{ATP}) channel opener, also restored the cardioprotective effects of IPC in Hhcy rat hearts as assessed in terms of reduction in myocardial infarct size (54.1±4.24% *vs* 38.7±4.17%) (Hhcy-Ischemic Preconditioned *vs* Diazoxide Treated Hhcy-Ischemic Preconditioned), LDH (304.8±17.4 *vs* 217.4±14.9 U/L) and CK (172.4±9.21 *vs* 94.8±7.43 U/L).

DISCUSSION

The magnitude of CK-MB elevation has been shown to correlate strongly with infarct size.[46] LDH is an enzyme that increases in myocardial infarction after reperfusion, which may be due to sustained ischemic injury.[47] The increase in infarct size and the release of LDH and CK are an index of I/R-induced myocardial injury.[48,49] In the present study, 30-min ischemia followed by 120-min reperfusion produced myocardial injury as shown by increased infarct size in the heart and elevated release of LDH and CK in the coronary effluent, which were consistent with earlier reports.[41,48] The maximal release of LDH was noted immediately after reperfusion, whereas the peak release of CK was observed after 5 min of reperfusion, which are in agreement with the results of earlier studies.[47,49]

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals steal electrons from the lipids in cell membranes, resulting in cell damage. TBARS are low-molecular-weight end products, the main component of which is malondialdehyde, that are formed during the decom-

Table 1. Effect of Tyrphostin AG490 and IPC on Coronary Flow Rate (CFR) (ml/min)

Groups	Basal	0 min	5 min	30 min	120 min
Normal Control	6.9±0.76	7.1±0.77	6.9±0.72	6.7±0.73	6.5±0.69
I/R Control	7.1±0.73	2.6±0.31	4.4±0.54	3.6±0.31	2.9±0.32[a]
Tyr *Per se* Normal Control	7.2±0.68	7.2±0.69	7.5±0.81	7.2±0.79	6.8±0.77
Ischemic Preconditioned	7.2±0.79	4.8±0.54	5.4±0.58	5.1±0.58	4.9±0.45[b]
Tyr Pretreated I/R Control	7.4±0.77	4.3±0.41	5.1±0.55	4.8±0.45	4.4±0.57[b]
Tyr Treated Ischemic Preconditioned	6.9±0.72	4.5±0.52	5.3±0.63	4.9±0.56	4.6±0.55[b]
Hhcy-Control	7.4±0.81	7.3±0.79	7.5±0.82	7.2±0.78	6.9±0.79
Hhcy-I/R Control	7.2±0.76	2.9±0.33	3.7±0.42	3.4±0.44	2.1±0.29[c,d]
Tyr *Per se* Hhcy-Control	7.1±0.78	7.3±0.69	7.5±0.78	7.9±0.82	7.4±0.69
Hhcy-Ischemic Preconditioned	7.4±0.81	3.1±0.49	3.5±0.41	2.8±0.25	2.3±0.32
Tyr Pretreated Hhcy-I/R Control	7.5±0.79	4.1±0.47	4.2±0.52	3.5±0.42	3.1±0.39[e]
Tyr Treated Hhcy-Ischemic Preconditioned	6.8±0.72	4.2±0.39	5.1±0.55	4.8±0.57	4.7±0.52[e,f]

Values are expressed as mean ±S.D. a=$p<0.05$ *vs* Normal Control; b=$p<0.05$ *vs* I/R Control; c=$p<0.05$ *vs* Hhcy-Control; d=$p<0.05$ *vs* I/R Control; e=$p<0.05$ *vs* Hhcy-IR Control; f=$p<0.05$ *vs* Hhcy-Ischemic Preconditioned.

position of lipid peroxidation products. Malondialdehyde reacts with TBA to form a fluorescent red adduct known as TBARS, which is the index of lipid peroxidation.[50,51] Accumulation of insoluble blue-colored formazan complex (reduced NBT) is an indicator of oxidative stress associated with generation of ROS, in particular O_2^-.[52] GSH is the principal intracellular low molecular-weight thiol and plays a critical role in the cellular defense against oxidative stress by directly scavenging ROS. The increase in lipid peroxidation and superoxide anion generation has been suggested to be an indicator of oxidative stress.[53,54] Glutathione forms an essential component of defense against increased oxidative stress and is usually decreased in oxidative stress.[55] Lipid peroxidation measured in terms of TBARS and superoxide anion generation assessed in terms of reduced NBT increase and the glutathione level decreases as a result of 30-min ischemia and 20-min reperfusion. This suggests the development of I/R-induced oxidative stress, which may be responsible for the noted I/R-induced myocardial injury in the present study. In the present study, a marked increase in infarct size and release of LDH and CK were noted in Hhcy rat hearts when compared with normal rat hearts subjected to I/R. Administration of L-methionine (1.7 g/kg/day, *p.o.*) in rats for 4 weeks produced Hhcy, which is consistent with our recent reports.[31,43] Hhcy has been reported to produce high oxidative stress in the heart by activating NADPH oxidase-mediated ROS generation.[29,56] Moreover, Hhcy downregulates NO bioavailability by upregulating asymmetric dimethylarginine (ADMA).[57] When oxygen is reintroduced during reperfusion, the conversion of excess hypoxanthine to xanthine and xanthine to uric acid by xanthine oxidase results in the formation of ROS.[29] Thus the observed marked increase in myocardial injury in Hhcy rat hearts may be due to the development of high degree of oxidative stress. This contention is supported by the fact that a marked increase in lipid peroxidation and superoxide anion generation and subsequent decrease in glutathione level were noted in Hhcy rat hearts compared with normal rat hearts subjected to I/R. IPC has been documented to produce myocardial protection against I/R-induced myocardial injury.[3] The mechanisms involved in the cardioprotective potential of IPC are activation of PI3K/Akt and eNOS, release of NO, closure of the MPTP, opening of mito K_{ATP} channels, and reduction in reperfusion-induced oxidative stress.[11,58] It has been noted that κ-opioid and δ-opioid receptors are involved in IPC-mediated reduction in infarction.[59] It has been reported that opioid receptors, like other PKC-receptors, participate in the triggering of preconditioning in the myocardium.[60] Moreover, PKC-ε and PKC-δ isoforms have been found to play a vital role in preconditioning-mediated attenuation of apoptosis and necrosis.[61,62] The opening of mito K_{ATP} channels is an important component of IPC-mediated cardioprotection in rat hearts.[63–66] It was reported that opening of mito K_{ATP} channels by diazoxide causes cardioprotection by potentiating ischemic inhibition of mitochondrial ATP synthase [F(1)F(0)-ATPase], which indicates that mito K_{ATP} channel opening and IPC share common mechanisms of cardioprotection.[67]

In the present study, IPC reduced I/R-induced myocardial injury in normal rat hearts as assessed in terms of reductions in infarct size, release of LDH and CK, and oxidative stress. However, the cardioprotective effects of IPC were not observed in Hhcy rat hearts with a high degree of oxidative stress. Thus it is strongly suggested that the high degree of oxidative stress developed in Hhcy rat hearts may be responsible for the observed paradoxical effects of IPC. Pretreatment with Tyrphostin AG490 (5 μM), a selective JAK-2 inhibitor did not alter the cardioprotective effects of IPC in normal rat hearts, but Tyrphostin AG490 pretreatment significantly restored the cardioprotective effects of IPC in Hhcy rat hearts. Tyrphostin AG490 has been reported to be a selective inhibitor of JAK-2.[36–38] Tyrphostin AG490 in the concentration of 5 μM has been reported to selectively inhibit JAK-2 in rat hearts.[36,68] Thus it is suggested that JAK-2 activation during reperfusion of the ischemic myocardium may play a pivotal role in the attenuation of the cardioprotective potential of IPC in Hhcy rat hearts. The signaling mechanisms such as activation of PI3K/Akt, subsequent activation of eNOS, and generation of NO have been implicated in IPC-mediated cardioprotection. Studies suggested that Hhcy downregulates eNOS and reduces the generation and bioavailability of NO by increasing oxidative stress.[28–30] Further, activation of JAK-2 has been reported to inhibit PI3K/Akt pathway.[35] Since Tyrphostin AG490 restores the cardioprotective effects of IPC in Hhcy rat hearts, JAK-2 may mediate the inactivation of the PI3K/Akt-eNOS pathway in

Hhcy rat hearts and be responsible for abolishing the cardioprotective potential of IPC. JAK-2 has been demonstrated to be activated by ROS.[33] It has been demonstrated that JAK/STAT signaling plays a role in the activation of IPC-induced survival signal in the myocardium subjected to I/R.[69–73] JAK-2 is required for erythropoietin-induced cell division and maturation, which play a role in cytoprotective signalling.[74,75] However, subsequent studies revealed the detrimental role of JAK-2 in cardiovascular function.[32,36] Reperfusion-induced ROS activates JAK-2 in the heart.[36] JAK-2 thus activated by ROS is associated with cardiac dysfunction during I/R.[36] Moreover, JAK-2 has been shown to increase the expression of proinflammatory cytokines and adhesion molecules like intracellular cell adhesion molecule-1 (ICAM-1)[76] and to be involved in oxidative stress-induced apoptotic cell death.[32,36] Thus the generation of high amounts of ROS in Hhcy rat hearts during reperfusion may activate JAK-2, which may abolish the cardioprotective effects of IPC, possibly by inhibiting the IPC-mediated cardioprotective PI3K/Akt/eNOS pathway. This contention is supported by the results obtained in the present study that pretreatment with Tyrphostin AG490 restored the cardioprotective and infarct size-limiting properties of IPC in Hhcy rat hearts. Opening of mito K_{ATP} channels is a major event considered to mediate the cardioprotective effects of IPC.[77] In the present study, treatment with diazoxide (30 μM), a K_{ATP} channel opener, also restored the cardioprotective effects of IPC in Hhcy rat hearts. This suggests that the closure of K_{ATP} channels occurs as a result of Hhcy, which may be an important abnormality in the downstream signaling mechanism of IPC in Hhcy rat hearts.

It may be concluded that the high oxidative stress produced in Hhcy rat hearts during reperfusion and consequent activation of JAK-2 and closure of K_{ATP} channels may be responsible for abolishing the cardioprotective potential of IPC against I/R-induced myocardial injury.

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