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Research Article

PROBABLE ROLE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR-ALPHA IN ATTENUATED CARDIOPROTECTIVE EFFECT OF ISCHEMIC PRECONDITIONING IN HYPERLIPIDEMIC RAT HEARTS

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ABSTRACT

Objectives: The present study has been designed in order to investigate the probable role of peroxisome proliferator activated receptor-alpha (PPAR-α) in hyperlipidemia-induced attenuation of cardioprotective effect of ischemic preconditioning (IPC).

Methods: Experimental hyperlipidemia was produced by feeding high fat diet to rats for a period of 28 days. Isolated langendorff's perfused normal and hyperlipidemic rat hearts were subjected to global ischemia for 30 min followed by reperfusion for 120 min. The myocardial infarct size was assessed macroscopically using triphenyltetrazolium chloride (TTC) staining. Coronary effluent was analyzed for lactate dehydrogenase (LDH) and creatine kinase (CK) release to assess the extent of cardiac injury. Moreover, the oxidative stress in heart was assessed by measuring thiobarbituric acid reactive substance (TBARS), superoxide anion generation and reduced form of glutathione (GSH).

Results: The ischemia-reperfusion (I/R) has been noted to induce oxidative stress by increasing TBARS, superoxide anion generation and decreasing reduced form of glutathione in normal and hyperlipidemic rat hearts. Moreover, I/R produced myocardial injury, which was assessed in terms of increase in myocardial infarct size, LDH and CK release in coronary effluent and decrease in coronary flow rate in normal and hyperlipidemic rat hearts. In addition, the hyperlipidemic rat hearts showed enhanced I/R-induced myocardial injury with 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, LDH, CK and oxidative stress. On the other hand, IPC mediated myocardial protection against I/R-injury was abolished in hyperlipidemic rat hearts. Treatment with Fenofibrate (100 mg/kg/day, i.p.), an activator of PPAR-α has not affected the cardioprotective effect of IPC in normal rat hearts, but its treatment markedly restored the cardioprotective potential of IPC in hyperlipidemic rat hearts.

Conclusion: It is suggested that the high degree of oxidative stress produced in hyperlipidemic rat heart during reperfusion and consequent down-regulation of PPAR-α may be responsible to abolish the cardioprotective potential of IPC against I/R induced myocardial injury.

Keywords: Hyperlipidemia, Ischemia-reperfusion injury, Ischemic preconditioning, Fenofibrate, Proliferator activated receptor-alpha.

INTRODUCTION

Coronary artery disease is the leading cause of morbidity and mortality, and its prevalence is continuously increasing worldwide [1]. Myocardial ischemia is a condition in which heart tissue gets inadequate blood flow, followed by inadequate oxygen and nutrient supply. The restoration of coronary blood flow to an ischemic myocardium is mandatory in order to avoid the myocardial damage. However, reperfusion of the previously ischemic myocardium is often, followed by detrimental changes in myocardial tissues, is 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) [3,4]. IPC has been noted to reduce I/R-induced myocardial injury by decreasing oxidative stress, limiting myocardial infarct size, decreasing neutrophil polymorphonuclear leukocytes accumulation, preserving coronary endothelial function and inhibiting apoptosis and necrosis [4-7]. Various mechanisms involved in the cardioprotective potential of IPC include activation of phosphatidylinositol-3-kinase (PI3K)/Akt pathway, generation of nitric oxide (NO), activation of mitochondrial ATP-sensitive K^+ channels (mito K_{ATP} channels) and closure of mitochondrial permeability transition pore (MPTP) [4,8,9]. However, the cardioprotective and infarct size limiting effect of IPC has been abolished in some pathological conditions such as diabetes, obesity, heart failure, hyperlipidemia (Hpl), ageing and hypertension [10-12]. Hpl, a condition of an elevated level of lipids and triglycerides in the blood, has been considered to be an independent risk factor for cardiovascular diseases [13,14]. Hpl has been shown to generate a high amount of reactive oxygen species (ROS) by activating nicotinamide adenine dinucleotide (NAD) phosphate oxidase [14-16]. It has been recently reported that Hpl decreased the eNOS mRNA expression, followed by increased oxidative stress and decreased bioavailability of NO occur to damage the vascular endothelium [17]. We have noted that the cardioprotective potential of IPC was abolished in the hyperlipidemic rat heart. However, the mechanism involved in the attenuation of cardioprotective effect of IPC in the hyperlipidemic rat heart is not known. We have noted that the hyperlipidemic rat heart produced a high degree of oxidative stress upon reperfusion when compared with the normal rat heart subjected to I/R. Thus, it was believed that the signaling mechanisms activated by high degree of oxidative stress may play a detrimental role in the attenuation of cardioprotective effect of IPC in the hyperlipidemic rat heart.

Peroxisome proliferator-activated receptor-α (PPAR-α) is a subfamily of the nuclear receptor superfamily naturally activated by ligands such as free fatty acids and eicosanoids [18]. PPARs are ligand-activated transcriptional factors that regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis. It has been reported that PPAR-α gets down regulated during high amount of oxidative stress [19,20]. Further, PPAR-α has been noted to activate PI3K/Akt pathway and activation

of PI3K/Akt pathway has been previously well-demonstrated to be involved in the cardioprotective effect of IPC [21,22]. Moreover, PPAR-α downregulation has been implicated in the pathogenesis of I/R-induced myocardial injury. Fenofibrate has been shown to be a selective activator of PPAR-α [23]. Therefore, the present study has been designed to investigate the effect of fenofibrate, an activator of PPAR-α, in the abrogated cardioprotective effect of IPC in hyperlipidemic rat hearts subjected to I/R.

MATERIALS AND METHODS

Wistar albino rats of either sex weighing about 180-220 g were employed in the present study. They were fed on standard chow diet (Ashirwad Industries Private Ltd., Punjab, India) and were provided water ***ad libitum***. They were housed in departmental animal house and were exposed to 12 hrs light and dark cycles. All animals were maintained as per the guidelines for the care and use of laboratory animals. The study protocol was approved by Institutional Animal Ethics Committee.

Diet-induced Hpl

Experimental Hpl was produced by feeding high-fat diet (casein, 200 g; coconut oil, 250 g; cholesterol, 10 g; cholic acid, 5 g; sucrose, 484 g; choline chloride, 2 g; DL-methionine, 4 g; vitamin mix, 10 g; mineral mix, 35 g were added to make 1.0 kg of diet) to rats for a period of 28 days [24]. Mineral mix was composed of NaCl, 5.57 g; KCl, 32 mg; $MgSO_4$, 2.29 g; $FeSO_4.7H_2O$, 108 g; $CaHPO_4$, 70 mg; $CuSO_4.5H_2O$, 0.1 mg; $MnSO_4.H_2O$, 0.01 mg; $ZnSO_4.H_2O$, 28.7 mg; KI, 0.025 mg; $COCl_2.6H_2O$, 9 mg and MgO, 0.15 mg. Moreover, vitamin mix was comprised of retinol acetate, 5000 IU; cholecalciferol, 400 IU; 7-dehydrocholesterol, 2000 IU; tocopheryl acetate, 15 mg; thiamine hydrochloride, 5 mg; riboflavin, 5 mg; nicotinamide, 45 mg; D-panthenol, 5 mg; pyridoxine hydrochloride, 2 mg; ascorbic acid, 75 mg; folic acid, 1000 µg and cyanocobalamin, 5 µg.

Assessment of diet-induced Hpl

Hpl was determined by estimating the levels of total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), Very LDL (VLDL) and triglycerides in blood serum using commercially available kits. Values were expressed in mg/dl.

Estimation of serum total cholesterol and HDL levels

Serum total cholesterol and HDL levels were estimated spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 505 nm by the method of Allain ***et al.*** (1974) using commercially available kit (Monozyme India Ltd., Secunderabad, India).

Total cholesterol level

Serum total cholesterol = Abs. of cholesterol test/Abs. of standard × 200

HDL level

Serum HDL level = Abs. of HDL test/Abs. of standard × 50

Estimation of serum triglyceride levels

Serum triglycerides were estimated spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 546 nm by enzymatic glycerol phosphate oxidase/peroxidase method (Werner ***et al.***, 1981) using commercially available kit (Kamineni Life Sciences Private Ltd., Hyderabad, India).

Triglyceride level

Serum triglyceride levels (mg/dl) = Abs. of test/Abs. of standard × 200

Estimation of VLDL and LDL levels

VLDL and LDL concentrations were calculated from the Friedewald equation [25].

VLDL level

Serum VLDL levels (mg/dl) = triglyceride level/5, and

LDL level

Serum LDL levels (mg/dl) = total cholesterol-(HDL level+VLDL level).

Isolated rat heart preparation

Heparin (500 U; i.p.) was administered about 20 minutes before sacrificing the animal by cervical dislocation. The heart was rapidly excised and immediately mounted on Langendorff apparatus [26]. The heart was enclosed in a double walled jacket, and the temperature of which was maintained at 37°C by circulating warm water. The preparation was perfused with Krebs Henseleit (K-H) solution (NaCl 118 mM; KCL 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4.7H_2O$ 1.2 mM; $NaHCO_3$ 25mM; KH_2PO_4 1.2 mM; $C_6H_{12}O_6$ 1 mM) of pH 7.4, maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The coronary flow rate (CFR) was maintained at around 7 ml/minutes by keeping the perfusion pressure at 80 mmHg. Global ischemia was produced for 30 minutes by blocking the inflow of physiological solution, and it was followed by reperfusion of 120 minutes after 10 minutes of stabilization. The CFR was noted at basal (before global ischemia), 0 minute (at the onset of reperfusion), 5 minutes, 30 minutes and 120 minutes of reperfusion.

IPC

Langendorff's perfused normal and hyperlipidemic hearts were subjected to four episodes of ischemia, followed by reperfusion, each comprising of 5 minutes occlusion and 5 minutes reperfusion, to produce IPC.

Assessment of myocardial injury

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

Estimation of LDH and CK-MB

The myocardial injury was assessed by measuring the release of LDH and CK-MB in the coronary effluent using the commercially available enzymatic kits (Vital Diagnostics, Thane, Maharastra, India). LDH was measured in the coronary effluent by 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 increase in absorbance due to a reduction of NAD. The LDH activity is expressed in U/L using the formula: LDH activity (U/L) = ΔA/minute × 3376. CK-MB was measured in the coronary effluent by immunoinhibition method, which is based on the principle that CK-M fraction of CK-MM in the sample is completely inhibited by CK-M antibody present in the reagent. Then, the activity of CK-B fraction is measured, and the CK-MB activity is expressed in U/L using the formula: CK-MB activity (U/L) = ΔA/minute × 6752.

Infarct size measurement

Hearts were removed from Langendorff's apparatus. Both auricles, root of aorta and right ventricle were excised and left ventricle was kept overnight at −4°C. Frozen ventricle was sliced into uniform sections of 2-3 mm in thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) solution in 0.1 M tris buffer, of pH 7.8, for 20 minutes at 37°C. 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. The infarcted cell that has lost dehydrogenase enzyme remains 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 volume method [27].

Assessment of oxidative stress

The left ventricle was minced and homogenized in 0.05 M ice cold phosphate buffer (pH 7.4) using a teflon homogenizer. The clear supernatant of homogenate was used to estimate thiobarbituric acid reactive substance (TBARS) and reduced form of glutathione (GSH).

Estimation of TBARS

The quantitative measurement of TBARS, an index of lipid peroxidation in heart was performed according to the method of Ohkawa ***et al.*** (1979). 0.2 ml of the supernatant homogenate was pipetted out in a test tube, followed by addition of 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 30% acetic acid (pH 3.5) and 1.5 ml of 0.8% of thiobarbituric acid and the volume was made up to 4 ml with distilled water. The test tubes were incubated for 1 hr at 95°C, then cooled and added 1 ml of distilled water, followed by addition of 5 ml of n-butanol-pyridine mixture (15:1 v/v). The test tubes were centrifuged at 4000 g for 10 minutes. The absorbance of developed pink color was measured spectrophotometrically (Thermo Double Beam Spectrophotometer, Thermo Electron Corporation, United Kingdom) 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 [28].

Estimation of superoxide anion generation

The heart was cut into transverse sections and placed in 5 ml of K-H solution buffer containing 100 μM of nitroblutetrazolium (NBT) and incubated at 37°C for 1.5 hrs. 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 40 mg/L di-ethylene triamine pentaacetic acid. The mixture was centrifuged at 20000 g for 20 minutes, and the resultant pellets were resuspended in 1.5 ml of pyridine and kept at 80°C for 1.5 hrs to extract formazon. The mixture was centrifuged at 10,000 g for 10 minutes, and the absorbance of formazon was determined spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) 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 minutes), Wt is blotted wet weight of the 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 in picomoles per minutes per milligram of wet tissue [29].

Estimation of reduced GSH

The reduced (GSH) content in heart was estimated using the method of Beutler ***et al.*** (1963). The supernatant of homogenate was mixed with trichloroacetic acid (10% w/v) in 1:1 ratio. The tubes were centrifuged at 1000 g for 10 minutes at 4°C. The supernatant obtained (0.5 ml) was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25 ml of 0.001 M freshly prepared (5,5'-dithiobis(2-nitrobenzoic acid)[DTNB] dissolved in 1% w/v citric acid) was added and the absorbance was noted spectrophotometrically (UV1 Spectrophotometer, Thermo Electron Corporation, England) at 412 nm. The standard curve was plotted using 5-50 μM of reduced form of GSH and results were expressed as micromoles of reduced GSH per milligram of wet tissue weight [30].

Experimental protocol

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

Group I (Normal control): Isolated normal rat heart was perfused for 150 minutes using K-H solution after 10 minutes of stabilization.

Group II (I/R-control): Isolated normal rat heart after 10 minutes of stabilization was subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion.

Group III (fenofibrate ***per se*** normal control): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was perfused for 150 minutes using K-H solution after 10 minutes stabilization.

Group IV (Ischemic preconditioned): After 10 minutes of stabilization, the normal rat heart was subjected to four episodes each comprised of 5 minutes of global ischemia, followed by 5 minutes of reperfusion to produce IPC. After four episodes of IPC, the heart was subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion.

Group V (fenofibrate treated I/R-control): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was then subjected to 30 minutes of global ischemia followed by 120 minutes of reperfusion after 10 minutes of stabilization.

Group VI (fenofibrate treated ischemic preconditioned): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated normal rat heart was subjected to IPC as mentioned in Group IV, followed by 30 minutes of global ischemia and 120 minutes of reperfusion.

Group VII (Hpl control): Isolated hyperlipidemic rat heart was perfused for 150 minutes using K-H solution after 10 minutes of stabilization.

Group VIII (Hpl-I/R control): Isolated hyperlipidemic rat heart was subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion after 10 minutes of stabilization.

Group IX (fenofibrate ***per se*** Hpl-control): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hyperlipidemic rat heart was perfused for 150 minutes using K-H solution after 10 minutes stabilization.

Group X (Hpl-Ischemic preconditioned): After 10 minutes of stabilization, the hyperlipidemic rat heart was subjected to IPC as mentioned in Group IV. After IPC, the heart was subjected to 30 minutes of global is chemia followed by 120 minutes of reperfusion.

Group XI (fenofibrate treated Hpl-I/R control): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hyperlipidemic rat heart was then subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion after 10 minutes of stabilization.

Group XII (fenofibrate treated Hpl-ischemic preconditioned): The rat was given fenofibrate (100 mg/kg/day, i.p.) for 2 weeks. After 2 weeks, the isolated hyperlipidemic rat heart was subjected to IPC as mentioned in Group IV, followed by 30 minutes of global ischemia and 120 minutes of reperfusion.

Statistical analysis

The results were expressed in mean±standard deviation. The data obtained from various groups were statistically analyzed using two-way ANOVA, followed by Tukey's multiple comparison test. $p<0.05$ were considered as statistically significant.

Drugs and chemicals

Fenofibrate was obtained from Ranbaxy Pvt. Ltd. India as ex-gratia samples. The LDH and CK-MB enzymatic estimation kits were purchased from vital diagnostics, Thane, Maharashtra, India. DTNB and NBT were obtained from Loba Chem, Mumbai, India. 1,1,3,3-tetramethoxy propane and reduced GSH were procured from Sigma-Aldrich, USA. HDL kits purchased from Monozyme Ltd., Secunderabad, India. Serum Triglyceride kits purchased from Kamineni Life Sciences Private Ltd., Hyderabad, India. TTC stain and high-fat diet purchased from Sanjay Biological, Amritsar, Punjab, India. All other reagents used in this study were of analytical grade.

RESULTS

Rat fed with high-fat diet for 28 days with oral gavage significantly increased serum concentrations of total cholesterol (272.1±24.4*),

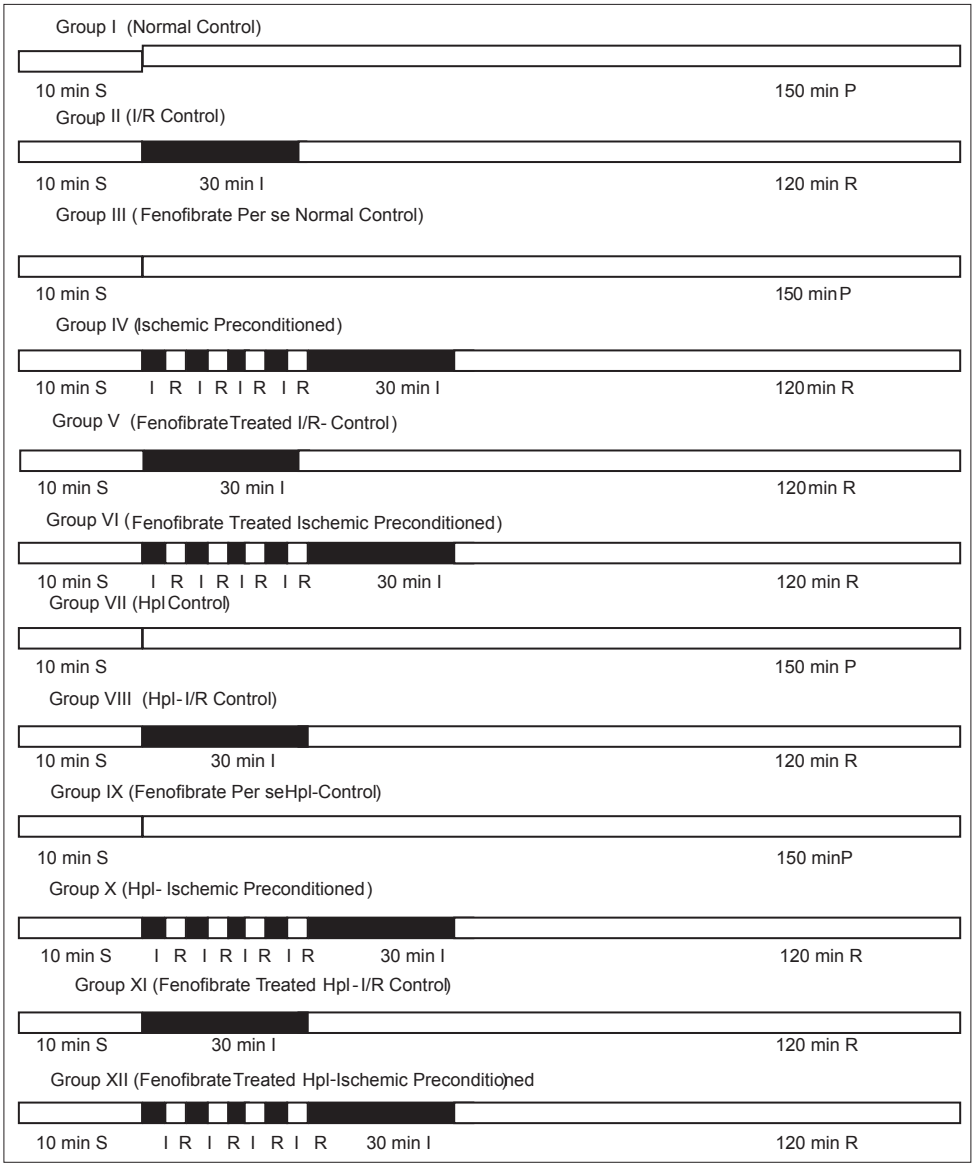

Fig. 1: Diagrammatic representation of experimental protocol: S indicates Stabilization; I indicates global ischemia; R indicates reperfusion with K-H solution; I/R indicates ischemia-reperfusion injury; Ischemic preconditioned indicates ischemic preconditioned normal rat heart; Hpl indicates hyperlipidemia

triglycerides (258.7±19.7*), LDL (190.86±18.8*), VLDL (51.74±5.2*) and HDL (29.5±4.1*) levels (mg/dl) leads to Hpl when compared with normal rats. Moreover, the serum concentration of HDL was significantly reduced in rats fed with high-fat diet for 28 days (Table 1).

The lipid peroxidation measured in terms of increased TBARS and superoxide anion generation with consequent decrease in GSH were noted in hyperlipidemic rat hearts subjected to 30 minutes of global ischemia and 120 minutes of reperfusion, as compared to normal (Tables 2-4). Moreover, hyperlipidemic rat hearts showed high oxidative stress when compared with normal rat hearts subjected to I/R (Tables 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 the consequent increase in reduced GSH. However, IPC mediated reduction in oxidative stress against I/R was markedly abolished in hyperlipidemic rat hearts (Tables 2-4).

Global ischemia followed by reperfusion significantly increased LDH and CK-MB levels in the coronary effluent in normal and hyperlipidemic rat hearts (Tables 5 and 6). Maximum release of LDH was noted immediately after reperfusion, whereas peak release of CK-MB was noted at 5 minutes of reperfusion. Further, I/R was noted to increase the infarct size in normal and hyperlipidemic rat hearts (Table 7). Moreover, hyperlipidemic rat hearts showed enhanced myocardial injury when compared with normal rat hearts subjected to I/R. The 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-MB levels and myocardial infarct

Table 1: Effect of high fat diet on serum lipid profile

S. no	Cholesterol	Normal control	High fat diet treated rats (hyperlipidemic rats)
1	Total cholesterol	98.22±8.2	272.1±24.4*
2	Triglycerides	107.25±8.9	258.7±19.7*
3	LDL	28.97±3.2	190.86±18.8*
4	VLDL	21.45±2.6	51.74±5.2*
5	HDL	47.8±4.3	29.5±4.1*

*p<0.05 versus control, LDL: Low density lipoprotein, HDL: High density lipoprotein, VLDL: Very low density lipoprotein

Table 2: Effect of fenofibrate and IPC in I/R-induced increase in TBARS level

Groups	TBARS (nM/g wet tissue weight)
Normal control	30.4±3.5
I/R control	76.7±5.7[a]
Feno ***per se*** normal control	36.4±3.1
IPC control	52.3±4.5[b]
Feno treated I/R control	52.4±4.1[b]
Feno treated IPC	57.8±3.3[b]
Hpl-control	38.7±3.3
Hpl-I/R control	99.8±6.8[c, d]
Feno ***per se*** Hpl-control	47.1±3.8
Hpl- IPC control	93.4±6.3
Feno treated Hpl-I/R control	83.3±5.5[e]
Feno treated Hpl-IPC	70.8±5.4[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning, TBARS: Thiobarbituric acid reactive substance

Table 3: Effect of fenofibrate and IPC in I/R-induced increase in superoxide anion level (expressed as reduced NBT)

Groups	Reduced NBT (pM/minute/mg wet tissue weight)
Normal control	19.8±2.2
I/R control	65.4±5[a]
Feno ***per se*** normal control	22.7±2.4
IPC control	40.2±3.8[b]
Feno treated I/R control	41.3±3.2[b]
Feno treated IPC	39.8±3.5[b]
Hpl-control	22.1±2
Hpl-I/R control	87.5±5[c, d]
Feno ***per se*** Hpl-control	24.1±2.6
Hpl-IPC control	78.1±5.1
Feno treated Hpl-I/R control	64.1±4.2[e]
Feno treated Hpl- IPC	53.1±3.5[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning, NBT: Nitroblutetrazolium

Table 4: Effect of fenofibrate and IPC in I/R-induced decrease in reduced GSH level

Groups	GSH (uM/mg wet tissue weight)
Normal control	0.763±0.033
I/R control	0.598±0.041[a]
Feno ***per se*** normal control	0.755±0.036
IPC control	0.892±0.045[b]
Feno treated I/R control	0.941±0.041[b]
Feno treated IPC	0.963±0.032[b]
Hpl-control	0.797±0.051
Hpl-I/R control	0.473±0.049[c, d]
Feno ***per se*** Hpl-control	0.679±0.043
Hpl-IPC control	0.539±0.046
Feno treated Hpl-I/R control	0.629±0.03[e]
Feno treated Hpl-IPC	0.866±0.04[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning, GSH: Glutathione

size (Tables 5 and 6). However, the IPC mediated the cardioprotection against I/R-injury was markedly abolished in hyperlipidemic rat hearts. Global ischemia, followed by reperfusion significantly decreased the amount of coronary perfuaste in normal and hyperlipidemic rat hearts

Table 5: Effect of fenofibrate and IPC in I/R-induced increase in CK-MB level

Groups	CK-MB (U/L)
Normal control	33.2±5.8
I/R control	166.5±11.1[a]
Feno ***per se*** normal control	38.1±4
IPC control	71.5±9[b]
Feno treated I/R control	75.6±6.6[b]
Feno treated IPC	81.2±6.9[b]
Hpl-control	29.9±5.6
Hpl-I/R control	199.2±15.1[c, d]
Feno ***per se*** Hpl-control	35.3±4.7
Hpl-IPC control	177.6±14.8
Feno treated Hpl-I/R control	141.3±8.9[e]
Feno treated Hpl-IPC	104.5±6.5[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning, CK: Creatine kinase

Table 6: Effect of fenofibrate and IPC in I/R-induced increase in LDH level

Groups	LDH (U/L)
Normal control	35.8±5.3
I/R control	255.1±14.9[a]
Feno ***per se*** normal control	38.3±4
IPC control	178.6±12.8[b]
Feno treated I/R control	191.3±13.7[b]
Feno treated IPC	182.6±15.3[b]
Hpl-control	40.3±6.1
Hpl-I/R control	292.1±18.9[c, d]
Feno ***per se*** Hpl-control	47.6±3.9
Hpl-IPC control	267.7±18.5
Feno treated Hpl-I/R control	230.6±15.8[e]
Feno treated Hpl-IPC	200.4±9.5[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning, LDH: Lactate dehydrogenase

Table 7: Effect of fenofibrate and IPC in I/R-induced increase in infarct size

Groups	Percentage of infarct size
Normal control	8±1.2
I/R control	47.5±3.1[a]
Feno ***per se*** normal control	8.1±2.1
IPC control	23.4±2.6[b]
Feno treated I/R control	25.7±2.3[b]
Feno treated IPC	23.6±1.8[b]
Hpl-control	8.8±1.6
Hpl-I/R control	59.2±4.8[c, d]
Feno ***per se*** Hpl-control	7.9±2.2
Hpl-IPC control	51.2±4.5
Feno treated Hpl-I/R control	40.4±3.1[e]
Feno treated Hpl-IPC	35.2±2.9[e, f]

Values are expressed as mean±SD. [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-IPC. SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning

(Table 8). In addition, the hyperlipidemic rat hearts showed a marked reduction in the coronary perfusate when compared with normal rat hearts (Table 8). The IPC significantly improved the CFR in normal rat hearts. On the other hand, the IPC has failed to improve the CFR in hyperlipidemic rat hearts subjected to I/R (Table 8).

Table 8: Effect of fenofibrate and IPC on CFR (ml/minute)

Groups	Basal	0 minute	5 minute	30 minute	120 minute
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]
Feno ***per se*** normal control	7.1±0.68	7.0±0.68	7.3±0.79	6.9±0.76	6.5±0.70
IPC control	7.2±0.79	4.8±0.54	5.4±0.58	5.1±0.58	4.9±0.45[b]
Feno treated I/R control	7.1±0.75	4.7±0.41	5.5±0.54	5.2±0.42	4.9±0.54[b]
Feno treated IPC	7.1±0.71	4.7±0.51	5.7±0.61	5.3±0.53	5.0±0.54[b]
Hpl-control	7.2±0.81	7.1±0.79	7.2±0.82	7.0±0.78	6.9±0.79
Hpl-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]
Feno ***per se*** Hpl-control	7.0±0.76	7.2±0.68	7.1±0.77	7.2±0.83	7.2±0.68
Hpl-IPC control	7.3±0.81	3.1±0.49	3.5±0.41	2.8±0.25	2.3±0.32
Feno treated Hpl-I/R control	7.1±0.77	4.1±0.47	4.4±0.52	3.7±0.41	3.0±0.38[e]
Feno treated Hpl-IPC	7.1±0.69	5.4±0.30	5.7±0.53	5.4±0.55	5.3±0.51[e, f]

Values are expressed as mean±SD, [a]p<0.05 versus normal control, [b]p<0.05 versus I/R control, [c]p<0.05 versus Hpl-control, [d]p<0.05 versus I/R control, [e]p<0.05 versus Hpl-IR control, [f]p<0.05 versus Hpl-ischemic preconditioned. CFR: Coronary flow rate, SD: Standard deviation, I/R: Ischemia-reperfusion, Hpl: Hyperlipidemia, IPC: Ischemic preconditioning

Effect of fenofibrate in I/R-induced oxidative stress and myocardial injury in normal and hyperlipidemic rat hearts

Hyperlipidemic rat hearts showed a high degree of oxidative stress and enhanced myocardial injury when compared with normal rat hearts subjected to I/R. Treatment with fenofibrate (100 mg/kg/day, i.p., for 2 weeks) markedly reduced the oxidative stress produced as a result of I/R in normal rat hearts as assessed in terms of reduction in TBARS and superoxide anion generation and consequent increase in reduced form of GSH; but its treatment partially reduced the I/R-induced oxidative stress in hyperlipidemic rat hearts (Tables 2-4). Treatment with fenofibrate (100 mg/kg/day, i.p., for 2 weeks) markedly reduced the I/R-induced myocardial injury in normal rat hearts as assessed in terms of reduction in myocardial infarct size, decrease in LDH and CK-MB levels and improvement in CFR (Table 8). On the other hand, fenofibrate treatment partially reduced I/R-induced myocardial injury in hyperlipidemic rat hearts (Tables 5-7).

Effect of fenofibrate IPC-mediated myocardial protection in normal rat hearts

Pretreatment with fenofibrate (100 mg/kg/day, i.p., for 2 weeks) has not affected the IPC-induced attenuation I/R-mediated oxidative stress in normal rat hearts. Moreover, its pretreatment has not modulated the IPC-induced reduction in infarct size, LDH and CK-MB levels and improvement in CFR in normal rat hearts subjected to I/R (Tables 2-7).

Effect of fenofibrate in abrogated cardioprotective potential of IPC in hyperlipidemic rat hearts

Treatment with fenofibrate (100 mg/kg/day, i.p., for 2 weeks) did not affect the cardioprotective effects of IPC in normal rat hearts subjected to I/R. On the other hand, its pretreatment markedly restored the cardioprotective potential of IPC in hyperlipidemic rat hearts subjected to I/R as assessed in terms of improvement in CFR and reduction in myocardial infarct size, LDH, CK-MB and oxidative stress (Tables 2-7).

DISCUSSION

Increase in infarct size, and the release of LDH and CK-MB are documented to be an index of I/R-induced myocardial injury [31]. In the present study, 30 minutes of ischemia followed by 120 minutes of reperfusion was noted to produce myocardial injury as assessed in terms of increased infarct size in the heart and elevated release of LDH and CK-MB in the coronary effluent, which were consistent with earlier reports [32]. The maximal release of LDH was noted immediately after reperfusion whereas the peak release of CK-MB was observed after 5 minutes of reperfusion, which are in accordance with earlier studies. Furthermore, the increase in lipid peroxidation and superoxide anion generation with a consequent decrease in the reduced GSH levels have been suggested to be the indicators of oxidative stress [33,34]. 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 significant decrease in CFR and a marked increase in infarct size, release of LDH and CK-MB were noted in hyperlipidemic rat hearts when compared with the normal rat hearts subjected to I/R.

High-fat diet for 28 days significantly increased serum concentrations of total cholesterol, triglycerides, LDL and VLDL. Moreover, the serum concentration of HDL was significantly reduced in rats fed with high-fat diet for 28 days. Hpl has been noted to modulate the severity of I/R-induced myocardial injury and interfere with the cardioprotective potential of IPC [35]. Moreover, Hpl possesses a major risk factor for coronary heart disease. Hpl has been reported to decrease myocardial NO concentration, causes the generation of ROS such as superoxide anion and peroxynitrite radical, activates apoptotic caspase-3 and lead to accumulation of cholesterol in the sarcolemmal and mitochondrial membranes [13,35], that may attenuate the cardioprotective effect of IPC in hyperlipidemic states. Thus, the observed marked increase in myocardial injury in hyperlipidemic 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 GSH level were noted in hyperlipidemic rat hearts when compared with normal rat hearts subjected to I/R.

IPC has been well-documented to produce myocardial protection against I/R-induced myocardial injury [3,7]. The mechanisms involved in the cardioprotective potentials of IPC are activation of PI3K/Akt and eNOS, release of NO, closure of MPTP, opening of K_{ATP}-channels and reduction in reperfusion-induced oxidative stress [36,37]. In the present study, IPC was noted to reduce I/R-induced myocardial injury in normal rat hearts as assessed in terms of reductions in infarct size, release of LDH and CK-MB and oxidative stress. However, the cardioprotective effect of IPC was insignificant in Hpl rat hearts with a high degree of noted oxidative stress. Thus, it is strongly suggested that the high degree of oxidative stress developed in Hpl rat hearts may be responsible for the observed paradoxical effect of IPC.

Pretreatment with fenofibrate (100 mg/kg/day, i.p., for 2 weeks) did not affect the cardioprotective effect of IPC in normal rat hearts; but its pretreatment significantly restored the cardioprotective effect of IPC in Hpl rat hearts. Fenofibrate has been well reported to be a selective synthetic agonist of PPAR-α [23,38]. Thus, it is suggested that activation of PPAR-α in ischemic myocardium may play a pivotal role in the attenuation of cardioprotective potential of IPC in Hpl rat hearts. The signaling mechanisms such as activation of PI3K/Akt, subsequent activation of eNOS and generation of NO have been well implicated in IPC mediated the cardioprotection. It has been well reported that Hpl down regulates eNOS and reduces the generation and bioavailability of NO [21]. Moreover, various experimental studies have reported that Hpl increase oxidative stress significantly [39]. Further, activation of PPAR-α has been reported to activate PI3K/Akt pathway [22]. Since, fenofibrate has restored the cardioprotective effect of IPC in Hpl rat hearts, it may be

suggested that PPAR-α mediated activation of PI3K/Akt-eNOS pathway in hyperlipidemic rat hearts may be responsible for the restoration of cardioprotective potential of IPC.

In addition, PPAR-α activation has been noted to diminish ROS generation and the postischemic cardiomyocytic apoptosis [22,41]. Reperfusion-induced ROS production has been noted to down-regulate PPAR-α expression that is detrimental for maintaining contractile function of the heart. Thus, it may be suggested that down regulation of PPAR-α by ROS may be associated with cardiac dysfunction in Hpl rat hearts subjected to I/R. Moreover, activation of PPAR-α has been shown to decrease the expression of pro-inflammatory cytokines and involve in oxidative stress-induced apoptotic cell death [38,41,42]. Thus, it could be suggested that PPAR-α activation during reperfusion may be responsible for the decrease in generation of the high amount of ROS in Hpl rat hearts possibly by involving the well-established IPC-mediated cardioprotective PI3K/Akt/eNOS pathway. This contention is supported by the results obtained in the present study that pretreatment with fenofibrate has restorted the cardioprotective and infarct size limiting properties of IPC in Hpl rat hearts as assessed in terms of reductions of CK-MB and LDH in coronary effluent along with decreased oxidative stress in Hpl rat hearts. Our study for the first time reports that the fenofibrate has a significant role in the restoration of abrogated cardioprtective effect of IPC in hyperlipidemic rat hearts. Hence, it can be postulated that the selective PPAR-α agonists may be the potential candidates for providing pharmacological preconditioning in hyperlipidemic patients in order to afford cardioprotection. However, further studies measuring the PPAR-α expression during the hyperlipidemic condition may be warranted.

Based on the above discussion, it may be concluded that there may be down regulation of PPAR-α signaling during the hyperlipidemic condition that consequently produced a high degree of oxidative stress, which may be responsible to abolish the cardioprotective potential of IPC against I/R induced myocardial injury in hyperlipidemic rat hearts. The PPAR-α activation by fenofibrate restored the attenuated cardioprotective effect of IPC in hyperlipidemic rat hearts.

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