Reduction of Ischemic, Pharmacological and Remote Preconditioning Effects by an Antioxidant N-Acetyl Cysteine Pretreatment in Isolated Rat Heart

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The present study was designed to investigate the possible role of free radicals in cardioprotective effects of ischemic, pharmacological and remote preconditioning. Isolated rat heart was perfused on Langendorff apparatus with Kreb’s Henseleit solution and subjected to 30 min global ischemia followed by 120 min reperfusion. To assess myocardial injury, coronary effluent was analyzed for lactate dehydrogenase and creatine kinase activity. Myocardial infarct size was estimated using triphenyl tetrazolium chloride staining. Ischemic preconditioning, pharmacological preconditioning (angiotensin II; H₂O₂), remote aortic preconditioning markedly attenuated I/R induced increase in lactate dehydrogenase and creatine kinase release and myocardial infarct size. Administration of N-Acetyl Cysteine (NAC), in vitro, during ischemic and pharmacological, and in vivo during remote preconditioning attenuated the cardioprotective effects of preconditioning. On the basis of these results, it may be concluded that sub threshold generation of Reactive Oxygen Species (ROS) may activate redox signaling which may be responsible for preconditioning induced cardioprotection.

Key words—reactive oxygen species; remote preconditioning; cardioprotection; ischemic preconditioning; pharmacological preconditioning

INTRODUCTION

Acute occlusion of major coronary artery, leading to myocardial infarction, represents a staggering economic burden on health care sources and poised to become the leading cause of death and morbidity in the industrialized and developed countries. Timely restoration of coronary blood flow by pharmacological agents and coronary angioplasty has been successfully used to limit myocardial infarct size. Furthermore, reperfusion of ischemic myocardium has its own inherent limitation to produce reperfusion injury. The adaptive phenomenon, in which short episodes of ischemia protect myocardium from deleterious effects of subsequent more prolonged ischemia and reperfusion, has been termed as "Ischemic Preconditioning".

Many pharmacological interventions such as endotoxin, monophosphoryl lipid A, interleukin-1, A1 receptor agonist, angiotensin, bradykinin, morphine, endothelin-1, hydrogen peroxide, volatile anesthetics such as halothane, desflurane, isoflurane, and sevoflurane have been observed to produce ischemic preconditioning like protection of heart. These conditions to protect subsequent ischemia have been termed as pharmacological preconditioning. Short occlusion of renal artery, abdominal aorta or mesenteric artery has been observed to produce ischemic preconditioning like protection. However, current mechanisms describe their protective effects also. Redox signaling is a regulating process by which reactive oxygen species (ROS) induced death signal is converted into survival signal. It has been implicated that ROS generated in the mitochondrial respiratory chain act as a trigger of ischemic preconditioning. Recently it has been reported that reactive oxygen species generated from Kupffer cells during ischemic preconditioning are responsible for protective effects in liver, as scavengers of reactive oxygen species reversed the effect of ischemic preconditioning. It has also been reported that ozone preconditioning induced protection of liver against ischemia/reperfusion (I/R) injury also involves reactive oxygen species. The role of reac-
tive oxygen species in the process of myocardial angiogenesis has been described, which may be contributing in ameliorating the function of the heart during ischemic stress. Adaptation of infant hearts to chronic hypoxia and in resistance to subsequent ischemia has been demonstrated to involve reactive oxygen and nitrogen species. The cardioprotective actions of diazoxide have reported to involve by generation of a pro-oxidant environment. Activation of mitochondrial K (ATP) channels and increased ROS production from mitochondria has been implicated in ACh-induced preconditioning in cardiomyocytes. Moreover, bradykinin, opioids, and phenylephrine have been documented to trigger preconditioning by generating free radicals. Further more, postconditioning induced protection has also been shown to involve an early free radical generation.

N-acetyl cysteine (NAC) is a precursor in the formation of the antioxidant glutathione in the body. The thiol (sulfhydryl) group confers antioxidant effects and it has been widely used as an antioxidant for unrevealing the role of free radicals in preconditioning induced tissue protective effects.

Although the involvement of reactive oxygen species triggered protection has been suggested, yet the comparative role of these in ischemic, pharmacological and remote aortic preconditioning induced cardioprotection is not explored. So, the present study was designed to investigate possible roles of reactive oxygen species in cardioprotective effects of different types of preconditioning in rats.

**MATERIALS AND METHODS**

Wistar albino rats (150–300 g) of either sex were employed in the present study. The animal experiments were conducted in accordance with the guidelines of the US National Institute of Health for care and use of laboratory animals. Angiotensin II (Sigma-Aldrich, Bangalore, India), N-Acetyl Cysteine (NAC) (SRL, Mumbai, India) and Hydrogen peroxide (Rankem, SAS Nagar; India) were dissolved in Kreb’s Henseleit solution. Exposed parts of the Langendorff perfusion assembly were covered with aluminum foil during perfusion with H2O2 in order to protect these chemicals from photolysis.

Remote Aortic Preconditioning Rats were anaesthetized with thiopental sodium (40 mg kg–1, i.p.). A 2 cm long incision was given on the abdomen. The abdominal aorta was isolated below the point of origin of renal artery and a silken suture (numbered 5/0) was used to make shoelace knot to occlude abdominal aorta and knot was untied for reperfusion. Four episodes of ischemia and reperfusion, each comprising of 5 min occlusion and 5 min reperfusion, were used to produce remote aortic preconditioning.

**Isolated Perfused Rat Heart** Rats were heparinised (500 IU, i.p.) about 20 m before sacrificing the animal by stunning. Heart was rapidly excised and immediately mounted on Langendorff apparatus. Isolated heart was retrogradely perfused at constant pressure of 80 mm Hg with Kreb’s Henseleit (K-H) solution pH 7.4, maintained at 37°C bubbled with 95% O2 and 5% CO2. Flow rate was maintained at 7–9 ml/min using Hoffman’s screw. The heart was enclosed in a double wall jacket, the temperature of which was maintained by circulating water heated to 37°C. Global ischemia was produced for 30 min by blocking the inflow of K-H solution. It was followed by reperfusion for 120 min. ECG (BPL MK801, Bangalore, India) was monitored on limb lead II using two silver electrodes fixed at left ventricular apex and right auricle.

**Assessment of Myocardial Injury** To determine the extent of myocardial injury, release of lactate dehydrogenase and creatine kinase was measured in the coronary effluent using 2,4-dinitrophenylhydrazine method and Hughes method, respectively. Values were expressed in international units (IU) per liter.

**Myocardial Infarct Size** After 120 min reperfusion, heart was removed from Langendorff apparatus. The auricles and the root of aorta was excised and heart was kept overnight at 0°C. Frozen heart was sliced into uniform sections of 2–3 mm thickness. The slices were incubated in 1% triphenyl tetrazolium chloride (TTC) at 37°C in 0.2 M Tris buffer (pH 7.4) for 20 min. The extent of myocardial infarct size was estimated macroscopically by volume and weight method.

**EXPERIMENTAL PROTOCOL**

In all groups, isolated rat heart was perfused with K-H solution and allowed to stabilize for 10 min.

**Group-I [Sham; n = 6]** Isolated rat heart was perfused with K-H solution for 195 min after stabilization for 10 min.
Group-II [Control; \(n=6\)] Isolated rat heart was perfused for 45 min with K-H solution after stabilization and then subjected to 30 min of global ischemia followed by 120 min of reperfusion with K-H solution.

Group-III [N-Acetyl Cysteine (NAC) (1 mM) Treated; \(n=6\)] Isolated rat heart preparation was perfused with K-H solution containing NAC (1 mM) for 45 min and then subjected to 30 min of global ischemia followed by reperfusion with K-H solution (containing no NAC) for 120 min.

Group-IV [Ischemic Preconditioning; \(n=6\)] Isolated perfused rat heart was subject to four episodes of ischemic preconditioning. Each episode comprised of 5 min global ischemia followed by 5 min of reperfusion with K-H solution, before subjecting the preparation to sustained global ischemia of 30 min and 120 min of reperfusion with K-H solution. In this group, coronary effluent was collected for 1 min, immediately after stabilization (Basal), before global ischemia (BGI) and immediately, 5 and 30 min after reperfusion for LDH and CK estimation.

Group-V [NAC (0.5 mM) in Ischemic Preconditioning, \(n=6\)] Isolated rat heart was perfused with K-H solution containing NAC (0.5 mM) for 5 min, before subjecting it to four episodes of ischemic preconditioning. Each episode comprised of 5 min global ischemia followed by 5 min of reperfusion with K-H solution containing NAC (0.5 mM) and the heart was subjected to 30 min of global ischemia followed by reperfusion with K-H solution (containing no NAC) for 120 min.

Group-VI [NAC (1 mM) in Ischemic Preconditioning, \(n=6\)] Isolated rat heart was perfused with K-H solution containing NAC (1 mM) for 5 min, before subjecting it to four episodes of ischemic preconditioning. Each episode comprised of 5 min global ischemia followed by 5 min of reperfusion with K-H solution containing NAC (1 mM) and the heart was subjected to 30 min of global ischemia followed by reperfusion with K-H solution (containing no NAC) for 120 min.

Group-VII [Angiotensin II (100 nM) Preconditioning; \(n=6\)] Isolated rat heart was perfused for 5 min with K-H solution containing angiotensin II (100 nM), followed by 5 min perfusion with K-H solution (containing no angiotensin II). This was repeated four times before subjecting heart to sustained ischemia of 30 min and reperfusion for 120 min. In this group, coronary effluent was collected for 1 min, immediately after stabilization (Basal), before global ischemia (BGI) and immediately, 5 and 30 min after reperfusion for LDH and CK estimation.

Group-VIII [NAC (0.5 mM) in Angiotensin II Preconditioning; \(n=6\)] Isolated rat heart was perfused for 5 min with K-H solution containing NAC before subjecting to four episodes of angiotensin II preconditioning. Each episode comprised of 5 min perfusion with K-H solution containing angiotensin II (100 nM) followed by 5 min perfusion with K-H solution containing NAC (0.5 mM) and then isolated heart was subjected to 30 min of global ischemia followed by reperfusion with K-H solution (containing no Angiotensin or NAC) for 120 min.

Group-IX [NAC (1 mM) in Angiotensin II Preconditioning; \(n=6\)] Isolated rat heart was perfused for 5 min with K-H solution containing NAC before subjecting to four episodes of angiotensin II preconditioning. Each episode comprised of 5 min perfusion with K-H solution containing angiotensin II (100 nM) followed by 5 min perfusion with K-H solution containing NAC (1 mM) and then isolated heart was subjected to 30 min of global ischemia followed by reperfusion with K-H solution (containing no Angiotensin or NAC) for 120 min.

Group-X [H\(_2\)O\(_2\) (100 \(\mu\)M) Preconditioning; \(n=6\)] Isolated rat heart was perfused for 5 min with K-H solution containing H\(_2\)O\(_2\) (100 \(\mu\)M) followed by 5 min perfusion with K-H solution (containing no H\(_2\)O\(_2\)). This was repeated four times and heart was then subjected to global ischemia for 30 min followed by reperfusion with K-H solution (containing no H\(_2\)O\(_2\)) for 120 min. In this group, coronary effluent was collected for 1 min, immediately after stabilization (Basal), before global ischemia (BGI) and immediately, 5 and 30 min after reperfusion for LDH and CK estimation.

Group-XI [NAC (0.5 mM) in H\(_2\)O\(_2\) Preconditioning; \(n=6\)] Isolated heart was perfused for 5 min with K-H solution containing NAC (0.5 mM) before subjecting it to 5 min perfusion with K-H solution containing H\(_2\)O\(_2\) (100 \(\mu\)M) and this was followed by 5 min of perfusion with K-H solution containing NAC (0.5 mM). Four such episodes were repeated before subjecting the preparation to sustained global ischemia of 30 min and reperfusion for 120 min with K-H solution (containing no H\(_2\)O\(_2\) or NAC) for 120 min.

Group-XII [NAC (1 mM) in H\(_2\)O\(_2\) Preconditioning;
n=6] Isolated heart was perfused for 5 min with K-H solution containing NAC (0.5 mM) before subjecting it to 5 min perfusion with K-H solution containing H₂O₂ (100 μM) and this was followed by 5 min of perfusion with K-H solution containing NAC (1 mM). Four such episodes were repeated before subjecting the preparation to sustained global ischemia of 30 min and reperfusion for 120 min with K-H solution (containing no H₂O₂ or NAC) for 120 min.

Group-XII [Sham Control Group; n=6] Rats were subjected to surgical procedures to isolate abdominal aorta and to pass ligature beneath it but aorta was not occluded. Hearts were excised 40 min after isolation of aorta and isolated hearts were perfused continuously on Langendorff apparatus for 160 min without subjecting them to global ischemia and reperfusion.

Group-XIII [Control Group; n=6] Rats were subjected to surgical procedures to isolate abdominal aorta but aorta was not occluded. Hearts were excised 40 min after the isolation of aorta and isolated hearts were perfused on Langendorff apparatus and after 10 min stabilization, hearts were subjected to global ischemia for 30 min followed by reperfusion for 120 min.

Group-XIV [NAC treated Control Group; n=6] Rats were administered NAC (200 mg kg⁻¹, i.v.), free radical scavenger before isolation of abdominal aorta. Rest of protocol was same as described in group XIV.

Group-XV [Remote Aortic Preconditioning Group; n=6] Rats were subjected to four episodes of remote aortic preconditioning. Hearts were excised immediately after the last episode of preconditioning, perfused on Langendorff apparatus and were subjected to global ischemia for 30 min followed by reperfusion for 120 min. In this group, coronary effluent was collected for 1 min, immediately after stabilization (Basal), immediately, 5 and 30 min after reperfusion for LDH and CK estimation.

Group-XVI [NAC (Low dose) Treated Remote Aortic Preconditioning Group; n=6] NAC (100 mg kg⁻¹, i.v.) was administered to rats 15 min before isolation of abdominal aorta. Rest of protocol was same as described in group XVI.

Group-XVII [NAC (High dose) Treated Remote Aortic Preconditioning Group; n=6] NAC (200 mg kg⁻¹, i.v.) was administered to rats 15 min before isolation of abdominal aorta. Rest of protocol was same as described in group XVI.

**STATISTICAL ANALYSIS**

All the results were expressed as mean±S.E.M. One way ANOVA followed by Tukey's test as post hoc test was employed for multiple comparisons between different groups. p<0.05 was considered to be statistically significant.

**RESULTS**

Effect of Pharmacological Interventions on Myocardial Infarct Size  Global ischemia for 30 min followed by reperfusion for 120 min significantly increased myocardial infarct size measured by volume and weight method (Fig. 1). Ischemic preconditioning, angiotensin II preconditioning, hydrogen peroxide preconditioning and remote aortic preconditioning significantly attenuated the ischemia and reperfusion induced increase in myocardial infarct size (Fig. 1). The N-acetyl cysteine (NAC) treatment produced no marked effect on ischemia and reperfusion induced increase in myocardial infarct size when given in vitro or in vivo. However, N-acetyl cysteine significantly prevented ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning induced decrease in myocardial infarct size (Fig. 1).

Effect of Pharmacological Interventions on Release of LDH in Coronary Effluent  Global ischemia for 30 min followed by reperfusion for 120 min markedly increased the release of LDH in coronary effluent (Fig. 2). Ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning significantly attenuated the ischemia and reperfusion induced increase in release of LDH (Fig. 2). The N-acetyl cysteine (NAC) treatment produced no marked effect on ischemia and reperfusion induced increase in release of LDH when given in vitro or in vivo in coronary effluent. However N-acetyl cysteine (NAC) significantly prevented ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning induced decrease in release of LDH (Fig. 2).

Effect of Pharmacological Interventions on Release of CK in Coronary Effluent  Global ischemia for 30 min followed by reperfusion for 120 min significantly increased the release of creatine kinase (CK) in coronary effluent (Fig. 3). Ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning significantly attenuated ischemia and reperfusion induced increases in release of CK (Fig. 3). The N-acetyl cysteine (NAC) treatment produced...
Fig. 1. Effect of Pharmacological Interventions on Myocardial Infarct Size by Volume and Weight Method

Values are mean ± SEM for six animals. a = p < 0.05 Vs Sham, b = p < 0.05 Vs Control, c = p < 0.05 Vs IPC, d = p < 0.05 Vs APC, e = p < 0.05 Vs H₂O₂ PC, f = p < 0.05 Vs RAPC Sham, g = p < 0.05 Vs RAPC Control. IPC-Ischemic Preconditioning, APC-Angiotensin Preconditioning, H₂O₂-Hydrogen Peroxide Preconditioning, RAPC-Remote Aortic Preconditioning.

Fig. 2. Effects of Pharmacological Interventions on Release of Lactate Dehydrogenase (LDH) in Coronary Effluent at Basal Level, Immediately and 30 min after Reperfusion

Values are mean ± SEM for six animals. a = p < 0.05 Vs Sham, b = p < 0.05 Vs Control, c = p < 0.05 Vs IPC, d = p < 0.05 Vs APC, e = p < 0.05 Vs H₂O₂ PC, f = p < 0.05 Vs RAPC Sham, g = p < 0.05 Vs RAPC Control. IPC-Ischemic Preconditioning, APC-Angiotensin Preconditioning, H₂O₂-Hydrogen Peroxide Preconditioning, RAPC-Remote Aortic Preconditioning.
no marked effect on ischemia and reperfusion induced release of CK when given in vitro or in vivo in coronary effluent. However, N-acetyl cysteine (NAC) significantly prevented ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning induced decrease in release of CK (Fig. 3).

**Effect of Pharmacological Interventions on Coronary Flow Rate**  
Global ischemia for 30 min followed by reperfusion for 120 min significantly decreased the amount of coronary perfusate (Table 1). Ischemic preconditioning, angiotensin II preconditioning, hydrogen peroxide preconditioning and remote aortic preconditioning significantly improved the ischemia and reperfusion induced decrease in amount of coronary perfusate (Table 1). The N-acetyl cysteine (NAC) treatment produced no marked effect on ischemia and reperfusion induced decrease in amount of coronary perfusate, when given in vitro or in vivo. However, N-acetyl cysteine significantly prevented ischemic, angiotensin II, hydrogen peroxide and remote aortic preconditioning induced improvement in amount of coronary perfusate (Table 1).

**DISCUSSION**

Langendorff preparation and working heart preparation are hemodynamically comparable to investigate the effects of pharmacological interventions on ischemia and reperfusion - induced myocardial injury.\(^6\) The isolated rat heart preparation perfused retrogradely on Langendorff apparatus has been employed in the present study.

Four episodes of ischemia and reperfusion (each comprising of 5 min ischemia followed by reperfusion for 5 min) are reported to produce ischemic preconditioning in rats\(^7\) and same protocol is followed in the present study. Release of LDH is a biochemical marker of cardiac injury and marked increase in LDH release is noted immediately or 30 min after reperfusion. It may be suggested that initial release of LDH occurring immediately after reperfusion may be due to ischemic injury. Similar observations have been reported from our own laboratory using rat heart preparation.\(^9,10\) CK release during reperfusion is a measure of rather myocyte injury and in present study peak increase was noted after 5 min reperfusion.
The infarct size has been assessed macroscopically because good correlation has been reported between macroscopic and microscopic assessment of infarct size.9 The NADH and dehydrogenase enzymes present in viable myocardium convert triphenyl tetrazolium chloride (TTC) to red formazone pigment and stained it deep red in color. However, infarct cells lost dehydrogenase enzyme and cofactor NADH and thus remained unstained or dull yellow.10 The infarcted cells have lost the enzyme and cofactor and thus remained unstained or dull yellow. The ventricular slices were placed between two glass plates. A transparent plastic grid with 100 squares in 1 cm² was placed above it. Average area of ventricular slice was calculated by counting the number of squares on either side. Similarly, numbers of square falling over non-stained dull yellow area were counted. Infarct size was expressed as percentage of average ventricular area. Whole of ventricular slices were weighed. Infarcted dull yellow part was dissected out and weighed. Infarct size was expressed as a percentage total ventricular weight. Moreover, reperfusion of 120 min employed in present study is sufficient to washout the NADH and dehydrogenase enzyme from infarcted cells.

Ischemic preconditioning, angiotensin II and hydrogen peroxide produced cardioprotective effect because it markedly reduced the release of LDH, CK and decreased the infarct size significantly as previously reported by our own laboratory.12 Short occlusions and reperfusion of abdominal aorta9,19 have demonstrated to produce cardioprotection against sustained ischemia and reperfusion. Similarly, in the present study, four episodes of abdominal aortic preconditioning have significantly attenuated ischemia and reperfusion induced increase in myocardial infarct size and release of CK and LDH. No significant effect on coronary flow rate in comparison to control was observed with N-acetyl cysteine (NAC) treatment and different types of preconditioning.

Reactive oxygen species (ROS) are generated during myocardial ischemia and reperfusion30 from mitochondria in cardiomyocytes.40 The excessive formation of ROS have been documented to produce cardiac damage and ultimately induce contractile dysfunction,42 however it has been reported that sub threshold amounts of ROS generated during and reperfusion is responsible to provide ischemic preconditioning induce cardioprotection.42 It has been reported that sub threshold amounts of ROS maintained redox balance in cardiomyocytes by activating various mediators.45

Reactive oxygen species (ROS) generated during angiotensin preconditioning40 and remote precon-
ditioning exert cardioprotective effect. The predominant source of ROS is membrane bound vascular NADH/NADPH oxidases\(^\text{(45)}\) which is responsible for redox signaling. In the present study the administration of N-acetyl cysteine (NAC), a free radical scavenger significantly attenuated the cardioprotective effect of ischemic preconditioning, angiotensin induced preconditioning, hydrogen peroxide preconditioning and remote preconditioning. On the other hand, per se treatment of NAC has not modulated induced preconditioning, angiotensin constructive effect of ischemic preconditioning, angiotensin scavenger significantly attenuated the cardioprotection which is attenuated by scavenger of reactive oxygen species.

On the basis of the above discussion it may be concluded that sub threshold levels of reactive oxygen species (ROS) generated during various preconditioning activates redox signaling and consequently provides cardioprotection which is attenuated by scavenger of reactive oxygen species \(i.e.,\) N-acetyl cysteine (NAC).

**CONCLUSIONS**

It may be concluded that generation of sub threshold levels of reactive oxygen species (ROS) trigger cardioprotection in ischemic, pharmacological and remote aortic preconditioning in rats.

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