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Effect of actinomycin D and cycloheximide on ischemic preconditioning-induced delayed cardioprotective effect in rats

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The present study was designed to investigate the effect of actinomycin D, a transcription inhibitor, and cycloheximide, a translation inhibitor, on the delayed cardioprotective effect of ischemic preconditioning. Left thoracotomy was performed in anaesthetized rats at 4th/5th intercostal space and polypropylene suture (5-0) was employed to occlude left common coronary artery. Ischemic preconditioning was produced by four episodes of 5 min of coronary artery occlusion followed by 5 min of reperfusion and thoracic cavity was sutured. Left thoracotomy was performed again after 24 hr of ischemic preconditioning and left coronary artery was occluded for 30 min followed by reperfusion for 120 min. Area at risk and infarct size was estimated by patent blue and TTC staining respectively. Total left ventricular RNA was isolated and estimated quantitatively. Ischemic preconditioning, 24 hr after its induction, produced significant decrease in myocardial infarct size occurred as a result of sustained ischemia and reperfusion but produced no marked effect on ventricular RNA content. Actinomycin D and cycloheximide only, in high dose, markedly attenuated ischemic preconditioning induced decrease in myocardial infarct size. However, no such effect was noted with low dose of cycloheximide. The results suggest that delayed cardioprotective effect of ischemic preconditioning may be mediated through denovo synthesis of protein(s) which is regulated both at transcriptional and translational level.

The cardioprotective effects of ischemic preconditioning are transient and wane off gradually. This transient cardioprotection that appears immediately after ischemic preconditioning is termed as first window of protection (FWOP) or "classical preconditioning"[1,2]. Moreover, the cardioprotective effects of ischemic preconditioning reappear on its own after 12-24 hr[3-5]. This delayed phase of cardioprotection observed many hours after preconditioning trigger, and quite distinct from the classical preconditioning, is termed as second window of protection (SWOP).

Brief episodes of ischemia and reperfusion are reported to enhance the levels of myocardial mRNA encoding for proto-oncogenes[6]. Moreover, mRNA concentration and activity of proteins such as stress proteins, iNOS and Mn-SOD have demonstrated a biphasic increase after 20 or 40 min. and 24 hr after ischemic preconditioning[7-10]. This biphasic pattern is directly correlated to the cardioprotective effect of ischemic preconditioning. The enhanced expression of iNOS is also proposed as a probable mediator for delayed cardioprotective effect of ischemic preconditioning[11,12]. Therefore, it may be possible that ischemic preconditioning induced late cardioprotection may involve enhanced formation or *de novo* synthesis of a cardioprotective protein and it may be of further interest to study the differential regulation of protein synthesis at transcriptional level and translational level. Therefore, the present study has been designed to investigate the effect of actinomycin D, a transcription inhibitor, and cycloheximide, a translation inhibitor, on the cardioprotective effect of SWOP of ischemic preconditioning.

Materials and Methods

Wistar rats (150-300 g) of either sex having free access to food and tap water were used. Rats were anaesthetized with thiopental sodium (40 mg kg^{-1} ip) The trachea was cannulated and animal was ventilated with air at tidal volume of 1.2 ml/100g body weight and at a rate of 60-70 strokes/min using rodent ventilator (UGO Basile, Italy). The ECG was recorded on limb lead II.

Experimental ischemic preconditioning—Left thoracotomy was performed at 4th/5th intercostal space and heart was exteriorized. A 5-0 polypropylene suture was passed beneath left common coronary artery and heart was repositioned back in the thoracic cavity with ends of sutures passed through a small plastic tubing. Pulling the ends of snare with constant weights produced ischemia, which was confirmed by

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ST segment elevation. Releasing the weights from the ends of snare instituted reperfusion and attenuated ST segment elevation. The animal was allowed to stabilize for 10 min before subjecting the hearts to ischemic preconditioning. Ischemic preconditioning was produced by four episodes of 5 min of coronary artery occlusion followed by 5 min of reperfusion. After ischemic preconditioning protocol, the thorax was evacuated and sutured in the layers. The trachea was extubated, sutured and the animals were allowed to recover from anaesthesia.

Sustained ischemia and reperfusion—Thoracotomy was performed again 24 hr after subjecting the rats to ischemic preconditioning.The coronary artery was occluded for 30 min as described earlier and it was followed by reperfusion for 120 min ECG was recorded at 5, 15 and 30 min after coronary artery occlusion and at 5, 15, 30, 60 and 120 min after reperfusion.

Assessment of area at risk (% AAR)—Fifteen min before the end of 120 min. reperfusion, rat was heparinised (200 I.U. iv) and the heart was isolated by sacrificing it with a high dose of anaesthesia .The isolated heart was retrogradely perfused through aorta with normal saline to remove blood. The snare around left coronary artery was retightened and the aorta was perfused with patent blue (0.5% w/v) for 2 min at a rate of 10 min/inch by using constant infusion pump (Inco Ambala , India) fitted with 10 ml syringe. The area under risk remained unstained and rest of ventricular mass was stained bluish green with patent blue.

Infarct size measurement (% IS)—Heart was removed from perfusion apparatus and left ventricle was separated from right ventricle, aorta and auricles. It was refrigerated overnight. Frozen ventricle was cut into transverse slices of about 2 mm thickness from apex to base. These slices were incubated at 37°C with 1% w/v solution of triphenyl tetrazolium (TTC) in tris buffer, *p*H 7.4 for 10-20 min[13,14]. The TTC was converted into brick red formazon by the viable cells whereas infarcted portion remained dull yellow[15]. Both the % AAR and % IS were measured by volume and weight method[16,17].

Ventricular RNA isolation—In separate group of animals, the left ventricle was separated and was homogenized with 1 ml guanidium thiocyanate solution at 4°C. The homogenate was used to isolate the total left ventricular RNA by Chomczynski and Sacchi method[18]. The RNA was estimated spectrophotometrically (Beckman DU640B, Switzerland) by taking the absorbance at 260 nm. The protein impurity was detected by taking the absorbance at 280 nm and DNA contamination was assessed by running the RNA samples on 0.8% agarose gel in a submarine electrophoresis unit (Pharmacea Biotech, Hongkong).

Experimental protocol—Ten groups were employed in the present study and each group consisted of 6 rats. Group I (control group, n=6) rats were subjected to surgical procedures as described under experimental ischemic preconditioning but animals were not subjected to ischemic preconditioning. Rats were subjected after 24 hr of surgical procedure to regional sustained ischemia for 30 min followed by reperfusion for 120 min. Group II (ischemic preconditioning group, n=6) rats were subjected to ischemic preconditioning and after a gap of 24 hr they were subjected to sustained regional ischemia for 30 min followed by reperfusion for 120 min. Group III (actinomycin D treated control group, n=6) rats were administered actinomycin D (1 mg/kg, ip) 15 min before surgery, and rest of the procedure was same as described in Group I. Group IV (actinomycin D treated and ischemic preconditioning group, n=6) rats were administered actinomycin D (1 mg/kg, ip) 15 min before subjecting them to ischemic preconditioning and rest of the procedure was same as described in group II. Group V (low dose cycloheximide treated control group, n=6) rats were administered cycloheximide (1 mg/kg, ip) 15 min before surgery and rest of the procedure was same as described in group I. Group VI (low dose of cycloheximide treated and ischemic preconditioning group, n=6) rats were administered cycloheximide (1 mg/kg, ip) 15 min before subjecting them to ischemic preconditioning and rest of the procedure was same as described in group II. Group VII (high dose cycloheximide treated control group, n=6) rats were administered cycloheximide (28 mg/kg, iv) 15 min before surgery and the rest of procedure was same as described in group I. Group VIII (high dose cycloheximide treated and ischemic preconditioning group, n=6) rats were administered cycloheximide (28 mg/kg ,iv) 15 min before subjecting them to ischemic preconditioning and rest of the procedure was same as described in group II. Group IX (control RNA group, n=6) rats were subjected to thoracotomy and sham operation. Coronary ligature was passed underneath the left coronary artery, but animal were not subjected to ischemic preconditioning. After 24 hr of sham operation, the heart was excised, left ventri-

cle was isolated and RNA was estimated. Group X (ischemic preconditioning RNA group, n=6) Rats were subjected to ischemic preconditioning. After 24 hr of ischemic preconditioning the heart was excised, left ventricle was isolated and RNA was estimated.

Statistical analysis—Values are expressed as means ± SE. One way analysis of variance (ANOVA) followed by student range test was applied to calculate the statistical significance between various groups. A value of $P<0.05$ was constituted to be statistically significant.

Results

Effect of ischemic preconditioning and pharmacological interventions on heart rate and area at risk—Left common coronary artery occlusion followed by reperfusion produced no significant change in heart rate. Ischemic preconditioning, administration of actinomycin D and cycloheximide produced no marked effect on heart rate.

Left common coronary artery ligation for 30 min followed by 120 min reperfusion in rat produced 59% area at risk measured by both volume and weight method. Ischemic preconditioning (after 24 hr) produced no change in percentage area at risk (% AAR) measured after sustained ischemia and reperfusion. Pretreatment with actinomycin D (1 mg/kg, ip) and cycloheximide (1 mg/kg ip; 28 mg/kg, iv) produced no significant change in the area at risk in control as well as ischemic preconditioned hearts (Figs 1 and 2).

Effect of ischemic preconditioning and pharmacological interventions on infarct size—Left common coronary artery ligation for 30 min. and reperfusion for 120 min produced 58% and 60% myocardial infarct size (expressed as % AAR) measured by volume and weight method respectively. Ischemic preconditioning, 24 hr after its induction, produced significant decrease in myocardial infarct size occurred as a result of sustained ischemia of 30 min followed by reperfusion for 120 min (Fig. 3).

Administration of actinomycin D (1 mg/kg, ip) produced no *per se* effect on myocardial infarct size occurred as result of sustained ischemia and reperfusion. Moreover, actinomycin D treatment markedly attenuated ischemic preconditioning induced decrease in myocardial infarct size (Fig. 3).

Administration of cycloheximide in low dose (1 mg/kg, ip) as well as high dose (28 mg/kg, ip) produced no *per se* effect on myocardial infarct size occurred as a result of sustained ischemia and reperfusion. Cycloheximide, in high dose, significantly prevented ischemic preconditioning induced decrease in myocardial infarct size, however, no such effect was noted with low dose cycloheximide (Fig. 4).

Effect of ischemic preconditioning on left ventricular RNA content—RNA concentration expressed as mg/g dry weight of ventricle was observed to be 5.289 ± 0.18. Moreover, ischemic preconditioning, 24 hr after its induction produced no marked effect on ventricular RNA content (5.3 ± 0.16).

Discussion

In the present study, four episodes of ischemia and reperfusion provided cardioprotection even after 24 hr against sustained ischemia and reperfusion induced injury. The present result are in accordance

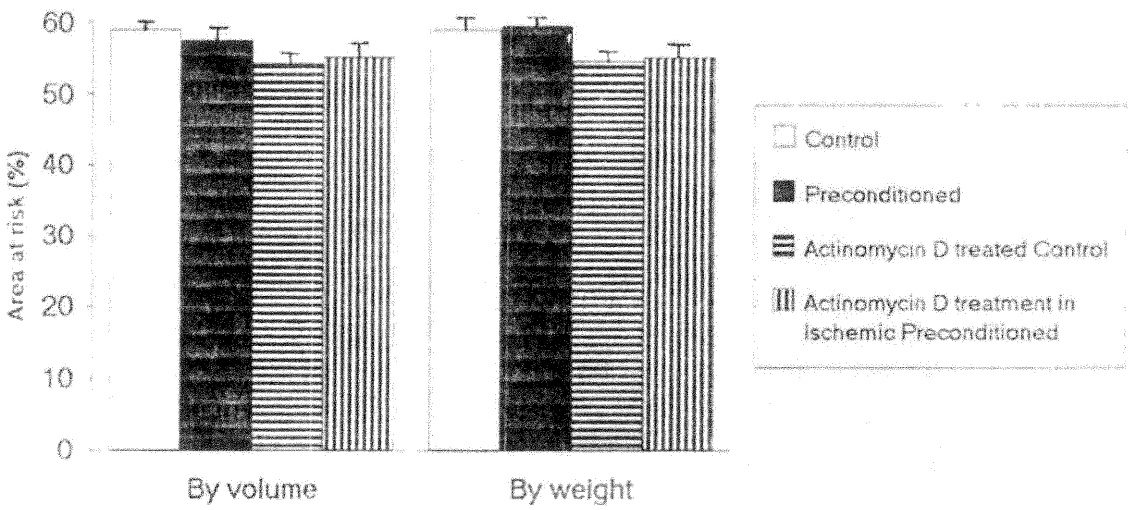

Fig. 1—Effect of ischemic preconditioning and actinomycin D (1 mg/kg, ip) treatment on area at risk (expressed as percent left ventricular vol./wt.) in rat heart (n=6) subjected to sustained ischemia-reperfusion 24 hr after ischemic preconditioning.

with those of Yamashita *et al.*[4] who demonstrated a similar delayed cardioprotective effect of ischemic preconditioning in rat. Ischemic preconditioning induced protection may involve enhanced formation or *de novo* synthesis of cardioprotective proteins[8-10]. It is, however, interesting to note that in the present study, ischemic preconditioning produced no significant change in total left ventricular RNA. It tentatively suggests that alteration in transcriptional activity may not be involved in the delayed cardioprotective effect of ischemic preconditioning. However, data in hand do not rule out the increase in selective mRNA responsible for transcriptional activity.

Actinomycin D, a polypeptide antibiotic, is a transcriptional inhibitor[19]. Actinomycin D (1 mg/kg ip) produced 80% inhibition of total RNA synthesis[20,21] In the present study, actinomycin D prevented the delayed cardioprotective effect of ischemic preconditioning. It suggests that delayed cardioprotective effect of ischemic preconditioning may involve increased rate of transcription of mRNA. Cycloheximide in low (1 mg/kg) as well as in high (28 mg/kg) dose is reported to produce 95% inhibition of total protein synthesis[22]. It is interesting to note that in the present study, only high dose of cycloheximide attenuated the delayed cardioprotective effect of ischemic preconditioning. It suggests that delayed cardioprotective effect of ischemic preconditioning may be mediated through a protein whose expression is regulated at both transcriptional and translational level. It is difficult to suggest the nature of cardioprotective protein from the present data . Transcrip-

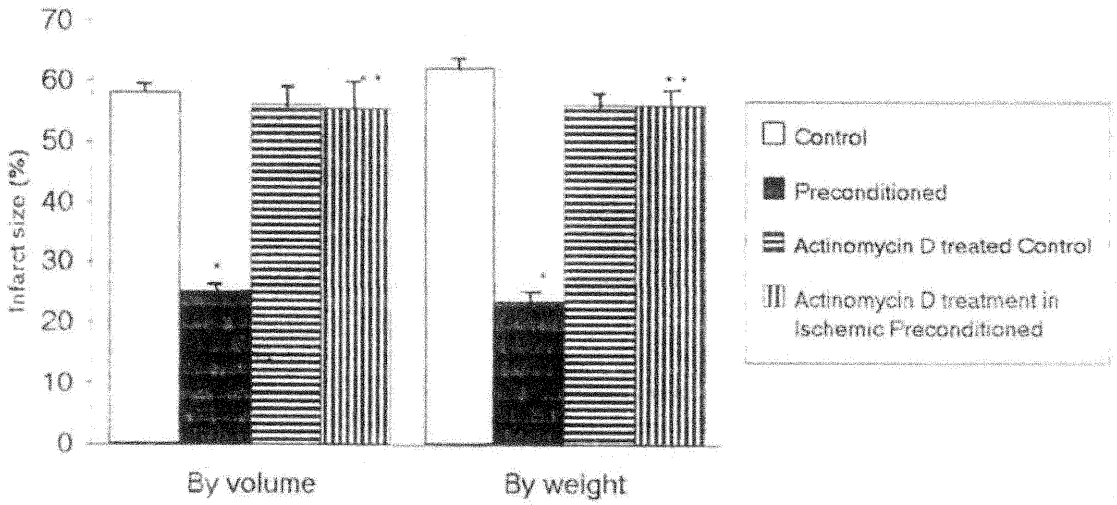

Fig. 2—Effect of ischemic preconditioning and cycloheximide treatment on area at risk (expressed as percent left ventricular vol./wt.) in rat heart subjected to sustained ischemia reperfusion 24 hr after ischemic preconditioning.

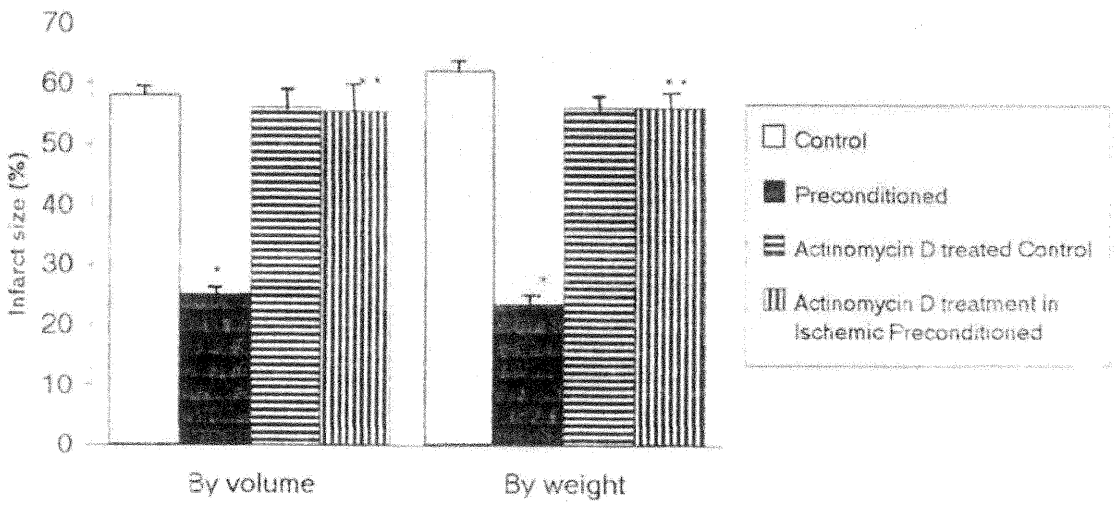

Fig. 3—Effect of ischemic preconditioning and actinomycin treatment (1 mg/kg ip) on infarct size (expressed as percent area at risk) in rat heart subjected to sustained ischemia reperfusion 24 hr after ischemic preconditioning. ($P < 0.05$: *vs Control; ** vs Preconditioned)

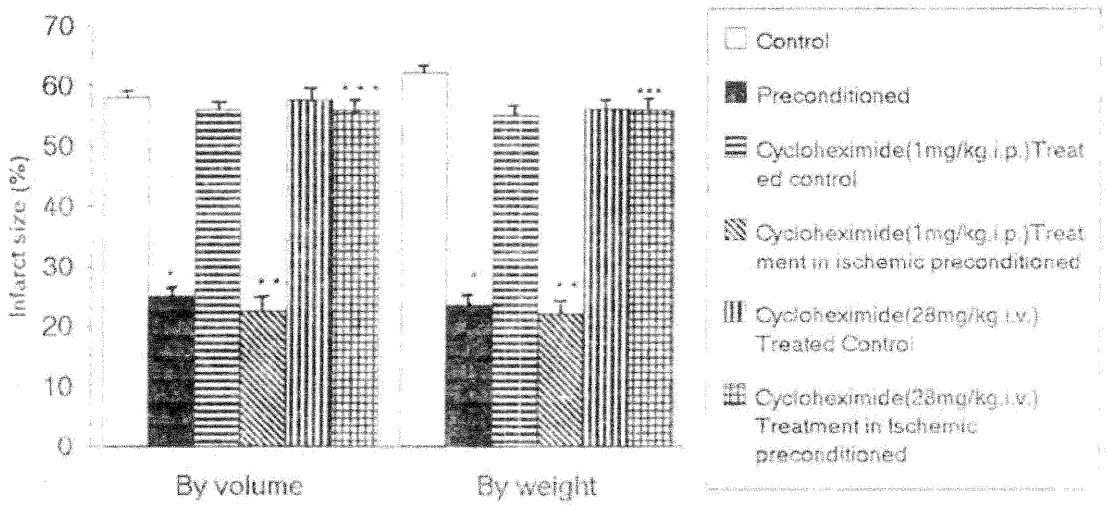

Fig. 4—Effect of ischemic preconditioning and cycloheximide treatment on infarct size (expressed as percent area at risk) in rat heart subjected to sustained ischemia reperfusion 24 hr after ischemic preconditioning.($P < 0.05$: * vs Control; **vs Cycloheximide treated Control; *** vs preconditioned)

tional inhibitor, actinomycin D, is reported to inhibit the synthesis of Mn-SOD and HSP[23]. Moreover, cycloheximide in low & in high dose does not or poorly inhibit Mn-SOD and HSP respectively[23]. The contention that Mn-SOD and HSP may be involved in the cardioprotective effect of ischemic preconditioning[7-9], is not supported by the present study because actinomycin D and cycloheximide in high dose have attenuated the cardioprotective effect of ischemic preconditioning.

Nitric oxide is proposed to be a mediator of delayed cardioprotective effect of ischemic preconditioning[10-12]. The high dose cycloheximide employed in the present study is reported to inhibit induction of iNOS[24] and this high dose of cycloheximide has completely attenuated the delayed cardioprotective effect of ischemic preconditioning. Moreover, iNOS expression is regulated both at transcriptional and translational level[25]. Therefore, iNOS appears to be a probable candidate to provide late cardioprotection as a result of ischemic preconditioning.

On the basis of above discussion, it may be concluded that delayed cardioprotective effect of ischemic preconditioning may be mediated through *de novo* synthesis of protein which is regulated both at transcriptional and translational level such as iNOS.

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