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Remote preconditioning by aortic constriction: Does it afford cardioprotection similar to classical or other remote ischaemic preconditioning? Role of inducible nitric oxide synthase

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Lactate dehydrogenase (LDH);
Inducible nitric oxide synthase (iNOS);
Cardioprotection

Summary
Purpose of the research: Does remote preconditioning by aortic constriction (RPAC) afford cardioprotection similar to classical or other remote ischaemic preconditioning stimulus? Moreover, the study was also designed to investigate the role of inducible nitric oxide synthase (iNOS) in remote preconditioning by aortic constriction. There are sufficient evidence that 'ischaemic preconditioning' has surgical applications and affords clinically relevant cardioprotection. Transient occlusion of the circumflex artery, renal artery, limb artery or mesenteric artery preconditions the myocardium against ischaemia/reperfusion injury in case of ischaemic heart disease leading to myocardial infarction. Here, the abdominal aorta was selected to produce RPAC.
The principal results: Four episodes of ischaemia/reperfusion of 5 min each to the abdominal aorta produced RPAC by assessment of infarct size, lactate dehydrogenase (LDH) and creatine phosphokinase (CK). These studies suggest RPAC produced acute (FWOP) and delayed (SWOP) cardioprotective effects. RPAC demonstrated a significant decrease in ischaemia/reperfusion-induced release of LDH, CK and extent of myocardial infarct size. L-NAME (nitro-L-arginine-

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methylester) (10 mg kg^{-1} I.V.), aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.) administered 10 min. before global ischaemia/reperfusion produced no marked effect. Aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.) pre-treatment after RPAC produced no significant effect on acute RPAC-induced decrease in LDH, CK and infarct size, whereas L-NAME (10 mg kg^{-1} I.V.) increased RPAC-induced decrease in LDH, CK and infarct size. The most interesting observation is with respect to delayed RPAC, where all NOS inhibitors' pre-treatment attenuate RPAC-induced decrease in LDH, CK and infarct size.

Major conclusions: RPAC affords cardioprotection similar to classical or other remote ischaemic preconditioning stimulus. Moreover, late or delayed phase of RPAC has been mediated iNOS, whereas it is not involved in acute RPAC.

Introduction

Myocardial infarct size is an established determinant of clinical complications and patient survival in an event of acute coronary occlusion. Ischaemic heart disease leading to myocardial infarction is considered to be one of the major causes of cardiovascular morbidity and mortality. Revascularisation of the ischaemic heart with thrombolytic agents, angioplasty or saphenous vein grafting is the primary requirement. Moreover, a delay to institute reperfusion deprives most of its beneficial effects as a direct function of time. Thus, attention has been focussed to understand the adaptive mechanisms that will make the myocardium more resistant to ischaemia of longer duration and to restore its viability on reperfusion. Repeated short episodes of ischaemia/reperfusion have been demonstrated to make the myocardium transiently more resistant to the deleterious effects of subsequent and prolonged ischaemic insult. This paradoxical form of myocardial adaptation has been termed as 'ischaemic preconditioning' (PC), which was reported to provide protection against infarct size and arrhythmias and improve post-ischaemic contractile function. Thus, ischaemic preconditioning has surgical applications and affords clinically relevant cardioprotection. PC has been reported to protect the myocardium even in diseased states, such as hypertrophy and diabetic myocardium [1—3].

Ischaemic PC is acquired by transient ischaemic stress in the same tissue or organ [4]. Short occlusions of renal artery [4] or mesenteric artery [5,6] or limb artery [7—9] also precondition the myocardium against ischaemia/reperfusion injury. Ischaemic stress in the remote regions is termed 'remote preconditioning'. The cardioprotective effects of ischaemic PC are biphasic, including an early effect, which lasts for 1—2 h and a delayed effect, which appears after 24 h. However, the delayed effect of remote preconditioning is not yet investigated [5].

The antiarrhythmic effect of classical ischaemic PC involves the activation of soluble guanylate cyclase through NO and subsequent elevation of cyclic guanosine monophosphate (cGMP) [10—12]. In contrast to the limited involvement of NO in classical ischaemic PC, substantial evidence implicates it in delayed ischaemic PC [13,14]. NO can act as a trigger as well as a mediator of the delayed phase of ischaemic PC. The delayed cardioprotective effect of ischaemic PC is accompanied by increased activity of nuclear factor Kappa-B (NF-κB) [15], which may, in turn, induce the expression of inducible nitric oxide synthase (iNOS). It is interesting to note that selective iNOS inhibitors, such as aminoguaidine and S-methyl-isothiourea, when administered before sustained ischaemia, abolish the PC-induced delayed cardioprotection against stunning [8] and infarction [16,17]. Moreover, the administration of endotoxin and non-toxic derivative MLA confers preconditioning such as delayed cardioprotection through iNOS induction [18,19]. Role of iNOS by intestinal ischaemia induces late PC against myocardial infarction [20].

The present study is designed to investigate the acute and delayed cardioprotective effect of remote aortic PC. Moreover, it is also envisaged to study the role of iNOS in the cardioprotective effect of remote aortic PC.

Materials and methods

Animals

Wistar albino rats (100—300 g) of either sex were employed in the present study. They were fed standard laboratory chow (Kisan Feeds Ltd., New Delhi, India) and had free access to tap water *ad libitum*. All the experimental protocols were performed according to Animals Ethical Committee, Gyan Vihar School of Pharmacy, and Protocol No. 42.

Remote aortic PC

Rats were anaesthetised with thiopental sodium (25 mg kg^{-1} I.V.). A 2-cm long incision was made on the abdomen. The lower portion of abdominal aorta was isolated and the suture (numbered 5/0) was passed beneath it away from the origin of renal arteries. The aorta was occluded by tying a shoe-lace knot and the knot was untied from reperfusion. The aorta was occluded for 5 min and was reperfused for 5 min. Four such episodes were used to produce PC [21]. In case the animals were to be used after 24 h of aortic PC, the abdomen was sutured in layers and the animals were allowed to recover.

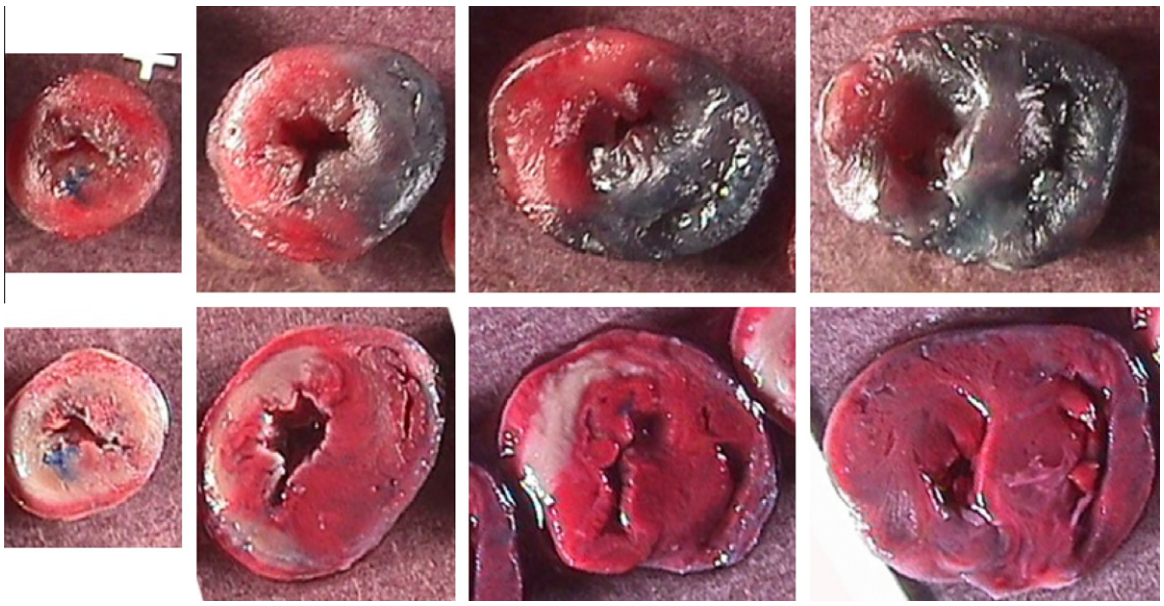

Figure 1 Heart slices showing pink portion as viable part and grey portion and infarcted part. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Global ischaemia and reperfusion in the isolated rat heart

Heparin (500 IU, i.p.) was administered nearly 20 min before sacrificing the animals. The heart was rapidly excised and immediately mounted on Langendorff's apparatus [22]. The aorta was retrogradely perfused at a constant pressure of 70 mm Hg with Kreb's Henseleit buffer (NaC1 118 mM; Kc1 4.7 mM; $CaCl_2$ 2.5 mM; $MgSO_4.7H_2O$ 1.2 mM; $NaHCO_3$ 25 mM; KH_2PO_4 1.2 mM; and $C_6H_{12}O_6$ 11 mM) pH 7.4, maintained at 37 °C bubbled with 95% O_2 and 5% CO_2. Flow rate was maintained between 6 and 9 ml min^{-1} using Hoffman's screw. The heart was enclosed by a double-walled jacket, the temperature of which was maintained by circulating water heated to 37 °C. Global ischaemia was produced for 30 min by blocking the in-flow Kreb's buffer. It was followed by reperfusion for 120 min. Electrocardiography (ECG) (BPL CARDIAART 108T-DIGI, New Delhi, India) was done using two silver electrodes fixed at the left ventricular apex and right auricle. ECG was recorded immediately after stabilisation, 5, 15 and 30 min during ischaemia and immediately, 5, 15, 30, 60 and 120 min after reperfusion. The coronary effluent was collected at the same time intervals during reperfusion for lactate dehydrogenase (LDH) and CK estimation.

Infarct size measurement

The heart was removed from Langendorff's apparatus. Both the auricles and the root of the aorta were exercised, and the ventricles were kept overnight at 4 °C. Frozen ventricles were sliced into uniform sections of 2—3 mm thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37° in 0.2 M Tris buffer (pH 7.4) for 20 min. TTC is converted to red formazone pigment by reduced nicotinamide adenine dinucleotide (NADH) and dehydrogenase enzyme and therefore, stained the viable cells deep red. The infarcted cells lost the enzyme and co-factor 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 cm^2 was placed above it. The average area of each ventricular slice was calculated by counting the number of squares on either side. Similarly, the number of squares falling over the non-stained dull yellow area was also counted. The infarcted area was expressed as a percentage of total ventricular area. All the ventricular slices were weighed. The infarcted dull yellow part was dissected and weighed. Infarct size was expressed as a percentage of total ventricular weight (Fig. 1).

Estimation of LDH

LDH was estimated in the coronary effluent by the 2,4-dinitrophenylhydrazine (2,4-DNPH) method [23].

Principle

LDH catalyses the following reaction:

$$\text{Lactate} + \text{NAD} \leftrightarrow \text{Pyruvate} + \text{NADH}.$$

The pyruvate so formed is coupled with 2,4-DNPH to give the corresponding hydrazone, which gives a brown colour in alkaline medium. The intensity of this colour is proportional to the amount of LDH activity and is measured spectrophotometrically at 440 nm.

Estimation of creatine phosphokinase (CK)

Creatine phosphokinase (CPK) was measured in the coronary effluent by the modified method of Hughes [24].

Principle

CPK catalyses the following reaction:

$$\text{Creatine phosphate} + \text{ADP} \leftrightarrow \text{Creatine} + \text{ATP}.$$

At pH 7.4, CPK catalyses the forward reaction. The creatine so formed, reacts with diacetyl and naphthol in alkaline medium to give a pink colour. The intensity of this colour is proportional to enzyme activity and is measured spectrophotometrically at 520 nm. Mg^{2+} and cysteine is added as an activator. P-chloromercuribenzoate stops the reaction by inactivating the enzyme.

Experimental protocol

Twenty-four groups of Wistar albino rats were employed in the present study.

Remote aortic PC-induced acute or first window of protection (FWOP): First window of protection (FWOP) was observed immediately after remote aortic PC.

Group I (FWOP control group; *n* = 6)

Rats were subjected to a surgical procedure for aortic isolation, but the aorta was not occluded. Hearts were excised 40 min after a sham operation. Isolated hearts were perfused on Langendorff's apparatus and were subjected to global ischaemia for 30 min, followed by reperfusion for 120 min.

Group II (FWOP remote preconditioning by aortic constriction (RPAC) group; *n* = 6)

Rats were subjected to remote preconditioning by aortic constriction (RPAC), as described earlier. Hearts were excised immediately after the last episode of reperfusion, perfused on Langendorff's apparatus and subjected to global ischaemia for 30 min, followed by reperfusion for 120 min.

Group III (FWOP L-NAME (nitro-L-arginine-methylester) (10 mg kg^{-1} I.V.) [25]-treated control group; *n* = 6)

Rats were administered 10 mg kg^{-1} of L-NAME (nitro-L-arginine-methylester) I.V., 10 min before excising the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group IV (FWOP L-NAME (10 mg kg^{-1} I.V.) treated RPAC group; *n* = 6)

L-NAME (10 mg kg^{-1} I.V.) was administered during the last episode of reperfusion during RPAC, that is, 10 min before isolating the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group V (FWOP aminoguanidine (150 mg kg^{-1} I.V.) [26,27]-treated control group; *n* = 6)

Rats were administered 150 mg kg^{-1} of aminoguanidine I.V. 10 min before excising the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group VI (FWOP aminoguanidine (150 mg kg^{-1} I.V.) treated RPAC group; *n* = 6)

Aminoguanidine (150 mg kg^{-1} I.V.) was administered during the last episode of reperfusion during RPAC, that is, 10 min before isolating the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group VII (FWOP aminoguanidine (300 mg kg^{-1} s.c.) [28]-treated control group; *n* = 6)

Rats were administered 300 mg kg^{-1} of aminoguanidine I.V. 10 min before excising the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group VIII (FWOP aminoguanidine (300 mg kg^{-1} I.V.)-treated RPAC group; *n* = 6)

Aminoguanidine (300 mg kg^{-1} I.V.) was administered during the last episode of reperfusion during RPAC, that is, 10 min before isolating the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group IX (FWOP S-methyl isothiourea (3 mg kg^{-1} I.V.) [28]-treated control group; *n* = 6)

Rats were administered S-methyl isothiourea (3 mg kg^{-1} I.V.) 10 min before excising the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group X (FWOP S-methyl isothiourea (3 mg kg^{-1} I.V.)-treated RPAC group; *n* = 6)

S-methyl isothiourea (3 mg kg^{-1} I.V.) was administered during the last episode of reperfusion during RPAC, that is, 10 min before isolating the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group XI (FWOP 1400W (1 mg kg^{-1} I.V.) [25]-treated control group; *n* = 6)

Rats were administered 1400W (1 mg kg^{-1} I.V.) 10 min before excising the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Group XII (FWOP 1400W (1 mg kg^{-1} I.V.)-treated RPAC group; *n* = 6)

1400W (1 mg kg^{-1} I.V.) was administered during the last episode of reperfusion during RPAC, that is, 10 min before isolating the heart for Langendorff's perfusion. The rest of the protocol was the same as described in Group I.

Remote aortic PC-induced delayed or second window of protection (SWOP): SWOP was observed 24 h after remote aortic PC.

Group XIII (SWOP control group; *n* = 6)

Rats were subjected to a surgical procedure for aortic isolation, but the aorta was not occluded. Hearts were excised 24 h after a sham operation. The rest of the protocol was the same as in Group I.

Group XIV (SWOP RPAC group; *n* = 6)

Rats were subjected to RPAC and hearts were excised 24 h after remote aortic PC. The rest of the protocol was the same as in Group I.

Group XV (SWOP L-NAME (10 mg kg^{-1} I.V.)-treated control group; *n* = 6)

Rats were subjected to the same protocol as described in group XIV except that L-NAME (10 mg kg^{-1} I.V.) was

administered 10 min before excising the heart for Langendorff's perfusion.

Group XVI (SWOP L-NAME (10 mg kg^{-1} I.V.)-treated RPAC group; n = 6)

Rats were subjected to the same protocol as described in group XIV except that L-NAME (10 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XVII (SWOP aminoguanidine (150 mg kg^{-1} I.V.)-treated control group; n = 6)

Rats were subjected to the same protocol as described in group XI except that aminoguanidine chloride (150 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XVIII (SWOP AMINOGUANIDINE (150 mg kg^{-1} I.V.)-treated RPAC group; n = 6)

Rats were subjected to the same protocol as described in group XIV except that aminoguanidine (150 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XIX (SWOP aminoguanidine (300 mg kg^{-1} I.V.)-treated control group; n = 6)

Rats were subjected to the same protocol as described in group XIII except that aminoguanidine chloride (300 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XX (SWOP aminoguanidine (300 mg kg^{-1} I.V.)-treated RPAC group; n = 6)

Rats were subjected to the same protocol as described in group XIV except that aminoguanidine (300 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XXI (SWOP S-methyl isothiourea (3 mg kg^{-1} I.V.)-treated control group; n = 6)

Rats were subjected to the same protocol as described in group XIII except that S-methyl isothiourea (3 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XXII (SWOP S-methyl isothiourea (3 mg kg^{-1} I.V.)-treated RPAC group; n = 6)

Rats were subjected to the same protocol as described in Group XIV except that S-methyl isothiourea (3 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XXIII (SWOP 1400W (1 mg kg^{-1} I.V.)-treated control group; n = 6)

Rats were subjected to the same protocol as described in Group XIII except that 1400W (1 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Group XXIV (SWOP 1400W (1 mg kg^{-1} I.V.)-treated RPAC group; n = 6)

Rats were subjected to the same protocol as described in Group XIV except that 1400W (1 mg kg^{-1} I.V.) was administered 10 min before excising the heart for Langendorff's perfusion.

Drugs and chemicals

Aminoguanidine hydrogen carbonate (Lancaster Chemicals, Chennai, India) and 1400W ([*N*-(3-(aminomethyl)benzyl)-acetamidine]sulphate) (a specific iNOS inhibitor) were from Acros Organics (Noisy le Grand, France), and were dissolved in distilled water immediately before use; S-methylthiourea sulphate and L-NAME (non-specific iNOS inhibitor) were from Sigma-Aldrich, USA; Tris buffer was prepared by adding 50 ml of 0.2 M Tris (CDH Chemicals, New Delhi, India) in 32.5 ml of 0.2 M HCl and the volume was made up to 200 ml with distilled water. All other reagents used in the study were analar grade of Qualigens (Glaxo, Mumbai, India).

Statistical analysis

Values for enzymatic data and infarct size were expressed as mean ± standard error of the mean (SEM). Statistical significance was calculated using one-way analysis of variance. Dunnett's test and Student's *t*-test were employed as *post hoc* tests for comparison with the control group and for multiple comparisons between groups, respectively. A value of $P < 0.05$ is considered to be statistically significant. Sigma10 software was used for statistical analysis.

Theory/calculation

There is sufficient evidence that ischaemic PC has surgical applications and affords clinically relevant cardioprotection. Transient occlusion of circumflex artery, renal artery, limb artery or mesenteric artery preconditions the myocardium against ischaemia/reperfusion injury in case of ischaemic heart disease leading to myocardial infarction. Here, the abdominal aorta was selected to produce RPAC. The present study was designed to investigate whether RPAC affords cardioprotection similar to classical or other remote ischaemic PC. The role of iNOS in acute and delayed RPAC was also investigated by administering specific and non-specific iNOS inhibitors.

Results

Effect of remote aortic PC and pre-treatment with NOS inhibitors on coronary flow rate and heart rate

Global ischaemia for 30 min produced a marked decrease in coronary flow rate (Table 1) and heart rate (Table 2) and this decrease persisted for the entire 120 min of reperfusion. NOS inhibitors (L-NAME(10 mg kg^{-1} I.V.), aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.)) pre-treatment and remote aortic PC produced no

Table 1 Acute effect (FWOP) and delayed effect (SWOP) of remote preconditioning by aortic constriction (RPAC) and pre-treatment with NOS inhibitors on coronary flow rate (ml/min) in isolated rat heart subjected to global ischaemia (30 min) and reperfusion (120 min).

Groups	Basal	Imm. *R*.	5 min. *R*.	15 min. *R*.	30 min. *R*.	60 min. *R*.	120 min. *R*.
(I) Sham (FWOP)	8.40 ± 0.13	4.40* ± 0.24	5.32* ± 0.05	4.98* ± 0.18	4.16* ± 0.42	2.90* ± 0.48	1.24* ± 0.5
(II) RAPC (FWOP)	8.22 ± 0.30	4.36* ± 0.37	4.94* ± 0.29	4.30* ± 0.50	4.92* ± 0.67	2.58* ± 0.56	1.96* ± 0.41
(III) L-NAME (10 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	10.38 ± 1.53	7.04* ± 0.72	4.92* ± 0.72	4.04* ± 0.71	3.92* ± 0.73	2.85* ± 0.40	2.49* ± 0.51
(IV) L-NAME (10 mg/kg I.V.) pretreatment immediately after RPAC (FWOP)	9.73 ± 0.49	5.98* ± 0.49	4.82* ± 1.06	3.93* ± 1.12	3.53* ± 0.20	3.19* ± 0.61	2.63* ± 0.24
(V) Aminoguanidine (150 mg/kg s.c) pretreatment immediately after sham operation (FWOP)	8.06 ± 0.63	4.78* ± 0.79	3.98* ± 0.75	3.70* ± 0.70	2.58* ± 0.65	2.02* ± 0.47	1.66* ± 0.42
(VI) Aminoguanidine (150 mg/kg s.c) pretreatment immediately after RPAC (FWOP)	9.66 ± 0.96	6.86* ± 1.19	6.46* ± 1.06	6.00* ± 0.71	5.20* ± 1.10	4.30* ± 0.74	3.46* ± 0.46
(VII) Aminoguanidine (300 mg/kg s.c) pretreatment immediately after sham operation (FWOP)	9.51 ± 0.35	5.79* ± 0.62	5.96* ± 0.41	5.06* ± 1.25	4.17* ± 0.83	4.06* ± 0.62	3.24* ± 0.82
(VIII) Aminoguanidine (300 mg/kg s.c) pretreatment immediately after RPAC (FWOP)	11.23 ± 0.59	5.29* ± 0.45	4.02* ± 0.37	3.95* ± 0.48	2.79* ± 0.63	2.86* ± 0.94	2.71* ± 0.93
(IX) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	9.67 ± 0.28	6.19* ± 1.02	5.39* ± 1.02	5.26* ± 0.63	3.82* ± 0.63	3.72* ± 0.49	3.43* ± 0.59
(X) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after RPAC (FWOP)	9.52 ± 0.69	7.03* ± 0.54	6.08* ± 0.97	5.49* ± 0.37	4.94* ± 0.47	4.62* ± 0.92	3.49* ± 0.80
(XI) 1400W (1 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	11.03 ± 0.63	7.84* ± 0.81	3.83* ± 0.49	3.94* ± 1.05	2.68* ± 0.64	2.40* ± 0.67	1.97* ± 0.62
(XII) 1400W (1 mg/kg I.V.) pretreatment immediately after RPAC (FWOP)	9.97 ± 0.96	5.86* ± 1.30	4.60* ± 0.28	3.98* ± 0.52	2.80* ± 0.27	2.73* ± 0.31	1.29* ± 0.53
(XIII) Sham (SWOP)	8.20 ± 0.12	4.84* ± 1.22	5.26* ± 0.95	4.04* ± 1.34	2.06* ± 0.52	2.62* ± 0.28	1.02* ± 0.17
(XIV) RAPC (SWOP)	8.18 ± 0.20	4.76* ± 0.93	5.20* ± 0.78	4.40* ± 1.09	4.06* ± 1.25	2.46* ± 1.04	1.30* ± 0.30
(XV) L-NAME (10 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	10.46 ± 0.64	6.04* ± 0.58	7.32* ± 0.30	4.78* ± 1.21	3.86* ± 0.94	3.04* ± 1.02	3.02* ± 0.41
(XVI) L-NAME (10 mg/kg I.V.) pretreatment 24 h. after RAPC	11.92 ± 0.28	5.49* ± 0.82	5.60* ± 0.25	3.93* ± 0.72	3.86* ± 0.63	2.97* ± 0.79	2.08* ± 0.53
(XVII) Aminoguanidine (150 mg/kg s.c) pretreatment immediately after sham operation (SWOP)	9.42 ± 0.25	5.12* ± 0.49	4.82* ± 0.46	3.92* ± 0.52	2.80* ± 0.40	2.08* ± 0.44	1.28* ± 0.10
(XVIII) Aminoguanidine (150 mg/kg s.c) pretreatment 24 h. after RAPC	10.96 ± 0.60	7.26* ± 0.92	7.68* ± 1.27	6.48* ± 1.18	4.20* ± 1.00	3.98* ± 0.89	2.18* ± 0.61
(XIX) Aminoguanidine (300 mg/kg s.c) pretreatment immediately after sham operation (SWOP)	10.80 ± 1.27	7.90* ± 1.03	7.79* ± 0.22	5.42* ± 0.23	4.54* ± 0.74	3.64* ± 0.62	3.50* ± 0.83
(XX) Aminoguanidine (300 mg/kg s.c) pretreatment immediately after RPAC (SWOP)	11.82 ± 0.44	5.92* ± 0.39	5.62* ± 1.20	5.39* ± 0.85	3.03* ± 0.59	2.91* ± 0.29	2.84* ± 0.67
(XXI) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	9.07 ± 0.73	4.97* ± 0.85	4.69* ± 0.82	4.49* ± 0.46	4.06* ± 0.52	3.05* ± 0.85	2.94* ± 1.03
(XXII) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after RPAC (SWOP)	10.57 ± 0.59	5.70* ± 0.31	5.62* ± 0.73	5.20* ± 0.72	3.98* ± 0.83	2.74* ± 0.94	2.79* ± 0.47
(XXIII) 1400W (1 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	9.73 ± 1.34	6.39* ± 0.76	6.17* ± 0.68	4.92* ± 0.64	2.99* ± 0.95	2.82* ± 0.42	1.74* ± 0.29
(XXIV) 1400W (1 mg/kg I.V.) pretreatment immediately after RPAC (SWOP)	8.94 ± 1.42	5.89* ± 1.07	5.18* ± 0.87	4.05* ± 0.77	3.97* ± 1.18	3.32* ± 0.79	2.59* ± 1.02

Vales are mean ± SEM ($n = 6$). Coronary flow rate was measured after stabilization (Basal), immediately (Imm. *R*), 5 (5 min. *R*), 15 (15 min. *R*), 30 (30 min. *R*), 60 (60 min. *R*) and 120 (120 min. *R*) min after reperfusion (*R*).

* $P < 0.05$ vs. Basal, Sham denotes sham operated.

Table 2 Acute Effect (FWOP) and delayed effect (SWOP) of remote preconditioning by aortic constriction (RPAC) and pre-treatment with NOS inhibitors on heart rate (beats/min) in isolated rat heart subjected to global ischaemia (30 min) and reperfusion (120 min).

Groups	Basal	5 min *R*	15 min.*R*	30 min.*R*	60 min. *R*	120 min. *R*
(I) Sham (FWOP)	205 ± 30	144* ± 20	174* ± 20	146* ± 15	132* ± 10	126* ± 15
(II) RAPC (FWOP)	228 ± 35	120* ± 30	180* ± 30	144* ± 30	156* ± 30	114* ± 19
(III) L-NAME (10 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	226 ± 18	146* ± 31	164* ± 08	152* ± 13	143* ± 22	111* ± 25
(IV) L-NAME (10 mg/kg I.V.) pretreatment immediately after RPAC (FWOP)	207 ± 22	122* ± 13	178* ± 26	143* ± 28	140* ± 07	119* ± 32
(V) Aminoguanidine (150 mg/kg) pretreatment immediately after sham operation (FWOP)	182 ± 12	124* ± 23	142* ± 15	136* ± 15	138* ± 20	124* ± 20
(VI) Aminoguanidine (150 mg/kg) pretreatment immediately after RAPC (FWOP)	216 ± 26	120* ± 19	114* ± 17	132* ± 08	120* ± 10	112* ± 10
(VII) Aminoguanidine (300 mg/kg) pretreatment immediately after sham operation (FWOP)	178 ± 23	136* ± 22	159* ± 18	153* ± 26	134* ± 06	125* ± 13
(VIII) Aminoguanidine (300 mg/kg) pretreatment immediately after RAPC (FWOP)	207 ± 24	148* ± 24	136* ± 25	132* ± 14	122* ± 16	118* ± 19
(IX) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	182 ± 19	147* ± 26	177* ± 28	141* ± 28	132* ± 24	131* ± 20
(X) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	221 ± 33	149* ± 31	168* ± 32	168* ± 21	152* ± 27	141* ± 28
(XI) 1400W (1 mg/kg I.V.) pretreatment immediately after sham operation (FWOP)	233 ± 21	130* ± 19	188* ± 25	171* ± 23	139* ± 22	102* ± 24
(XII) 1400W (1 mg/kg I.V.) pretreatment immediately after RPAC (FWOP)	194 ± 16	127* ± 12	152* ± 34	166* ± 11	127* ± 30	126* ± 18
(XIII) Sham (SWOP)	228 ± 30	120* ± 20	138* ± 10	139* ± 20	126* ± 14	113* ± 12
(XIV) RAPC (SWOP)	252 ± 30	126* ± 15	180* ± 20	120* ± 08	114* ± 15	125* ± 10
(XV) L-NAME (10 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	203 ± 16	147* ± 31	179* ± 18	152* ± 13	133* ± 24	108* ± 16
(XVI) L-NAME (10 mg/kg I.V.) pretreatment immediately after RPAC (SWOP)	225 ± 18	132* ± 24	158* ± 30	138* ± 20	143* ± 19	122* ± 21
(XVII) Aminoguanidine (150 mg/kg s.c.) pretreatment 24 h. after sham operation	218 ± 09	140* ± 12	146* ± 11	134* ± 05	123* ± 08	96* ± 13
(XVIII) Aminoguanidine (150 mg/kg s.c.) pretreatment 24 h. after RAPC	234 ± 11	122* ± 16	152* ± 16	140* ± 11	144* ± 15	118* ± 07
(XIX) Aminoguanidine (300 mg/kg s.c.) pretreatment immediately after sham operation (SWOP)	223 ± 15	146* ± 17	183* ± 27	159* ± 16	149* ± 07	123* ± 19
(XX) Aminoguanidine (300 mg/kg s.c.) pretreatment immediately after RAPC (SWOP)	193 ± 23	135* ± 21	177* ± 15	148* ± 19	113* ± 12	116* ± 07
(XXI) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	202 ± 08	149* ± 29	169* ± 14	161* ± 27	151* ± 23	121* ± 14
(XXII) S-methyl isothiourea (3 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	189 ± 26	138* ± 06	168* ± 22	182* ± 06	147* ± 15	128* ± 10
(XXIII) 1400W (1 mg/kg I.V.) pretreatment immediately after sham operation (SWOP)	253 ± 07	128* ± 31	176* ± 25	173* ± 15	128* ± 19	108* ± 12
(XXIV) 1400W (1 mg/kg I.V.) pretreatment immediately after RPAC (SWOP)	199 ± 28	142* ± 22	149* ± 17	141* ± 27	132* ± 06	126* ± 22

Vales are mean ± SEM ($n = 5$). Heart rate was measured after stabilization (Basal), 5 (5 min. *R*), 15 (15 min. *R*), 30 (30 min. *R*), 60 (60 min. *R*) and 120 (120 min. *R*) min after reperfucion (*R*).
* $P < 0.05$ vs. Basal, Sham denotes sham operated.

Table 3 Acute effect (FWOP) of remote preconditioning by aortic constriction and pre-treatment with NOS inhibitors on lactate dehydrogenase (LDH) release in coronary effluent of isolated rat heart subjected to global ischaemia (30 min) followed by reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	197 ± 6.653
		RAPC	98.5 ± 4.976*
		L-NAME pretreatment in Sham	180.833 ± 3.419
		L-NAME pretreatment in RAPC	179.5 ± 0.582*
	Immediate reperfusion	Sham	1310.167 ± 22.511*
		RAPC	336 ± 10.906*
		L-NAME pretreatment in Sham	807.833 ± 10.406
		L-NAME pretreatment in RAPC	808.031 ± 4.379*
	30 min Reperfusion	Sham	999 ± 34.438
		RAPC	207.833 ± 10.134*
		L-NAME pretreatment in Sham	902.833 ± 10.613
		L-NAME pretreatment in RAPC	896.243 ± 7.892*
Aminoguanidine (150 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	197 ± 6.653
		RAPC	98.5 ± 4.976*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	180.833 ± 3.419
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RAPC	83.3 ± 0.601*
	Immediate reperfusion	Sham	1310.167 ± 22.511*
		RAPC	336 ± 10.906*
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	807.833 ± 10.406
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RAPC	347.333 ± 5.690*
	30 min Reperfusion	Sham	999 ± 34.438
		RAPC	207.833 ± 10.134*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	902.833 ± 10.613
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RAPC	231.333 ± 6.448*
Aminoguanidine (300 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	216.947 ± 2.756
		RAPC	106.621 ± 8.796*
		Aminoguanidine(300 mg/kg s.c.) pretreatment in Sham	188.639 ± 12.137
		Aminoguanidine(300 mg/kg s.c.) pretreatment in RAPC	198.723 ± 7.105
	Immediate reperfusion	Sham	1402.172 ± 25.347*
		RAPC	342.926 ± 14.488*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	827.833 ± 17.786
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RAPC	346.342 ± 6.780*
	30 min Reperfusion	Sham	1062.415 ± 24.368*
		RAPC	219.623 ± 15.462*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	1102.833 ± 15.134
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RAPC	230.372 ± 7.822*

S-methyl isothiourea (3 mg/kg I.V.) pretreatment FWOP)	Basal	Sham	239.452 ± 17.531
		RAPC	111.133 ± 12.673*
		S-methyl isothiourea pretreatment in Sham	179.937 ± 14.762
		S-methyl isothiourea pretreatment in RAPC	211.385 ± 14.178
	Immediate reperfusion	Sham	1382.194 ± 23.848*
		RAPC	337.963 ± 19.863*
		S-methyl isothiourea pretreatment in Sham	836.623 ± 27.637
		S-methyl isothiourea pretreatment in RAPC	352.262 ± 13.840*
	30 min Reperfusion	Sham	1069.415 ± 24.368*
		RAPC	223.377 ± 18.682*
		S-methyl isothiourea pretreatment in Sham	1104.373 ± 24.345
		S-methyl isothiourea pretreatment in RAPC	226.252 ± 17.223*
1400W (1 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	239.452 ± 17.531
		RAPC	111.133 ± 12.673*
		1400W pretreatment in Sham	185.357 ± 10.652
		1400W pretreatment in RAPC	223.539 ± 18.768
	Immediate reperfusion	Sham	1382.194 ± 23.848*
		RAPC	337.963 ± 19.863*
		1400W pretreatment in Sham	836.623 ± 27.637
		1400W pretreatment in RAPC	352.262 ± 13.840*
	30 min Reperfusion	Sham	1069.415 ± 24.368*
		RAPC	223.377 ± 18.682*
		1400W pretreatment in Sham	1131.468 ± 4.485
		1400W pretreatment in RAPC	231.723 ± 22.846*

LDH was estimated after stabilisation (Basal), immediately (Imm) and 30 min (30′) after reperfusion, Sham denoted that rats were not subjected to remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.

* $P < 0.05$ vs. Sham as compared to its respective Basal value.

Table 4 Acute effect (SWOP) of remote preconditioning by aortic constriction and pre-treatment with NOS inhibitors on lactate dehydrogenase (LDH) release in coronary effluent of isolated rat heart subjected to global ischaemia (30 min) followed by reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (SWOP)	Basal	Sham	207.294 ± 0.420
		RAPC	83.667 ± 0.529*
		L-NAME pretreatment in Sham	196 ± 1.358
		L-NAME pretreatment in RAPC	198.667 ± 0.831
	Immediate reperfusion	Sham	902.583 ± 15.290*
		RAPC	131.806 ± 5.333*
		L-NAME pretreatment in Sham	821.463 ± 13.472
		L-NAME pretreatment in RAPC	792.762 ± 7.036
	30 min Reperfusion	Sham	204.343 ± 5.533*
		RAPC	92.427 ± 1.587*
		L-NAME pretreatment in Sham	187.385 ± 6.333
		L-NAME pretreatment in RAPC	190.563 ± 3.416
Aminoguanidine (150 mg/kg s.c.) pretreatment (SWOP)	Basal	Sham	199.667 ± 0.760
		RAPC	89.667 ± 0.667*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	193 ± 1.065
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RAPC	183.667 ± 0.333
	Immediate reperfusion	Sham	852.5 ± 12.529*
		RAPC	101.333 ± 5.806*
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	803.333 ± 11.427
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RAPC	783.667 ± 5.596
	30 min Reperfusion	Sham	198.333 ± 8.815*
		RAPC	80.333 ± 1.358*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	177.5 ± 4.233
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RAPC	182.5 ± 2.141
Aminoguanidine (300 mg/kg s.c.) pretreatment (SWOP)	Basal	Sham	207.294 ± 0.420
		RAPC	83.667 ± 0.529*
		Aminoguanidine(300 mg/kg s.c.) pretreatment in Sham	196 ± 1.358
		Aminoguanidine(300 mg/kg s.c.) pretreatment in RAPC	198.667 ± 0.831
	Immediate reperfusion	Sham	902.583 ± 15.290*
		RAPC	131.806 ± 5.333*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	821.463 ± 13.472
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RAPC	792.762 ± 7.036
	30 min Reperfusion	Sham	204.343 ± 5.533*
		RAPC	92.427 ± 1.587*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	187.385 ± 6.333
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RAPC	190.563 ± 3.416

S-methyl isothiourea (3 mg/kg I.V.) pretreatment (SWOP)	Basal	Sham	214.954 ± 14.230
		RAPC	102.340 ± 12.425*
		S-methyl isothiourea pretreatment in Sham	176 ± 11.538
		S-methyl isothiourea pretreatment in RAPC	189.727 ± 10.127
	Immediate reperfusion	Sham	1002.583 ± 14.231*
		RAPC	124.318 ± 4.395*
		S-methyl isothiourea pretreatment in Sham	848.634 ± 11.437
		S-methyl isothiourea pretreatment in RAPC	824.527 ± 17.327
	30 min Reperfusion	Sham	192.463 ± 13.334*
		RAPC	98.241 ± 13.552*
		S-methyl isothiourea pretreatment in Sham	192.331 ± 6.459
		S-methyl isothiourea pretreatment in RPAC	188.693 ± 12.156
1400W (1 mg/kg I.V.) pretreatment (SWOP)	Basal	Sham	207.294 ± 0.420
		RPAC	83.667 ± 0.529*
		1400W pretreatment in Sham	204.593 ± 1.871
		1400W pretreatment in RPAC	213.429 ± 0.361
	Immediate reperfusion	Sham	902.583 ± 15.290*
		RPAC	131.806 ± 5.333*
		1400W pretreatment in Sham	816.361 ± 14.792
		1400W pretreatment in RPAC	832.642 ± 16.386
	30 min Reperfusion	Sham	204.343 ± 5.533*
		RPAC	92.427 ± 1.587*
		1400W pretreatment in Sham	203.865 ± 19.592
		1400W pretreatment in RPAC	225.724 ± 12.426

LDH was estimated after stabilisation (Basal), immediately (Imm) and 30 min (30') after reperfusion, Sham denoted that rats were not subjected to remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.

* $P < 0.05$ vs. Sham as compared to its respective Basal value.

Table 5 Acute Effect (FWOP) of Remote Preconditioning by Aortic Constriction and pre-treatment with NOS inhibitors on Creatine Kinase (CK) Release in coronary Effluent of Isolated Rat Heart Subjected to Global Ischaemia (30 min) followed by Reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	20.167 ± 0.601
		RPAC	2.167 ± 1.078*
		L-NAME pretreatment in Sham	22.333 ± 1.116
		L-NAME pretreatment in RPAC	23.927 ± 0.475
	5 min Reperfusion	Sham	140.5 ± 4.015*
		RPAC	30.5 ± 2.172*
		L-NAME pretreatment in Sham	126.5 ± 3.731
		L-NAME pretreatment in RPAC	133.5 ± 2.232
Aminoguanidine (150 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	20.167 ± 0.601
		RPAC	2.167 ± 1.078*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	22.333 ± 1.116
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RPAC	07 ± 0.683*
	5 min Reperfusion	Sham	140.5 ± 4.015*
		RPAC	30.5 ± 2.172*
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	126.5 ± 3.731
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RPAC	43.5 ± 2.232
Aminoguanidine (300 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	20.167 ± 0.601
		RPAC	2.167 ± 1.078*
		Aminoguanidine(300 mg/kg s.c.) pretreatment in Sham	22.333 ± 1.116
		Aminoguanidine(300 mg/kg s.c.) pretreatment in RPAC	07 ± 0.683*
	5 min Reperfusion	Sham	140.5 ± 4.015*
		RPAC	30.5 ± 2.172*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	126.5 ± 3.731
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RPAC	59.5 ± 2.232*
S-methyl isothiourea (3 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	22.627 ± 0.652
		RPAC	3.263 ± 1.738*
		S-methyl isothiourea pretreatment in Sham	24.333 ± 1.346
		S-methyl isothiourea pretreatment in RPAC	08.934 ± 0.832*
	5 min Reperfusion	Sham	152.563 ± 3.515*
		RPAC	33.725 ± 4.259*
		S-methyl isothiourea pretreatment in Sham	134.5 ± 4.318
		S-methyl isothiourea pretreatment in RPAC	36.5 ± 3.542
1400W (1 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	20.167 ± 0.601
		RPAC	2.167 ± 1.078*
		1400W pretreatment in Sham	22.333 ± 1.116
		1400W pretreatment in RPAC	07 ± 0.683*
	5 min Reperfusion	Sham	140.529 ± 4.015*
		RPAC	63.084 ± 2.172*
		1400W pretreatment in Sham	126.821 ± 3.731
		1400W pretreatment in RPAC	67.284 ± 2.232

LDH was estimated after stabilisation (Basal), immediately (Imm) and 30 min (30′) after reperfusion, Sham denoted that rats were not subjected to remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.

* $P < 0.05$ vs. Sham as compared to its respective Basal value.

significant change in the flow rate (Table 1) and the heart rate (Table 2).

Effect of pre-treatment with NOS inhibitors on ischaemia/reperfusion-induced myocardial injury

The peak increase in release of LDH in the coronary effluent of isolated rat heart subjected to global ischaemia and reperfusion was noted immediately and 30 min after reperfusion (Table 3), whereas peak increase in the release of CK was noted after 5 min of reperfusion. L-NAME (10 mg kg^{-1} I.V.), aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.) administered 10 min before removing the heart for ischaemia/reperfusion study using Langendorff's apparatus produced no marked effect on ischaemia/reperfusion-induced release of LDH (Table 3), CK (Table 5) and myocardial infarct size (Table 7).

Table 6 Delayed Effect (SWOP) of Remote Preconditioning by Aortic Constriction and pre-treatment with NOS inhibitors on Creatine Kinase (CK) Release in coronary Effluent of Isolated Rat Heart Subjected to Global Ischaemia (30 min) followed by Reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	40.167 ± 2.937
		RPAC	18.333 ± 2.565*
		L-NAME pretreatment in Sham	38.167 ± 2.428
		L-NAME pretreatment in RPAC	23.421 ± 1.751*
	5 min Reperfusion	Sham	233.5 ± 19.155*
		RPAC	43.5 ± 2.012*
		L-NAME pretreatment in Sham	203 ± 9.856
		L-NAME pretreatment in RPAC	121.647 ± 12.246*
Aminoguanidine (150 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	40.167 ± 2.937
		RPAC	18.333 ± 2.565*
		Aminoguanidine(150 mg/kg s.c.) pretreatment in Sham	38.167 ± 2.428
		Aminoguanidine(150 mg/kg s.c.) pretreatment in RPAC	36 ± 1.751
	5 min Reperfusion	Sham	233.5 ± 19.155*
		RPAC	43.5 ± 2.012*
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	203 ± 9.856
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RPAC	237.167 ± 3.842
Aminoguanidine (300 mg/kg s.c.) pretreatment (FWOP)	Basal	Sham	40.167 ± 2.937
		RPAC	18.333 ± 2.565*
		Aminoguanidine(300 mg/kg s.c.) pretreatment in Sham	38.167 ± 2.428
		Aminoguanidine(300 mg/kg s.c.) pretreatment in RPAC	36 ± 1.751
	5 min Reperfusion	Sham	233.5 ± 19.155*
		RPAC	43.5 ± 2.012*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	203 ± 9.856
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RPAC	237.167 ± 3.842
S-methyl isothiourea (3 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	43.617 ± 3.657
		RPAC	17.426 ± 4.585*
		S-methyl isothiourea pretreatment in Sham	40.524 ± 3.258
		S-methyl isothiourea pretreatment in RPAC	39.469 ± 2.541
	5 min Reperfusion	Sham	236.5 ± 9.553*
		RPAC	45.483 ± 3.126*
		S-methyl isothiourea pretreatment in Sham	205.930 ± 10.567
		S-methyl isothiourea pretreatment in RPAC	229.167 ± 5.835
1400W (1 mg/kg I.V.) pretreatment (FWOP)	Basal	Sham	40.167 ± 2.937
		RPAC	18.333 ± 2.565*
		1400W pretreatment in Sham	37.616 ± 2.428
		1400W pretreatment in RPAC	36.83 ± 1.751
	5 min Reperfusion	Sham	233.5 ± 19.155*
		RPAC	43.5 ± 2.012*
		1400W pretreatment in Sham	213 ± 9.856
		1400W pretreatment in RPAC	226.167 ± 2.242

LDH was estimated after stabilisation (Basal), immediately (Imm) and 30 min (30′) after reperfusion, Sham denoted that rats were not subjected to remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.

* $P < 0.05$ vs. Sham as compared to its respective Basal value.

Acute (FWOP) and delayed (SWOP) effects of RPAC on ischaemia/reperfusion-induced myocardial injury

Rat heart isolated immediately (acute/FWOP) or 24 h (delayed/SWOP) after RPAC demonstrated a significant decrease in ischaemia/reperfusion-induced release of LDH (Tables 3 and 4), CK (Tables 5 and 6) and extent of myocardial infarct size (Tables 7 and 8). RPAC produced acute (FWOP) and delayed (SWOP) cardioprotective effects.

Effect of NOS inhibitors on acute (FWOP) and delayed (SWOP) RPAC in ischaemia/reperfusion-induced myocardial injury

In the rat heart isolated immediately after RPAC (Acute/FWOP) and pre-treatment with aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.) produced no significant effect on PC-induced decrease in LDH (Table 3), CK (Table 5) release and myocardial infarct size

Table 7 Acute Effect of Remote Preconditioning by Aortic Constriction and pre-treatment with NOS inhibitors on Myocardial Infarct Size in Isolated Rat Heart Subjected to Global Ischaemia (30 min) followed by Reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (FWOP)	% Infarct by volume	Sham	41.167 ± 1.662
		RPAC	24.934 ± 1.238*
		Aminoguanidine pretreatment in Sham	45.5 ± 1.979
		Aminoguanidine pretreatment in RPAC	46.167 ± 0.872
	% Infarct by weight	Sham	42.5 ± 2.592
		RPAC	27.833 ± 1.662*
		Aminoguanidine pretreatment in Sham	47 ± 1.414
		Aminoguanidine pretreatment in RPAC	46.667 ± 1.308
Aminoguanidine (150 mg/kg s.c.) pretreatment (FWOP)	% Infarct by volume	Sham	41.167 ± 1.662
		RPAC	24 ± 1.238*
		Aminoguanidine pretreatment in Sham	45.5 ± 1.979
		Aminoguanidine pretreatment in RPAC	30.167 ± 0.872*
	% Infarct by weight	Sham	42.5 ± 2.592
		RPAC	27.833 ± 1.662*
		Aminoguanidine pretreatment in Sham	47 ± 1.414
		Aminoguanidine pretreatment in RPAC	27.667 ± 1.308*
Aminoguanidine (300 mg/kg s.c.) pretreatment (FWOP)	% Infarct by volume	Sham	42.274 ± 3.732
		RPAC	23.429 ± 2.368*
		Aminoguanidine pretreatment in Sham	46.425 ± 2.079
		Aminoguanidine pretreatment in RPAC	30.290 ± 0.977*
	% Infarct by weight	Sham	42.578 ± 2.392
		RPAC	28.333 ± 1.766*
		Aminoguanidine pretreatment in Sham	47.472 ± 1.741
		Aminoguanidine pretreatment in RPAC	26.733 ± 2.468*
S-methyl isothiourea (3 mg/kg I.V.) pretreatment (FWOP)	% Infarct by volume	Sham	40.333 ± 4.724
		RPAC	24.728 ± 2.383*
		Aminoguanidine pretreatment in Sham	45.541 ± 2.799
		Aminoguanidine pretreatment in RAPC	31.673 ± 1.726*
	% Infarct by weight	Sham	42.500 ± 3.924
		RAPC	26.833 ± 2.674*
		Aminoguanidine pretreatment in Sham	47.000 ± 2.414
		Aminoguanidine pretreatment in RPAC	26.667 ± 2.338*
1400W (1 mg/kg I.V.) pretreatment (FWOP)	% Infarct by volume	Sham	43.67 ± 1.662
		RAPC	24.859 ± 1.238*
		Aminoguanidine pretreatment in Sham	45.5 ± 1.979
		Aminoguanidine pretreatment in RPAC	44.167 ± 0.872
	% Infarct by weight	Sham	42.5 ± 2.592
		RPAC	27.833 ± 1.662*
		Aminoguanidine pretreatment in Sham	47 ± 1.414
		Aminoguanidine pretreatment in RPAC	48.667 ± 1.308

Sham denoted that rats were not subjected to remote remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.
* $P < 0.05$ vs. Sham.

(Table 7). L-NAME (10 mg kg^{-1} I.V.) administration increased release of LDH and CK (Table 3) in coronary effluent and also increased myocardial infarct size (Table 5) in acute/FWOP RPAC.

On the other hand, administration of L-NAME (10 mg kg^{-1} I.V.), aminoguanidine (150 mg kg^{-1} s.c.), aminoguanidine (300 mg kg^{-1} s.c.), S-methyl isothiourea (3 mg kg^{-1} I.V.) and 1400W (1 mg kg^{-1} I.V.) 24 h after subjecting rat heart to remote aortic PC (delayed/SWOP), attenuated RPAC-induced decrease in LDH (Table 4), CK (Table 6) release in coronary effluent and myocardial infarct size (Table 8).

Discussion

In the present study, four episodes of occlusion of aorta followed by reperfusion markedly protected the rat heart against sustained ischaemia/reperfusion-induced myocardial injury. The observed acute and delayed cardioprotective effect of RPAC has been supported by our earlier observation noted with remote renal PC [4–6]. It has been clarified that there is no limitation of this experimental condition, as *ex vivo* experimental results could be directly extrapolated into clinical settings as well as *in vivo* animal

Table 8 Delayed Effect of Remote Preconditioning by Aortic Constriction and pre-treatment with NOS inhibitors on Myocardial Infarct Size in Isolated Rat Heart Subjected to Global Ischaemia (30 min) followed by Reperfusion (120 min).

L-NAME (10 mg/kg I.V.) pretreatment (SWOP)	% Infarct by volume	Sham	45.167 ± 1.424
		RPAC	24.5 ± 0.764*
		L-NAME pretreatment in Sham	45 ± 1.238[a]
		L-NAME pretreatment in RPAC	45.333 ± 1.430[a]
	% Infarct by weight	Sham	45 ± 0.966
		RPAC	24.167 ± 0.872*
		L-NAME pretreatment in Sham	45.333 ± 1.116[a]
		L-NAME pretreatment in RPAC	47 ± 1.461
Aminoguanidine (150 mg/kg s.c.) pretreatment (SWOP)	% Infarct by volume	Sham	45.167 ± 1.424
		RPAC	24.5 ± 0.764[a]
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	45 ± 1.238
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RPAC	45.333 ± 1.430
	% Infarct by weight	Sham	45 ± 0.966
		RPAC	24.167 ± 0.872*
		Aminoguanidine (150 mg/kg s.c.) pretreatment in Sham	45.333 ± 1.116
		Aminoguanidine (150 mg/kg s.c.) pretreatment in RPAC	47 ± 1.461
Aminoguanidine (300 mg/kg s.c.) pretreatment (SWOP)	% Infarct by volume	Sham	44.675 ± 2.434
		RPAC	24.568 ± 1.767*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	45.783 ± 1.253
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RPAC	46.232 ± 1.630
	% Infarct by weight	Sham	45.024 ± 1.667
		RPAC	24.667 ± 0.762*
		Aminoguanidine (300 mg/kg s.c.) pretreatment in Sham	45.933 ± 3.121
		Aminoguanidine (300 mg/kg s.c.) pretreatment in RPAC	47.672 ± 2.346
S-methyl isothiourea (3 mg/kg I.V.) pretreatment (SWOP)	% Infarct by volume	Sham	48.528 ± 2.246
		RPAC	24.558 ± 1.674*
		S-methyl isothiourea pretreatment in Sham	45.000 ± 2.283
		S-methyl isothiourea pretreatment in RPAC	45.533 ± 4.436
	% Infarct by weight	Sham	45.824 ± 0.966
		RPAC	23.783 ± 0.724*
		S-methyl isothiourea pretreatment in Sham	45.333 ± 1.116
		S-methyl isothiourea pretreatment in RPAC	47.000 ± 4.244
1400W (1 mg/kg I.V.) pretreatment (SWOP)	% Infarct by volume	Sham	45.167 ± 1.424
		RPAC	24.5 ± 0.764*
		1400W pretreatment in Sham	45 ± 1.238
		1400W pretreatment in RPAC	45.333 ± 1.430
	% Infarct by weight	Sham	45 ± 0.966
		RPAC	24.167 ± 0.872*
		1400W pretreatment in Sham	45.333 ± 1.116
		1400W pretreatment in RPAC	47 ± 1.461

Sham denoted that rats were not subjected to remote preconditioning by aortic constriction and RPAC denoted that rats were subjected to remote preconditioning by aortic constriction. Values are mean ± SEM of six experiments.
* $P < 0.05$ vs. Sham.

conditions supported by findings by Hausenloy et al. [7] Bøtker et al. [8] and Hausenloy et al. [9]. In the present study, the delayed cardioprotective effect of RPAC is attenuated with aminoguanidine, L-NAME, S-methyl isothiourea and 1400W (NOS inhibitors). Aminoguanidine is reported to attenuate endotoxin-induced delayed cardioprotection [19].

1400W, a selective iNOS inhibitor, was used to determine whether this isozyme was involved in the cardioprotective mechanism. 1400W was chosen because of its higher selectivity for iNOS (5000 times more selective for iNOS than for eNOS) [25]. A specific role of iNOS-derived NO as a mediator of delayed cardioprotection has also been reported for ischaemic PC. Thus, iNOS induction was shown to be necessary for the development of delayed protection conferred by ischaemic PC in anaesthetised rabbit models of myocardial infarction and stunning [25]. Vegh et al. have also demonstrated that iNOS inhibition prevents the development of delayed PC against arrhythmias, in the dog. Using iNOS knockout mice, Guo et al. have shown that targeted disruption of the iNOS gene completely abrogates the infarct-sparing effect of late IP, demonstrating that the

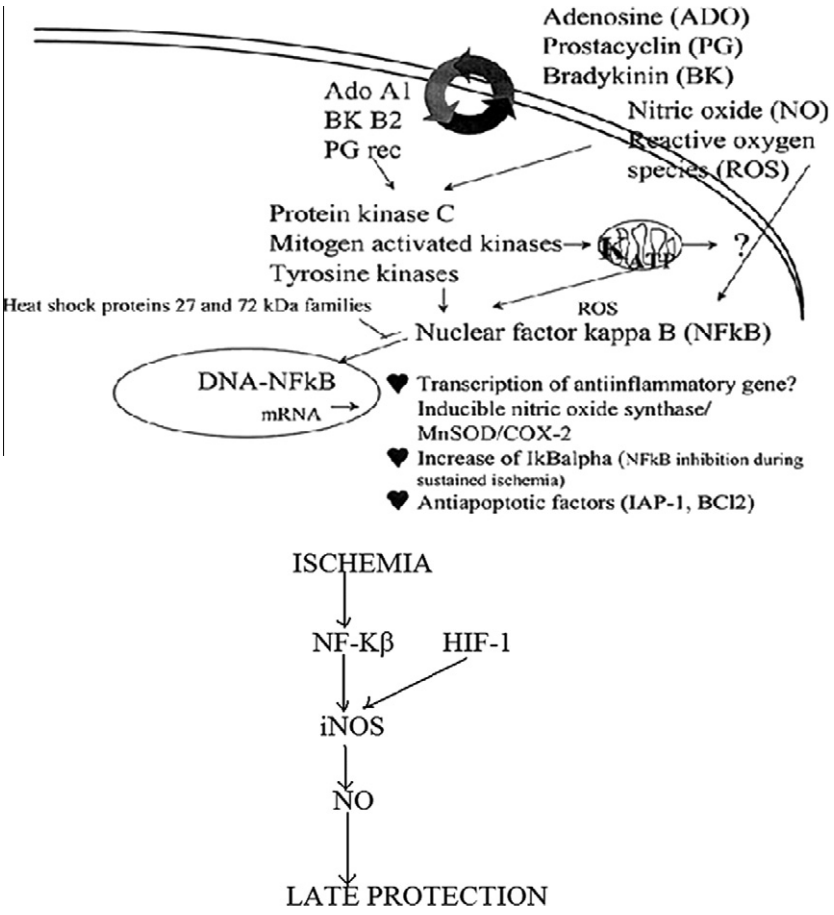

Figure 2 Mechanism of cardioprotection: release nitric oxide synthase by ischeamic preconditioning.

activity of iNOS is indispensable for this cardioprotective phenomenon to occur [25]. Furthermore, iNOS appears to be a final mediator of several other forms of delayed myocardial PC, such as that induced by NO donors, endotoxin derivatives and exercise. Although recent studies suggest that adenosine A1-receptor—agonist-induced cardioprotection occurs independently of either early generation of NO or induction of iNOS, an implication of iNOS has also been shown in this form of pharmacological PC [25].

Recent evidence has shown that the cardioprotection afforded by the late phase of ischaemic PC is mediated by up-regulation of iNOS. However, the specific cardiac cell type(s) that express(es) iNOS in response to ischaemic PC remain(s) unknown. Thus, mice underwent a sequence of six cycles of 4-min coronary occlusion/4-min reperfusion, which induces late PC, and tissue samples were collected at serial times for measurement of messenger RNA (mRNA) (Northern) and protein levels (Western). In addition, whole heart samples were cryosectioned for *in situ* hybridisation and immunohistochemistry. The steady-state levels of iNOS mRNA in the ischaemic regions started to increase at 1 h after ischaemic PC, peaked at 3 h (201 ± 31% of Sham, $n = 5$, $P < 0.01$) and remained elevated at 24 h (177 ± 22% of Sham, $n = 5$, $P < 0.01$). In accordance with these data, iNOS protein expression was increased at 24 h (219 ± 41% of Sham, $n = 5$, $P < 0.01$). By contrast, neither endothelial nitric oxide synthase (eNOS) mRNA levels nor its protein expression changed at any time point [26].

Administration of aminoguanidine (300 mg kg^{-1}, s.c.) or S-methylisothiourea sulphate (3 mg kg, I.V.), both relative iNOS inhibitors, 60 or 30 min before sustained myocardial ischaemia not only abolished the late PC afforded by intestinal ischaemia, but also inhibited the ability of intestinal ischaemia PC to significantly reduce neutrophil infiltration. A change in iNOS activity was not observed in normal myocardium 24 h after intestinal ischaemia, but 30 min of coronary occlusion significantly increased the iNOS activity in the preconditioned group, which was abolished by aminoguanidine or S-methylisothiourea sulphate. In conclusion, the above data provide pharmacological evidence that induction of iNOS, following intestinal ischaemia, is associated with increased myocardial tolerance to infarction 24 h later [20]. The same is confirmed in the present study which showed that aminoguanidine (300 mg kg^{-1}, s.c.) or S-methylisothiourea sulphate (3 mg kg^{-1}, I.V.), both relative iNOS inhibitors, abolished the late PC afforded by RPAC.

Aminoguanidine in the dose employed is reported to inhibit selectively iNOS [27,28]. The late phase of ischaemic PC is a delayed adaptive response that renders the heart relatively resistant to sustained ischaemia and reperfusion. NO is identified as an initial signal for triggering the late cardioprotective effect of classical ischaemic PC [15,16,29]. Acti-

vation of pro-inflammatory mediators such as cytokines and iNOS have been shown to contribute to myocardial injury after ischaemia and reperfusion [30—32]. Up-regulation of iNOS may account for preconditioning of the heart by brief ischaemic stress. Different induction of mRNA for iNOS in rat smooth muscle cells in culture and in aortic strips has been found. One of the transcription factors that could activate gene expression in response to ischaemic PC is nuclear factor Kappa-B (NF-κB). This oxidant-sensitive transcription factor plays a critical role in the immediate — early activation of a multitude of genes encoding signalling and defence proteins expressed in response to various stressful situations, and therefore appears to be a general mediator of cellular responses to stress [33—35]. It is well established that the 5′ flanking region of the iNOS gene contains a consensus sequence that is NF-κβ and that the activation of NF-κβ is a central mechanism controlling the induction of iNOS in several cell types, including cardiac myocytes [31,36—38]. Therefore, it may be possible that the noted delayed cardioprotective effect of RPAC may be due to the up-regulation of iNOS due to shear stress exerted on the myocardium by aortic occlusions. This contention is supported by the results of the present study because aminoguanidine, a selective iNOS inhibitor, attenuated the delayed cardioprotective effect of remote aortic PC [27,28]. Our results are supported by observations, which implicate the role of iNOS in the delayed cardioprotective effect of classical ischaemic PC [15,16,19]. It is further supported that pacing-induced delayed protection against arrhythmias is attenuated by aminoguanidine, an inhibitor of NOS [39]. Delayed or second-window PC induced by adenosine A1-receptor activation is independent of early generation of NO or late induction of iNOS [40].

The induction of iNOS requires a lag phase. The acute cardioprotective effect of RPAC was observed immediately after the PC stimulus. Therefore, the involvement of iNOS in it may be remote because the induction of iNOS requires some lag time [26,27]. Moreover, this contention is further supported by our results, which have demonstrated that aminoguanidine, S-methyl isothiourea and 1400W, in the dosage employed, have produced no notable effect on the acute cardioprotective effect of remote PC afforded by aortic constriction. Therefore, it may be possible that the acute cardioprotective effect of RPAC may be mediated through the activation of eNOS perhaps as result of shear stress [32,41], and is confirmed by the present study because L-NAME, a non-specific NOS inhibitor, abolished the acute cardioprotective effect produced by RPAC.

Over the past decade, an enormous number of studies (>100) have focussed on the role of NO in myocardial ischaemia. It is important to distinguish the function of NO in unstressed (non-preconditioned) myocardium from its function in preconditioned myocardium (i.e., myocardium that has shifted to a defensive phenotype in response to stress). The time has come to translate this enormous body of experimental evidence into clinically useful therapies by harnessing the cytoprotective properties of NO [42—46] (Fig. 2).

PC is, in experimental studies, the most powerful mode of cardioprotection known. The signal transduction pathways involve a variety of trigger substances, mediators, receptors and effectors. The studies of PC in cardiac surgery provide conflicting results, but the majority show that ischaemic PC is an effective adjunct to myocardial protection. However, ischaemic PC with repeated clamping of the aorta will never have widespread use. If the 'preconditioning response' is to be exploited in cardiac surgery, targeting the underlying molecular mechanisms must provide easily applicable techniques or drugs, which are shown in large-scale clinical studies to be beneficial [47]. Remote ischaemic PC increases the tolerance of the myocardium to ischaemia, reduces ischaemic chest discomfort during coronary balloon occlusion and reduces the prevalence of cardiac troponin I (cTnI) release after elective PCI [48]. The data add to the growing number of studies suggesting that remote ischaemic PC is a safe, effective, non-invasive, and cost-effective strategy for reducing ischaemic cardiac damage in settings where myocardial ischaemic damage is expected [49].

Conclusions

On the basis of the above results and discussion, it can be concluded that RPAC has produced acute and delayed cardioprotective effects as found earlier with classical and other remote PC stimuli. Recent evidence has shown that the cardioprotection afforded by the late phase of ischaemic PC is mediated by iNOS. The steady-state levels of iNOS mRNA in the ischaemic regions started to increase at 1 h after ischaemic PC, peaked at 3 h and remained elevated at 24 h. In accordance with these data, iNOS protein expression was increased at 24 h [26]. In the present study, iNOS was involved in the delayed cardioprotective effect afforded by RPAC, as supported by the above findings. On the other hand, activation of iNOS may not participate in the acute cardioprotective effect of RPAC.

Conflict of interest

There is no conflict of interest.

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