L-Arginine given after ischaemic preconditioning can enhance cardioprotection in isolated rat hearts

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Abstract

**Objective:** Ischaemic or pharmacological preconditioning with L-arginine has been reported to be insufficient for optimal cardioprotection. The ability of nitric oxide (NO) to enhance ischaemic preconditioning was assessed, and the role of L-arginine-induced ischaemic preconditioning in myocardial protection was determined. **Methods:** Isolated rat hearts were prepared and divided into six groups: control hearts (control, \(n = 6\)) were perfused without global ischaemia at 37°C for 160 min; global ischaemia hearts (GI, \(n = 6\)) were subjected to ischaemia for 20 min and reperfusion for 120 min; ischaemic preconditioned hearts (IP, \(n = 6\)) received 2 min of zero-flow global ischaemia followed by 5 min reperfusion, before 20 min of global ischaemia; L-arginine hearts (ARG, \(n = 6\)) received 1 mmol/l L-arginine for 5 min, before 20 min of global ischaemia; ischaemic preconditioning plus nitro-L-arginine methyl ester hearts (IP + L-NAME, \(n = 6\)) received 2 min of ischaemic preconditioning and 5 min reperfusion with 3 mmol/l L-NAME in Krebs–Henseleit buffer, before 20 min of global ischaemia; and ischaemic preconditioning plus L-arginine hearts (IP + ARG, \(n = 6\)) received 2 min of ischaemic preconditioning and 5 min reperfusion with 1 mmol/l L-arginine in Krebs–Henseleit buffer. Haemodynamic parameters and coronary flow were recorded continuously. Nitrites and nitrates (NO\(_x\)) were measured 5 and 60 min after reperfusion, and infarct size was also determined. **Results:** In the IP + ARG group, significant amelioration and preservation of left ventricular peak developed pressure and coronary flow was observed compared with the GI, IP, ARG and IP + L-NAME groups. Infarct size in the IP + ARG group was reduced significantly compared with that in the GI, IP, ARG and IP + L-NAME groups. Significant preservation of NO\(_x\) was observed during reperfusion in the IP + ARG group compared with the GI group. **Conclusions:** Inhibition of NO synthase with L-NAME had little impact on ischaemic preconditioning, suggesting that endogenous NO is not a major mediator of ischaemic preconditioning. Nevertheless, enhancement of the effects of ischaemic preconditioning can be achieved with L-arginine, a precursor of NO, improving post-ischaemic functional recovery and infarct size in the isolated rat heart. © 2001 Elsevier Science B.V. All rights reserved.

**Keywords:** Ischaemic preconditioning; L-Arginine; Nitric oxide; Cardioprotection; Isolated rat heart

1. Introduction

Since Murry et al. [1] reported a reduction in myocardial necrosis after repeated periods of ischaemia and reperfusion, known as ischaemic preconditioning, in an in vivo canine model of transient coronary occlusion, there have been several reports of similar results in other animal species [2–5]. Endogenous adaptation of the heart to prolonged ischaemic injury was observed by exposing the myocardium to a brief period of ischaemia before the prolonged event. Although protein kinase C and adenosine triphosphate-sensitive potassium (K\(^\text{+}\)-ATP) channel-mediated mechanisms have been suggested to play a role as mediators of myocardial protection [6], the exact details of the mechanisms and the role of ischaemic preconditioning remain unclear.

Nitric oxide (NO) is a strong endogenous vasodilator whose mode of action mimics ischaemic preconditioning. Vegh et al. [7] reported that NO contributed to the anti-arrhythmic effects of ischaemic preconditioning in the canine myocardium. However, a few reports [8] have contradicted these results and have suggested that NO does not play a role in ischaemic preconditioning. In this study, we examined the theory that NO does not play a major role in ischaemic preconditioning, but that it does enhance cardioprotection. L-Arginine, a precursor of NO,
was administered to isolated rat hearts under Langendorff perfusion by a short reperfusion method.

2. Materials and methods

2.1. Animals

All animals in this study received human care according to the guidelines laid down in the Principles of Laboratory Animal Care formulated by the National Society for Medical Research, and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). In addition, animals were used in accordance with the guidelines of the University of Tokyo Institutional Animal Care and Protocol of Animal Preparation.

2.2. Langendorff perfusion

Hearts were obtained from male Wistar rats (300–350 g body weight). Briefly, rats were anaesthetized with ether, and heparin (1000 units/kg, intravenously) was administered into the left femoral vein. The heart was excised rapidly and used for Langendorff perfusion. A water-filled latex balloon connected to a Statham pressure transducer was inserted into the left ventricle for measurement of left ventricular pressure (end-diastolic pressure and peak developed pressure). Left ventricular pressure was set to 5 mmHg by adjusting the volume of the balloon. This balloon volume was maintained for the duration of the experiment. Coronary flow of perfusion fluid was continuously measured with an extracorporeal electromagnetic flow probe. Haemodynamic variables were measured using the DTP-200 and WT 625G systems (Nihon Kohden Co., Tokyo, Japan).

The aorta was perfused with oxygenated (95% O₂ + 5% CO₂, pH 7.4) Krebs–Henseleit buffer (glucose 11.0 mmol/l, NaCl 118.5 mmol/l, KCl 4.8 mmol/l, MgSO₄ 1.2 mmol/l, KH₂PO₄ 1.2 mmol/l, NaHCO₃ 25.0 mmol/l and CaCl₂ 2.5 mmol/l) at 37°C and a root pressure of 100 mmHg. Before use, the buffer was filtered through a 5.0-μm porosity filter to remove any particulate matter. Aortic root pressure was maintained at 100 mmHg except during ischaemic periods.

2.3. Experimental protocol

The experimental protocol used is shown in Fig. 1. Hearts were perfused for 20 min to establish equilibrium haemodynamics. Equilibrium was ceased when left ventricular systolic pressure, diastolic pressure and coronary flow were maintained at the same level for three continuous periods of measurement timed 5 min apart. Hearts not meeting these criteria were not used in the study. After 20 min of perfusion, the hearts were divided into six groups: control hearts (control, n = 6) were perfused without global ischaemia at 37°C for 160 min; global ischaemia hearts (GI, n = 6) were subjected to 20 min ischaemia and 120 min reperfusion. Global ischaemia was achieved by cross-clamping of the root tube connected to the aorta; ischaemic preconditioned hearts (IP, n = 6) were subjected to 2 min of ischaemia and 20 min ischaemia preconditioning (IP+L-NAME, n = 6) were subjected to 2 min ischaemia and 120 min reperfusion. Global ischaemia was achieved by cross-clamping of the root tube connected to the aorta; ischaemic preconditioned hearts (IP, n = 6) were subjected to 2 min of

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**Fig. 1.** Experimental protocol.
zero-flow global ischaemia followed by 5 min reperfusion with Krebs–Henseleit buffer, before 20 min of global ischaemia and 120 min reperfusion; l-arginine hearts (ARG, n = 6) received 1 mmol/l l-arginine in Krebs–Henseleit buffer 5 min before 20 min of global ischaemia and 120 min reperfusion; ischaemic preconditioning plus an inhibitor of NO synthesis, nitro-l-arginine methyl ester (l-NAME), hearts (IP + l-NAME, n = 6) received 2 min ischaemic preconditioning and 5 min reperfusion with 3 mmol/l l-NAME in Krebs–Henseleit buffer, before 20 min of global ischaemia and 120 min reperfusion; hearts exposed to ischaemic preconditioning plus l-arginine hearts (IP + ARG, n = 6) received 2 min of ischaemic preconditioning and 5 min reperfusion with 1 mmol/l l-arginine in Krebs–Henseleit buffer, before 20 min of global ischaemia and 120 min reperfusion. A single 2-min episode of ischaemia was chosen for the optimal period of ischaemic preconditioning, and the dosage of l-arginine was determined as described previously [9,10] After the right atrium had been incised to prevent arrhythmia, the hearts were paced continuously through the right atrium at 370 beats/min throughout the experiment using a SEN-3301 (Nihon Kohden). Left ventricular end-diastolic pressure, peak developed pressure and coronary flow were expressed as percentages of the initial values in the experiments.

2.4. Measurement of infarct size

At the end of the experiment, the hearts were sliced across the long axis of the left ventricle into 1-mm-thick transverse sections and incubated in 1% triphenyl tetrazolium chloride (Sigma Chemical Co., St. Louis, MO) in phosphate buffer (pH 7.4) at 37°C for 20 min. Infarct areas were enhanced by storage in 10% formaldehyde solution for 24 h before measurement [11]. In the GI heart, the whole ventricle was at risk of infarction and, therefore, collateral flow and estimation of the area at risk was not required. All sections were photographed with a digitized camera and transferred to a personal computer. The area of the left ventricle and that of the infarcted tissue was measured by planimetry by an independent, blinded observer. The volumes of the infarcted zone and the area at risk were calculated by multiplying the planimetered areas by the slice thickness. Infarct volume was expressed as a percentage of left ventricular volume for each heart [11].

2.5. Measurement of nitrite and nitrate (NOx)

The initial coronary flow was collected with a sterilized plastic syringe. The assay was carried out with an NO-analyzing system (ECO-20; Eicom Corp., Kyoto, Japan) as described previously [12]. In brief, nitrite and nitrate were separated on a polystyrene polymer column, and the nitrite was reduced by passage through a cadmium column to form nitrite. Nitrite was mixed with a Griess reagent to form a purple azo dye. The absorbance of the colour of the dye product was measured at 540 nm with a flow-through spectrophotometer. The concentration of NOx was measured by assessing the peak area of the absorbance changes with a computer (PowerChrom; Eicom). The minimal concentration of NOx detectable was approximately 0.01 μM. All results are expressed as a percentage of the pre-ischaemic value.

2.6. Statistical analysis

Statistical analysis was performed using the StatView (Version 5) software package (SAS Institute Inc., NC). Data are expressed as mean ± standard deviation. Haemodynamic variables were analyzed by a two-way repeated-measures analysis of variance (ANOVA) (time and group). Infarct sizes and NOx during reperfusion were analyzed with one-way ANOVA followed by the Student’s t-test for unpaired data with Bonferroni correction. Significant values at P < 0.05 were noted.

3. Results

In this study, four hearts could not be used as they did not meet the criteria due to aortic dissection during the first 20 min of perfusion.

3.1. Left ventricular pressure

The effects of GI, IP, ARG, IP + l-NAME and IP + ARG on left ventricular pressure (end-diastolic pressure and peak developed pressure) in the isolated hearts during equilibrium, after 20 min of normothermic global ischaemia and after 120 min reperfusion, are shown in Figs. 2 and 3. Significant differences in left ventricular end-diastolic pressure were observed between the GI and IP + ARG groups during the reperfusion period. In addition, throughout reperfusion, the rise in left ventricular...
peak developed pressure in the IP + ARG group was significantly greater than those in the GI, ARG and IP + l-NAME groups. No significant difference was found between the IP and IP + NAME groups.

3.2. Coronary flow

Coronary flow in the IP, IP + l-NAME and IP + ARG groups decreased to 0% of the pre-ischaemic level, and returned to 92.6 ± 2.61% of the pre-ischaemic level in the IP group, 85.8 ± 2.66% in the IP + NAME group and 95.2 ± 3.35% in the IP + ARG group, after ischaemic preconditioning (Fig. 4). Significant differences were observed in coronary flow between the IP + ARG and both the GI and the ARG group during reperfusion. No significant difference was observed between the IP and IP + l-NAME groups.

3.3. Myocardial infarct size

Infarct size, expressed as a percent of ventricular myocardial volume, was 57.8 ± 2.80% in the GI group and 5.5 ± 0.97% in the controls (Fig. 5). Infarct size in the IP (36.9 ± 5.47%), ARG (42.3 ± 4.40%) and IP + l-NAME (40.2 ± 2.38%) groups was significantly less than that in the GI group. Furthermore, infarct size in the IP + ARG group (11.3 ± 1.68%) was significantly less than that in the GI, IP, ARG and IP + l-NAME groups, and there was no significant difference between the IP + ARG and control groups. In addition, no significant difference was found between the IP and IP + l-NAME groups.

3.4. Measurement of NOx

The level of NOx after 5 min reperfusion is shown in Fig. 6. NOx decreased to 63.5 ± 6.04% and 53.6 ± 3.35% of the pre-ischaemic levels in the control and GI groups, respectively. However, there was no significant difference between the control and GI groups. NOx in the IP and ARG groups increased slightly to 67.9 ± 5.54% compared with the control, but the difference was not statistically significant. There was no significant difference between the IP and IP + l-NAME groups. On the other hand, the NOx concentration in the IP + ARG group (98.4 ± 19.53%) was significantly greater than those in the GI and IP + l-NAME groups.

Fig. 7 shows NOx levels measured after 60 min reperfu-
reperfusion at reperfusion time. Each value is expressed as a percentage of the pre-ischaemic value. Results are shown as the mean and standard deviation of the mean for each group (n = 6). Significant differences during reperfusion at P < 0.05 vs. IP + ARG are designated by *.

Fig. 6. The effects of global ischaemia (GI), ischaemic preconditioning (IP), L-arginine (ARG), ischaemic preconditioning plus L-NAME (IP + L-NAME), and ischaemic preconditioning plus L-arginine (IP + ARG) on NOX 5 min after reperfusion. Each value is expressed as a percentage of the pre-ischaemic value. Results are shown as the mean and standard deviation of the mean for each group (n = 6). Significant differences during reperfusion at P < 0.05 vs. IP + ARG are designated by *.

4. Discussion

Our study using rat hearts shows that L-arginine can augment the effect of ischaemic preconditioning, ameliorating post-ischaemic functional recovery, coronary flow preservation and infarct size. Although there are experimental reports documenting that L-arginine can function as a cardioprotective agent [13], L-arginine alone had only a slight cardioprotective effect as compared with ischaemic preconditioning alone, in our experiment. On the other hand, L-NAME did not reduce the effect of ischaemic preconditioning. These results suggest that NO is not a major mediator of ischaemic preconditioning, but that the effect of ischaemic preconditioning can be enhanced by a precursor of NO. This agrees with the observation that the IP + ARG protocol preserved a significantly higher level of Nox, after global ischaemia, than the other protocols.

Ischaemic preconditioning is the phenomenon whereby repetitive brief periods of ischaemia result in tolerance to subsequent longer ischaemic episodes and reduce ischaemia-reperfusion injury. This phenomenon has been reported to limit infarct size [1], to attenuate the progression of the ischaemia-induced metabolic disorders and cell necrosis [2], and to reduce the occurrence of arrhythmia after ischaemia-reperfusion [3]. The cardioprotective effects of ischaemic preconditioning are dependent on the duration and frequency of this preconditioning. On the other hand, the efficacy of ischaemic preconditioning in terms of infarct sparing and repression of myocardial stunning reportedly differs among species [2–5]. Although the mechanism involved in ischaemic preconditioning is not yet clear, several mediators, including adenosine, bradykinin and NO, have been suggested [14–16].

Recently, Vegh et al. [7] reported that activation of NO elevated cyclic GMP and contributed to the anti-arrhythmic effects of ischaemic preconditioning in the canine myocardium. Furthermore, Yin et al. [17] reported that the mechanism of preconditioning in the ischaemic liver may involve generation of NO.

Conversely, Weselcouch et al. reported that inhibition of NO synthase had no influence on ischaemic preconditioning in rat and rabbit hearts, and concluded that ischaemic preconditioning in isolated rat hearts has no effect on the L-arginine-NO pathway [8]. The present results demonstrate that L-NAME does not attenuate the effect of ischaemic preconditioning. Therefore, it is suggested that NO does not play a major role in ischaemic preconditioning of the rat heart.

NO is well known as an endothelium-derived relaxing factor and is released from vascular endothelial cells, regulating vascular tone. Moreover, NO or its second messenger, cGMP, is known to attenuate the influx of calcium into cardiomyocytes, antagonize the effect of adrenergic stimulation, decrease oxygen consumption by the myocardium, and open K⁺-ATP channels [18]. These actions are thought to improve calcium overload and deplete high-energy phosphates that are considered to be the main perpetrators of myocardial ischaemia–reperfusion insult.

There are some possible explanations for administration of L-arginine, a precursor of NO, enhancing the cardioprot-
tective effect of ischaemic preconditioning. Ischaemic preconditioning induces the expression of inducible NO synthase (iNOS) via activation of protein kinase C [19]. Furthermore, adenosine stimulates the production of NO in the artery through a receptor-mediated mechanism. As a result, it may be reasonable that the maximal protective effects of NO on the ischaemic myocardium will be achieved by supplemental L-arginine given just after ischaemic preconditioning. On the other hand, one of the major mechanisms thought to play an important role in ischaemia-reperfusion injury is the activation of oxygen free radicals.

Supplemental NO exerts a positive cardioprotective effect by inhibiting the release of superoxide radicals or by quenching superoxide radicals produced by neutrophils [20]. Therefore, in addition to the cardioprotection induced by ischaemic preconditioning, an additional cardioprotective effect may be produced by NO through such a mechanism. However, further experimental study is needed to reveal the mechanism of this enhancement effect attributed to NO and to determine the optimal dose of L-arginine for producing the maximal effect.

The animals utilized for our study, aside from the differences among animal species, also had normal heart as well as being free of coronary artery lesions and/or collateral circulation. In addition, the clinical effect of ischaemic preconditioning in humans is still unknown [21,22]. However, pharmacological enhancement of ischaemic preconditioning by L-arginine may play this role in a limited clinical setting such as coronary artery bypass grafting without cardiopulmonary bypass or percutaneous transluminal coronary angioplasty.

4.1. Experimental limitations

The current study has several limitations. First, this model uses isolated crystalloid-perfused heart preparations to allow for comparison with previous studies which have used these preparations [23,24]. However, this model does not account for the intervening variables associated with in situ blood-perfused heart preparations. It is well known that both iNOS and eNOS exist in cardiomyocytes and vascular endothelial cells, and that iNOS is present in vascular smooth muscle cells. Moreover, nNOS exists in platelets, and eNOS in leukocytes. In vivo, as opposed to in vitro, NO produced by these NO synthases is thought to play various roles. These include inhibition of platelet adhesion and aggregation, and reduction of neutrophil aggregation and adherence to the vascular endothelium, but not endothelium-dependent vasorelaxation. In fact, Node et al. [25] reported that coronary arterio–venous differences in NOx concentrations increased within 40 min, and then increased even further after reperfusion in an in vivo canine model. Their results apparently differ from ours in that NOx levels fell to pre-ischaemic values following reperfusion, and this discrepancy may be partly due to differences between crystalloid and blood-perfused hearts. The concentration of L-arginine used may be toxic, and L-arginine itself may diminish cardiac function due to enhanced peroxynitrite production. Second, a rat model was used in the present study, and the effect of ischaemic preconditioning is known to differ among animal species. It is difficult to compare our results with those of other studies, and further data are clearly required before this technique can be applied in a clinical setting. Finally, in this study all groups underwent 120 min reperfusion after 20 min of global ischaemia, and the time course and relation between the effect of early and late ischaemic preconditioning were not examined.

In conclusion, inhibition of NO synthase with L-NAME had little effect on ischaemic preconditioning, suggesting that endogenous NO is not involved in the mechanism of ischaemic preconditioning. L-Arginine significantly enhanced the cardioprotective effects afforded by ischaemic preconditioning in post-ischaemic functional recovery and in limiting infarct size by augmenting NO release in isolated rat hearts. In contrast with these protective effects, high-dose L-arginine plays a detrimental role due to the reaction with peroxynitrate. Therefore, careful investigations are required to determine the optimal protocol for ischaemic preconditioning and the most effective dose of L-arginine in clinical practice.

References


