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Contribution of peroxynitrite to the beneficial effects of preconditioning on ischaemia-induced arrhythmias in rat isolated hearts

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Abstract

We studied the effects of urate, a peroxynitrite scavenger, on ischaemia- and peroxynitrite-induced preconditioning in rat isolated hearts. Isolated hearts perfused with Krebs–Henseleit solution were preconditioned either by 3 min of coronary artery occlusion or by peroxynitrite administration (1 μM) for 3 min, followed by 10 min of reperfusion and 30 min of coronary artery occlusion. Both ischaemia and peroxynitrite produced a marked reduction in arrhythmias. Urate (1 mM) added to the perfusate 10 min prior to ischaemic preconditioning or peroxynitrite infusion and maintained until coronary artery occlusion, markedly reversed the beneficial effects in the ischaemic and peroxynitrite-treated groups. Urate administration in the peroxynitrite-treated group increased the incidence of ventricular tachycardia from 57% ($n = 11$) to 100% ($n = 6$) and total ventricular fibrillation from 0% ($n = 0$) to 44% ($n = 4$). Similarly, urate augmented the incidence of ventricular tachycardia from 47% ($n = 8$) to 85% ($n = 6$) in the ischaemic preconditioning group. On its own, urate did not affect the severity of cardiac arrhythmias. Peroxynitrite infusion caused a marked increase in the effluent nitrate levels, from 0.05 ± 0.1 μM ($n = 5$) to 0.4 ± 0.2 μM ($n = 6$), and urate significantly decreased these levels to 0.08 ± 0.03 μM ($n = 9$). These results suggest that peroxynitrite at low concentrations contributes to the beneficial effects of preconditioning on ischaemia-induced arrhythmias in rat isolated hearts. © 2001 Published by Elsevier Science B.V.

Keywords: Preconditioning; Peroxynitrite; Ischaemia-induced arrhythmia; Urate; Nitrate level

1. Introduction

Brief episodes of ischaemia–reperfusion exert powerful endogenous cardioprotection against subsequent prolonged ischaemic injury, which has been known as ischaemic preconditioning (Murry et al., 1986). Preconditioning protects the heart from ischaemic damage by reducing infarct size (Murry et al., 1986), by suppressing ischaemia- and reperfusion-induced ventricular arrhythmias (Vegh et al., 1992a; Altup et al., 2000) and by enhancing recovery of contractile function (Cave and Hearse, 1992). The exact mechanism responsible for preconditioning has not been fully elucidated despite intensive investigation. There is a growing amount evidence that free radicals, adenosine, bradykinin, nitric oxide (NO) and opioids may play a pivotal role in ischaemic preconditioning (Nakano et al., 2000).

Recent studies suggest that the initial signal or signals responsible for triggering the development of early and late preconditioning after the ischaemic stimulus may involve the formation of NO and reactive oxygen species (Baines et al., 1997; Qiu et al., 1997). It has been suggested that mitochondrial reactive oxygen species participate in the initiation of hypoxic preconditioning in cardiomyocytes (Vanden Hoek et al., 1998). Simultaneous release of NO and superoxide has been shown to be accelerated in the early phase of reperfusion (Wang and Zweier, 1996). It is well known that NO can react with superoxide at a nearly diffusion-limited rate to form peroxynitrite (Beckman et al., 1990). Peroxynitrite formation has also been observed in piglet hearts during hypoxia and reoxygenation (Morita et al., 1994), and during ischaemia–reperfusion in rat and dog hearts (Zweier et al., 1995; Wang and Zweier, 1996; Yasmin et al., 1997). Although peroxynitrite can react with a wide range of molecules and produce toxic effects at high concentrations, it has some beneficial effects at low micromolar concentrations such as inhibiting platelet aggregation (Moro et al.,

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1994) or producing relaxation of coronary arteries (Liu et al., 1994). It has been demonstrated that peroxynitrite exerts cardioprotective effects on myocardial ischaemia–reperfusion injury by reducing myocardial infarct size, preserving coronary endothelial function, and inhibiting platelet–neutrophil and neutrophil–endothelium interactions (Moro et al., 1994; Lefer et al., 1997; Nossuli et al., 1997, 1998). We have also observed cardioprotective effects of peroxynitrite against reperfusion arrhythmias in rat isolated hearts (Altup et al., 1999). We have recently demonstrated that exogenous peroxynitrite administration can precondition the rat isolated heart, suggesting that peroxynitrie might be one of the mediators of ischaemic preconditioning (Altup et al., 2000). The aim of this study was to examine the effects of the potent peroxynitrite scavenger, urate, on ischaemia- and peroxynitrite-induced preconditioning in rat isolated heart. Measurements of nitrate levels during peroxynitrite and ischaemic preconditioning were also part of this study.

2. Materials and methods

2.1. Peroxynitrite synthesis

Peroxynitrite was synthesised in our laboratory from acidified nitrite and hydrogen peroxide (H_2O_2) according to the method of Beckman et al. (1994). Briefly, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M H_2O_2 containing 0.7 M HCl and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H_2O_2 was removed by addition of manganese dioxide (MnO_2) powder to the peroxynitrite solution. The mixture was then shaken for 5 min and then MnO_2 was removed by passage over a cellulose acetate disposable filter. The final concentration of peroxynitrite was determined spectrophotometrically ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$) as described previously (Altup et al., 1999, 2000). Fresh dilutions were made with Krebs–Henseleit solution without glucose and sodium pyruvate just before use and the pH of these solutions was adjusted to 8.4 by addition of an appropriate volume of 0.1 N NaOH (Nossuli et al., 1997). The stock solutions were aliquoted and stored at -20°C for a week.

2.2. Preparation of isolated hearts

Male Wistar rats obtained from the animal unit of our department, weighing 200–400 g, were housed with a 12-h light, 12-h dark cycle at 25°C and supplied with standard laboratory diet and tap water ad libitum. The animals were anaesthetised with an i.p. injection of thiopental (60 mg kg^{-1}). After the induction of anaesthesia, the abdomen was opened and heparin (500 U) was given through the renal vein. After 3 min of heparin injection, the abdominal aorta was cut to reduce the blood volume in the heart. Then the thorax was opened and the heart was quickly isolated and put into a Petri dish containing an ice-cold Krebs–Henseleit solution of the following composition (mM): NaCl 118; KCl 3.2; $CaCl_2$ 2.52; $MgSO_4$ 1.66; $NaHCO_3$ 26.88; KH_2PO_4 1.18; glucose 5.55 and sodium pyruvate 2.0 (Altup et al., 2000). The pH of the solution was adjusted to 7.4. The solution was then perfused retrogradely via the aorta by means of a modified Langendorff apparatus at a constant flow of a 8–10 ml min^{-1} which was determined according to animal weight using the formula: flow (ml min^{-1}) $= x^{0.56} \times 7.43$ (x is the heart weight), heart weight $= 0.0027y + 0.6$ (y is the body weight) at 37°C with the Krebs–Henseleit solution gassed with 95% O_2 and 5% CO_2 (Piacentini et al., 1993). A loose ligature was immediately placed round the left anterior descending coronary artery; both ends of the ligature were then passed through a short piece of polythene tubing (1 mm i.d. and 1.5 mm long) to form a snare. Following the stabilisation period of 15 min, the snare around the left anterior descending coronary artery was tightened and held in place with a small clip. An increase in perfusion pressure indicated successful ligation, likewise a decrease in perfusion pressure indicated successful reperfusion. The electrocardiogram (ECG) was recorded by two electrodes placed on the right atrium and the apex throughout the experiment by using a computerised data acquisition system (TDA 95, Commat, Maycom, Turkey) (Altup et al., 1999, 2000). Coronary perfusion pressure was measured by a pressure transducer attached to the system, and was recorded continuously with this same system.

2.3. Experimental protocol for isolated hearts

After completion of surgical procedures, all hearts were allowed to stabilise for 15 min prior to the experimental protocol. These protocols are shown in Fig. 1. In the first group of experiments (protocol 1, control, $n = 22$), the hearts were subjected to 30 min of left anterior descending coronary artery occlusion. In the second group of experiments (protocol 2, ischaemic preconditioning, $n = 17$), the hearts were preconditioned with a single 3 min of occlusion of left anterior descending coronary artery followed by 10 min of reperfusion and a subsequent 30 min of occlusion. In the third group of experiments (protocol 3, peroxynitrite-induced preconditioning, $n = 19$), the hearts were subjected to 3 min of infusion of peroxynitrite at 1 μM concentration followed by 10 min washout and then 30 min of occlusion. The concentration of 1 μM was chosen since it had been shown that this concentration could precondition the heart in our previous study (Altup et al., 2000). Peroxynitrite was infused into the perfusion solution through the rubber tubing placed just proximal to the heart. Stock solutions of peroxynitrite were kept on ice, and the infusion lines were wrapped in aluminium foil to reduce exposure to light. In protocols 4 and 5, the perox-

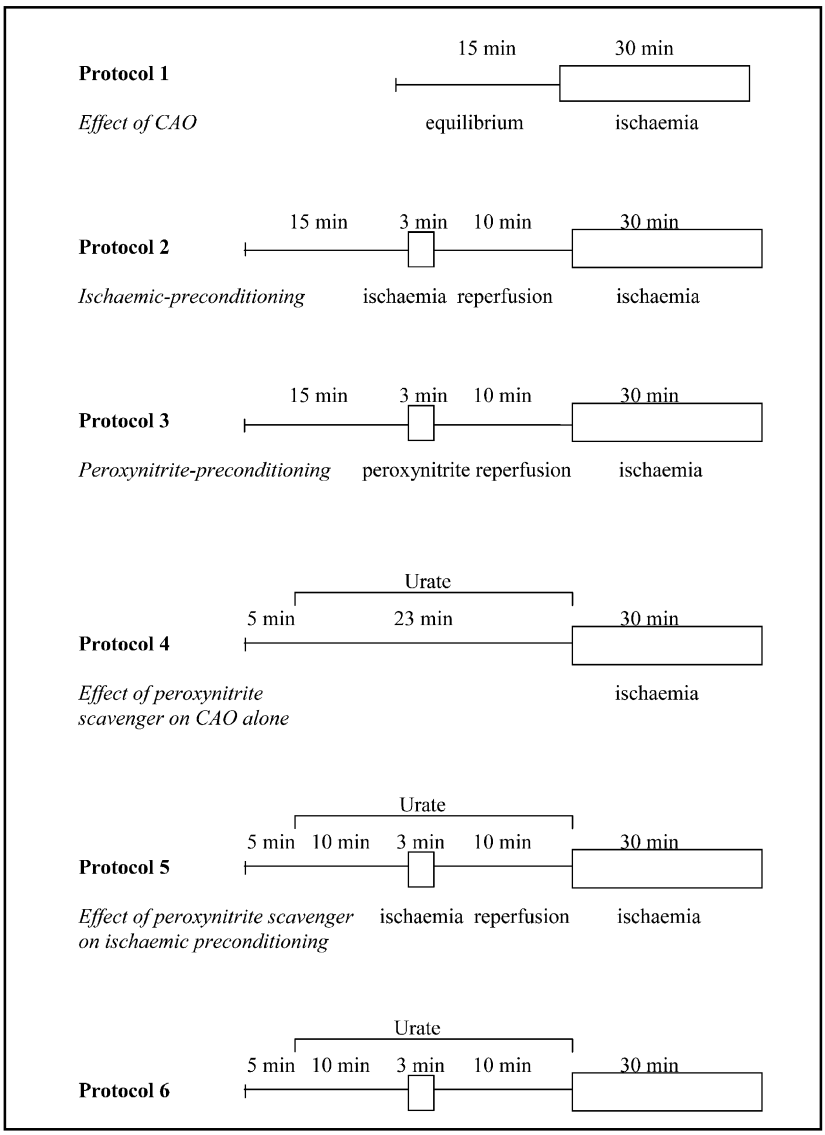

Fig. 1. Experimental protocols for the study. Isolated rat hearts perfused with Krebs–Henseleit solution were subjected to a 30-min ischaemia (protocol 1). Rat isolated hearts were preconditioned against 30 min of ischaemia by a 3-min period of coronary occlusion and 10-min reperfusion (protocol 2). A 1-μM concentration of peroxynitrite for 3 min was infused in order to precondition the heart (protocol 3). Urate (1 mM), a peroxynitrite scavenger, was added to the perfusate for 23 min prior to the ischaemic period (protocol 4), 10 min prior to the ischaemic preconditioning (protocol 5) or peroxynitrite infusion (protocol 6) and maintained until the starting of 30 min of occlusion (total 23 min).

ynitrite scavenger, urate (1 mM), was infused either alone ($n = 6$) or in combination with ischaemic preconditioning protocol ($n = 7$), respectively. For the last series of the experiments (protocol 6, urate + peroxynitrite-induced preconditioning, $n = 9$), the hearts were preconditioned with peroxynitrite as in protocol 3, but received urate for 23 min, starting from 10 min prior to peroxynitrite infusion and continuing until the start of the 30-min occlusion period.

2.4. Parameters measured

For all the groups, heart rate was measured from the recordings of electrocardiogram and the incidences of arrhythmias were registered, in accordance with the Lambeth conventions (Walker et al., 1988), as ventricular tachycardia, ventricular fibrillation, and ventricular ectopic beats. Ventricular ectopic beats are defined as discrete and identifiable premature QRS complexes. Ventricular tachycardia is defined as four or more consecutive ventricular ectopic beats. Ventricular fibrillation as diagnosed the ECG recording showed chaotic activity with an amplitude less than that of the normal ECG. Irreversible ventricular fibrillation was defined as ventricular fibrillation which did not reverse within the 10-min period of reperfusion. The onset and duration of arrhythmias were also measured. At the end of experiments, the left anterior descending coronary artery was occluded again at the same site as previously,

and 3 ml of a 2% solution of Evans blue was infused into the aortic cannula to estimate the area at risk. This was expressed as a percentage of left ventricular free wall (Altup et al., 2000).

2.5. Nitrate measurements

Capillary electrophoresis experiments were conducted using a SpectraPhoresis 100 system equipped with high-voltage power supply, a modular injector and a SpectraFOCUS scanning detector (Thermo Separation Products, CA, USA) connected to an Etacomp 486 DX4-100 computer processing the data using PC 1000 (version 2.6) working with the OS/2 Warp Program (version 3.0). The measurements were done in a 75-μm ID fused-silica column 82 cm total and 43 cm effective length (Phenomenex, CA, USA). The pH of the solutions was measured with Multiline P4 pH meter with SenTix glass electrode (WTW, Weilheim, Germany). All the solutions including the sample were degassed with a B-220 model ultrasonic bath (Branson, CT, USA) and used after centrifugation. The column was washed and conditioned by rinsing with, in turn, 5 min each, 0.1 M NaOH, double-distilled water and background electrolyte. A 2-min wash with background electrolyte was also done between each of the experiments.

A fused silica capillary was filled with the background electrolyte consisting of 200 mM lithium chloride, 10 mM borate buffer at pH 8.5. All the solutions were prepared in nitrate-free double-distilled water. The dilutions of nitrate were made from the stock solutions of 1.06×10^{-3} M KNO_3 and 4.39×10^{-4} M KBr as internal standard. The final concentration of internal standard was always 2.92×10^{-4} M in the calibration and sample solutions. Detection was done at 214 nm, where monochromatic light is absorbed maximally by the related anions. The injection time was 50 ms (corresponds to almost 25 nl) using the vacuum injection mode and a reversed polarity controlled current of 200 μA corresponding to 12.7 kV was applied. Samples (6 ml) obtained after the pharmacological processes were withdrawn and transferred to the tubes. They were left in an oven at 85°C for 2–3 days to evaporate them to dryness. Then, 100 μl distilled water and 200 μl acetonitrile, which contained the internal standard, were added to dissolve the residue. Thus, samples were concentrated 20-fold. They were mixed with a vortex and the clear samples were injected into the capillary electrophoresis system. The data obtained were used to calculate the nitrate amount from the calibration equation.

2.6. Chemicals

Urate, sodium nitrite and Evans blue were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide was purchased from Merck (Darmstat, Germany). For nitrate measurements: all the chemicals (Merck) and acetonitrile were of analytical and high performance liquid chromatography grade (Carlo Erba, Rodano, Italy). Double-distilled water was prepared in our laboratory using glassware only.

2.7. Statistical analysis

Values are presented as means ± S.E.M. or as percentage incidence. A Fischer's extract Chi-square test was used to detect significant differences in the incidence of ventricular tachycardia, ventricular fibrillation, and irreversible ventricular fibrillation between control and treated groups. Statistical comparison of more than two groups was performed by a one-way analysis of variance followed by Student–Newman–Keuls test. In all tests, P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of ischaemic and peroxynitrite-induced preconditioning on ischaemic arrhythmias

Preconditioning hearts with 3 min of ischaemia suppressed arrhythmias during the 30-min occlusion period. The incidence of ventricular tachycardia and of ventricular fibrillation was also significantly lower than in the control hearts (Table 1). Peroxynitrite infusion for 3 min mimicked the effect of ischaemic preconditioning and reduced the total number of ventricular ectopic beats. The incidence of ventricular tachycardia declined, while ventricular fibrillation was abolished both in peroxynitrite- and ischaemic-preconditioning groups. Peroxynitrite administration did not cause any significant change in coronary perfusion pressure throughout the experimental protocol (Table 2). Similarly, the pressure rate index was not modified by peroxynitrite administration (data not shown).

Table 1
Effects of ischaemic (3 min) and peroxynitrite (1 μM, 3 min)-induced preconditioning on ischaemic (30 min) arrhythmias, and effects of urate (1 mM) infusion for 23 min on ischaemia- and peroxynitrite-induced preconditioning

	n	Total VEBs	%VT	%VF	%irr. VF
Control	22	750 ± 66	100 (22)	45 (10)	14 (3)
Preconditioning	17	178 ± 26[a]	47 (8)[a]	0 (0)[a]	0 (0)
Peroxynitrite (1 μM)	19	204 ± 28[a]	57 (11)[a]	0 (0)[a]	0 (0)
Urate	6	677 ± 101	100 (6)	50 (3)	17 (1)
Urate + preconditioning	7	479 ± 105[b]	85 (6)[b]	57 (4)[b]	0 (0)
Urate + peroxynitrite (1 μM)	9	350 ± 91	100 (9)[c]	44 (4)[c]	11 (1)

VEBs: ventricular ectopic beats defined as discrete and identifiable premature QRS complexes; VT, ventricular tachycardia; VF, ventricular fibrillation; irr. VF, irreversible ventricular fibrillation. Numbers in parentheses are the number of hearts that exhibited that particular type of arrhythmia.

[a] $P < 0.05$ compared to control group.

[b] $P < 0.05$ compared to preconditioning group.

[c] $P < 0.05$ compared to peroxynitrite group.

Table 2
Mean coronary perfusion pressure values (mm Hg) during coronary occlusion and reperfusion in rat isolated hearts

	n	Baseline	Occlusion or peroxynitrite		Reperfusion or washout		Permanent occlusion	
			1 min	3 min	1 min	10 min	1 min	30 min
Control	22	33 ± 1	–	–	–	–	65 ± 1[a]	66 ± 1[a]
Preconditioning	17	35 ± 2	63 ± 1[a]	64 ± 1[a]	37 ± 2	36 ± 2	63 ± 1[a]	62 ± 1[a]
Peroxynitrite	19	30 ± 2	31 ± 2	31 ± 2	30 ± 2	29 ± 2	58 ± 2[a]	61 ± 2[a]
Urate	6	30 ± 2	–	–	–	–	60 ± 3[a]	60 ± 3[a]
Urate + preconditioning	7	33 ± 2	62 ± 2[a]	60 ± 2[a]	31 ± 2	31 ± 2	61 ± 2[a]	62 ± 2[a]
Urate + peroxynitrite	9	34 ± 2	36 ± 2	39 ± 3	38 ± 3	37 ± 3	65 ± 3[a]	69 ± 4[a]

Rat isolated hearts were subjected to 3 min of coronary occlusion followed by 10 min of reperfusion in the preconditioned group. The other three groups received peroxynitrite (1 μM) for 3 min followed by a 10-min washout. All groups were then subjected to 30 min of coronary occlusion.
[a] $P < 0.05$ compared to pre-occlusion values.

3.2. Effects of urate on ischaemic and peroxynitrite-induced preconditioning

Administration of the peroxynitrite scavenger, urate (1 mM), on its own (Fig. 1) did not show any marked effect on the arrhythmias that occurred during the 30 min of coronary artery occlusion. Urate reversed the beneficial effects of ischaemic preconditioning on ischaemia-induced arrhythmias (Table 1). Both ventricular tachycardia and total ventricular fibrillation times were increased from 15 ± 5 s ($n = 17$) to 126 ± 17 s ($n = 7$) and from none to 130 ± 45 s ($n = 5$), respectively. Similarly, urate (1 mM) reversed the beneficial effects of peroxynitrite-induced preconditioning on ischaemia-induced arrhythmias. It increased the incidence of ventricular tachycardia from 57% ($n = 11$) to 100% ($n = 9$) and total ventricular fibrillation from 0% ($n = 0$) to 44% ($n = 4$) (Table 1). Also the total ventricular fibrillation time was increased from 0 to 76 ± 49 s ($n = 4$).

Urate administration did not cause any significant change in coronary perfusion pressure (Table 2) and in the pressure rate index measured at various time points throughout the experiment (data not shown).

3.3. Effects of ischaemia- and peroxynitrite-induced preconditioning on nitrate levels

After 30 min of ischaemia, nitrate levels in the perfusate were increased from 0.15 ± 0.1 μM ($n = 7$) to 0.31 ± 0.1 μM ($n = 7$) in the control group, but this increase did not reach statistical significance. Ischaemic preconditioning decreased this elevation in nitrate levels (0.08 ± 0.03 μM, at the end of the 30-min coronary artery occlusion, $n = 7$). Nitrate levels were significantly increased from 0.05 ± 0.1 μM ($n = 5$) to 0.4 ± 0.2 μM ($n = 6$) at the end of infusion of 1 μM peroxynitrite for 3 min.

3.4. Effects of urate on nitrate levels in ischaemia- and peroxynitrite-induced preconditioning

Administration of urate prior to 30 min of coronary artery occlusion did not produce any effect on the nitrate levels. Urate administration markedly decreased the nitrate levels from 0.4 ± 0.2 μM ($n = 6$) to 0.08 ± 0.03 μM ($n = 9$) at the end of the 3-min peroxynitrite infusion. Nitrate levels were 0.16 ± 0.07 μM ($n = 6$) after 30 min of ischaemia in the peroxynitrite group, and these values were reduced to 0.06 ± 0.02 μM ($n = 8$) in the urate + peroxynitrite group. Nitrate levels did not change markedly during ischaemic preconditioning and urate administration did not modify the nitrate levels.

3.5. Area at risk measurements

No significant differences were noted in the left ventricular area at risk between control hearts (31 ± 3%) and those infused with 1 μM peroxynitrite for 3 min (33 ± 4%). The area at risk was similar in hearts from preconditioned (33 ± 4%), urate alone (25 ± 3%), urate + preconditioning (31 ± 2%) or urate + peroxynitrite preconditioning groups (36 ± 3%).

4. Discussion

We have now shown that peroxynitrite at low concentrations is able to mimic the beneficial effects of ischaemic preconditioning, confirming our previous observation (Altup et al., 2000). We have found that 1 μM peroxynitrite exerted significant cardioprotective effects and reduced the number of ventricular ectopic beats, the incidence of ventricular fibrillation and ventricular tachycardia during a 30-min occlusion period. The peroxynitrite-induced reduction in the severity of ischaemia-induced arrhythmias was lost in the presence of the peroxynitrite scavenger, urate. This observation is also in line with our previous data obtained in the presence of *N*-2-mercaptopropionylglycine (MPG) (Altup et al., 2000). These results further support the conclusion that peroxynitrite is a potential contributor to cardiac preconditioning.

We have previously shown that effects of peroxynitrite infusion on ischaemic preconditioning were concentration-dependent (Altup et al., 2000). In that study, three different concentrations (0.1, 1, 10 μM) of peroxynitrite

were tested. All concentrations of peroxynitrite studied decreased the ventricular ectopic beat number, but the most pronounced cardioprotective effect was seen with 1 μM. Therefore, this concentration was chosen for the present experiments. It has been proposed that the maximal achievable concentration of peroxynitrite formed under in vivo conditions is in the range of 2–5 μM (Nossuli et al., 1998). Therefore, the concentration of peroxynitrite (1 μM) used in this study was likely to be achieved under in vivo conditions. Detrimental effects can be observed at higher concentrations of peroxynitrite.

It has been documented that many endogenous substances are released and/or operative during ischaemic preconditioning, including reactive oxygen species, adenosine, bradykinin, opioids and NO which have all been identified either as triggers or mediators (Nakano et al., 2000). There are several potential sources of peroxynitrite formation in the heart exposed to ischaemia–reperfusion. The vascular endothelial cells generate NO constitutively and produce a burst of superoxide formation on reperfusion, suggesting that endothelial cells may generate peroxynitrite following ischaemia–reperfusion. It has been reported that NO and superoxide levels are increased and nitrotyrosine formation is detected during myocardial ischaemia–reperfusion in rat hearts (Liu et al., 1997), suggesting that a significant amount of peroxynitrite can be generated. However, the most significant source of peroxynitrite is likely to be mitochondria. It is documented that the mitochondrial electron transport chain is an important source of free radicals in isolated cells, and that mitochondrial superoxide generation during brief hypoxia can activate signalling cascades involved in protecting cardiomyocytes from subsequent ischaemia–reperfusion injury (Vanden Hoek et al., 1998). It has become evident that nitric oxide synthase (NOS) also exists in mitochondria, indicating that mitochondria produce, and are exposed to, significant amounts of peroxynitrite (Ghafourifar et al., 1999). This conclusion is further supported by the observation that mitochondrial membrane fragments are induced to generate superoxide in the presence of NO, thus forming peroxynitrite (Packer et al., 1996). It has been demonstrated that production of reactive oxygen species during preconditioning may trigger a protective response that renders the endothelial cells more resistant to subsequent reperfusion injury (Kaeffer et al., 1997). Endothelial cells are also capable of generating peroxynitrite in response to anoxia–reoxygenation (Zulueta et al., 1997) or agonist stimulation (Kooy and Royall, 1994). Exposure to low concentrations of oxygen radicals generated from hypoxanthine or purine plus xanthine oxidase reaction can reproduce the beneficial effects of ischaemic preconditioning on infarct size and post-ischaemic recovery of left ventricular function in rabbit hearts (Baines et al., 1997). Additionally, NO formation is also markedly elevated during ischaemia and/or reperfusion in rat isolated heart (Zweier et al., 1995; Liu et al., 1997) probably through activation of constitutive heart NO synthase (Depre et al., 1997). It has been shown that ischaemic preconditioning can be blocked both by inhibiting NOS (Vegh et al., 1992b) and by scavenging superoxide (Tanaka et al., 1994). Furthermore, L-arginine has been reported to precondition the heart through NO generation, and this response is mediated through a cGMP-dependent mechanism (Horimoto et al., 2000). Therefore, it seems likely that both superoxide and NO are produced, and that peroxynitrite is formed in myocardial ischaemia–reperfusion. Administration of a NOS inhibitor has been shown to decrease peroxynitrite formation in rat heart (Wang and Zweier, 1996; Yasmin et al., 1997), suggesting that pharmacological inhibition of cardiac NO biosynthesis may abolish the development of endogenous cardioprotective mechanisms. Peroxynitrite can freely cross phospholipid membranes (Marla et al., 1997) and is capable of diffusing across erythrocyte membranes via anion channels and passive diffusion (Denicola et al., 1998). Therefore, it is considered that peroxynitrite is a significant biological effector molecule, not only because of its reactivity, but also because of its high diffusibility.

It has been shown that peroxynitrite can be formed as early as with 20 s of reperfusion and reaches a maximum at 40 s (Wang and Zweier, 1996). Although we have not measured endothelial dysfunction in our experiments, results of several studies indicate that 2.5 min of reperfusion resulted in depressed acetylcholine responses and this effect may be due to the action of superoxide free radicals (Tsao and Lefer, 1990). It can be said that low concentrations of peroxynitrite generated in the early phase, i.e before 2.5 min, are responsible for the protective effect seen in our experiments. It is also expected that peroxynitrite formation can decrease in parallel to NO generation and this concentration of peroxynitrite can still be enough to exert a protective effect as observed in our experiments. All these studies evidenced endothelial dysfunction after a long (30–90 min), but not after a short (3 min), ischaemic period. The ischaemic period should be considered as an important factor to determine the endogenous concentration of peroxynitrite generated.

Peroxynitrite can be scavenged effectively by urate (Skinner et al., 1998). The reaction of urate with peroxynitrite not only reduces the peroxynitrite concentration but also regenerates NO (Skinner et al., 1998), producing a further protective effect. This may explain the incomplete attenuation of the protective effects of peroxynitrite and ischaemic preconditioning in the presence of urate in our experiments. Our results are in agreement with the observation in both in situ and in vitro rabbit hearts that the peroxynitrite scavenger, MPG, abolished the protection afforded by a single cycle of preconditioning (Baines et al., 1997).

We have measured nitrate levels of the perfusion solution leaving the isolated heart by capillary electrophoresis. There was a tendency towards an increase in nitrate levels

at the end of the 30-min ischaemic period but not during ischaemic preconditioning. This is in agreement with the results of Csonka et al. (1999) who showed a marked increase in myocardial NO content of the rat isolated heart after 30 min of ischaemia as assessed by electron spin resonance, whereas there was no apparent increase following three cycles of ischaemic preconditioning. Preconditioning improved post-ischaemic myocardial function and markedly decreased ischaemia–reperfusion-induced NO accumulation. Since nitrate is also a metabolite of peroxynitrite at a physiological pH, we measured nitrate levels and observed a marked elevation in nitrate levels at the end of the peroxynitrite infusion. Peroxynitrite-induced accumulation of nitrate was effectively scavenged by urate. These results show that (1) although NO synthesis by the heart is necessary to trigger early preconditioning, preconditioning in turn attenuates the accumulation of NO during ischaemia–reperfusion, probably by generating peroxynitrite, and (2) blockade of ischaemia–reperfusion-induced accumulation of cardiac NO by preconditioning or by an appropriate concentration of NOS inhibitor alleviates ischaemia–reperfusion injury, as demonstrated by the enhanced post-ischaemic function (Csonka et al., 1999). Accordingly, on the basis of the present results and of previous studies (Csonka et al., 1999; Altup et al., 2000), we propose that brief myocardial ischaemia leads to generation of peroxynitrite in the heart.

It is suggested that peroxynitrite preserves coronary endothelial function, decreases P-selectin expression, and attenuates polymorphonuclear leukocyte adherence to the vascular endothelium in a cat model of ischaemia–reperfusion (Nossuli et al., 1998). It has been reported that isolated perfused rat hearts were subjected to global ischaemia and reperfusion with rat polymorphonuclear leukocytes, which resulted in profound cardiac depression. Infusion of peroxynitrite reversed the myocardial contractile dysfunction of ischaemic–reperfused rat hearts to near baseline levels, and markedly attenuated the accumulation of polymorphonuclear leukocytes in the post-ischaemic heart. Therefore, these studies provide strong evidence that low concentrations of peroxynitrite both inhibit leukocyte-endothelial cell interactions and exert cytoprotective effects in myocardial ischaemia–reperfusion injury. Although the exact mechanism is not known, there are several possible mechanisms whereby peroxynitrite could induce a cardioprotective action. Firstly, peroxynitrite can *S*-nitrosylate glutathione or other thiol-containing substances in tissues, causing the formation of *S*-nitrosothiols (Moro et al., 1995; Wu et al., 1994). *S*-nitrosothiols can directly activate guanylyl cyclase and also release NO over sustained periods of time (Wu et al., 1994). Secondly, peroxynitrite forms intermediates that act as NO donors in the presence of plasma, proteins, glucose or glutathione (Moro et al., 1994, 1995). Thirdly, peroxynitrite causes vasodilation of vascular smooth muscle via direct activation of guanylyl cyclase (Tarpey et al., 1995) or poly(ADP-ribose) synthase (Chabot et al., 1997). Lastly, peroxynitrite might also activate other cardioprotective mechanisms. In this regard, Wei et al. (1996) have shown that peroxynitrite is able to activate K_{ATP} channels in vascular smooth muscle. Therefore, activation of K_{ATP} channels in peroxynitrite-induced preconditioning could be involved in cardioprotection. Since peroxynitrite administration did not cause any significant change in coronary perfusion pressure and pressure rate index measured at various time points throughout the experiment, the cardioprotective effect seen with peroxynitrite administration was not due to an effect on these parameters.

In conclusion, the results of this study demonstrated that peroxynitrite at low concentrations can be released upon reperfusion and is involved in the antiarrhythmic effects of the early phase of ischaemic preconditioning. Furthermore, we observed that urate, a peroxynitrite scavenger, given prior to initial occlusion, also prevented the protective effects of ischaemic preconditioning in rat isolated heart. Thus, it is likely that formation of peroxynitrite is an important step in the development of preconditioning in the rat and that peroxynitrite might be one of the possible triggers and/or mediators of ischaemic preconditioning.

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