



Evidence for the involvement of peroxynitrite in ischaemic preconditioning in rat isolated hearts

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1 The aim of this study was to investigate the involvement of peroxynitrite, reactive metabolite originating from nitric oxide and superoxide, in preconditioning of the ischaemic myocardium in rat isolated hearts.

2 Isolated hearts perfused with Krebs-Henseleit solution were preconditioned either by 3 min of coronary artery occlusion (CAO) or by peroxynitrite administration at three different concentrations (0.1, 1, 10 μM) for 3 min, followed by 10 min reperfusion and 30 min of CAO. Peroxynitrite, at 1 μM concentration, decreased the incidence of VT from 100% ($n=14$) to 62% ($n=13$) and abolished the occurrence of VF (50% in the control group).

3 N-2-mercaptopropionylglycine (MPG, 1 μM –10 mM) produced a concentration-dependent inhibition of peroxynitrite signals in luminol chemiluminescence and $67 \pm 1\%$ inhibition was observed at 100 μM ($n=7$). MPG (at 300 μM , $n=7$) added to the perfusate 10 min prior to ischaemic preconditioning or peroxynitrite infusion and maintained until CAO, significantly reversed the beneficial effects of the ischaemic and peroxynitrite-treated groups. MPG administration in the peroxynitrite-treated group increased the incidence of VT from 62% ($n=13$) to 100% ($n=10$) and total VF from 0% ($n=0$) to 67% ($n=10$). Similarly, MPG elevated the incidence of VT from 50% ($n=10$) to 100% ($n=8$) in the ischaemic preconditioned group. On its own, MPG did not affect the severity of cardiac arrhythmias.

4 These results suggest that endogenously produced peroxynitrite plays a significant role in the antiarrhythmic effect of ischaemic preconditioning in the rat isolated hearts.

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Abbreviations: CAO, coronary artery occlusion; CL, chemiluminescence; ECG, electrocardiogram; H_2O_2 , hydrogen peroxide; LAD, left anterior descending; MnO_2 , manganese dioxide; MPG, N-2-mercaptopropionylglycine; NO, nitric oxide; VEB, ventricular ectopic beat; VF, ventricular fibrillation; VT, ventricular tachycardia

Introduction

Brief episodes of myocardial ischaemia followed by periods of reperfusion increase the resistance to further ischaemic damage. This is known as myocardial ischaemic preconditioning which was first described by Murry *et al.* (1986). Preconditioning is a powerful mechanism which protects the heart from ischaemic damage (Murry *et al.*, 1986; Liu & Downey, 1992), reduces the incidence of arrhythmias (Hagar *et al.*, 1991; Vegh *et al.*, 1992a) and preserves contractile function (Kimura *et al.*, 1992). Although the exact mechanism responsible for preconditioning has not been fully elucidated, there is a growing body of evidence which suggests that endogenous myocardial protective substances like adenosine, bradykinin, prostaglandins and nitric oxide (NO) may play a pivotal role (Parratt & Vegh, 1996).

NO formation is increased and, at the same time, endothelial production of superoxide radical is accelerated in the early phase of reperfusion (Wang & Zweier, 1996). Beckman *et al.* (1990) have shown that NO and superoxide radical can rapidly combine to form peroxynitrite which is a powerful oxidant and can exert many cytotoxic effects (Beckman & Koppenol, 1996). It is generally accepted that during the early reperfusion period peroxynitrite formation occurs, since reperfusion of ischaemic myocardium is a good source of NO and superoxide. This concept is supported by a

number of observations demonstrating increasing formation of NO and/or peroxynitrite 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 & Zweier, 1996; Yasmin *et al.*, 1997). In contrast to the cytotoxic effects of peroxynitrite, it has recently been demonstrated that it also exerts beneficial effects, at low micromolar concentrations, including inhibition of platelet aggregation (Moro *et al.*, 1994), and relaxation of coronary arteries (Liu *et al.*, 1994). It has also been shown that peroxynitrite exerts significant cardioprotective effects on myocardial ischaemia-reperfusion injury by reducing myocardial infarct size, preserving coronary endothelial function, and inhibition of neutrophil-endothelium interactions (Lefer *et al.*, 1997; Nossuli *et al.*, 1997; 1998). Our previous observation in rat isolated hearts showed that exogenous administration of peroxynitrite is able to reduce the severity of reperfusion arrhythmias (Altuğ *et al.*, 1999). Thus, it might be hypothesized that exposure to peroxynitrite formed after brief periods of ischaemia might be one mechanism underlying preconditioning.

The involvement of peroxynitrite during ischaemic preconditioning has not been investigated. The aims of the present study, therefore, were to determine the effect of exogenously administered peroxynitrite on ischaemia-induced arrhythmias, and to explore the role of ischaemic preconditioning in the rat isolated hearts by the use of the thiol compound N-2-mercaptopropionylglycine (MPG), a peroxynitrite scavenger.

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Methods

Peroxynitrite synthesis

Peroxynitrite was synthesized in our laboratory from acidified nitrite and hydrogen peroxide (H₂O₂) 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 H₂O₂ containing 0.7 M HCl and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H₂O₂ was removed by addition of manganese dioxide (MnO₂) powder to the peroxynitrite solution. The mixture was then shaken for 5 min and MnO₂ was removed by passage over a cellulose acetate disposable filter. The final concentration of peroxynitrite was determined spectrophotometrically ($\epsilon_{302} = 1670 \text{ M}^{-1} \text{ cm}^{-1}$) as described previously (Yildiz *et al.*, 1998). Fresh dilutions were made with Krebs-Henseleit solution (lacking glucose and sodium pyruvate) just before use and the pH of this solution 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.

Chemiluminescence

Luminol-enhanced chemiluminescence (CL) was measured as described previously (Yildiz *et al.*, 1998). Phosphate buffered saline (10 mM KH₂PO₄ and 150 mM NaCl, pH 7.4) was mixed with luminol (250 μM) in a cuvette. After adding catalase (50 u ml⁻¹) to the cuvette to remove H₂O₂ left after MnO₂ treatment, peroxynitrite at 20 nM was injected and the CL produced was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The CL generated was measured continuously and recorded on a computer using the Luminometer 1250 program (version 1.12, Bio-Orbit) for 3 min. The effects of various concentrations of MPG were examined by addition to the mixture before peroxynitrite.

Preparation of isolated hearts

Male Wistar rats (200–400 g) were acclimatized with 12 h light/dark cycles at a room temperature of 25°C, and supplied with standard laboratory diet and tap water *ad libitum*. Animals were anaesthetized intraperitoneally with injection of thiopental (60 mg kg⁻¹). After 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. The thorax was opened and the heart was quickly excised and put into a petri dish containing an ice cold Krebs-Henseleit solution of the following composition (mM): NaCl 118; KCl 3.2; CaCl₂ 2.52; MgSO₄ 1.66; NaHCO₃ 26.88; KH₂PO₄ 1.18; glucose 5.55 and sodium pyruvate 2.0 (Piacentini *et al.*, 1993). The pH of the solution was corrected to 7.4. Then it was perfused retrogradely *via* aorta by means of a modified Langendorff apparatus at a constant flow by a peristaltic pump (Harward Apparatus, Model 1203, Kent, England) of between 8 and 10 ml min⁻¹ with a Krebs-Henseleit solution maintained at 37°C and gassed with 95% O₂ and 5% CO₂ (Piacentini *et al.*, 1993). The flow rate 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). A loose ligature was immediately placed round the left anterior descending (LAD) 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 a stabilization period of 15 min, the snare around the LAD coronary artery was tightened and held in place with a small clip. An increase in coronary perfusion pressure indicated successful ligation, likewise a decrease in perfusion pressure indicated successful reperfusion. The electrocardiogram (ECG) was recorded by two electrodes placed on right atrium and apex throughout the experiment by using a computerized data acquisition system (TDA 95 Maycom, Turkey) (Altuğ *et al.*, 1999). Coronary perfusion pressure was measured *via* a pressure transducer and recorded continuously by the same data acquisition system.

Experimental protocol for isolated hearts

After completing surgical procedures, all hearts were allowed to stabilize for 15 min prior to the experimental protocol. These protocols are diagrammatically represented in Figure 1. In the first group of experiments (protocol 1, control, $n = 14$), hearts were subjected to 30 min LAD coronary artery

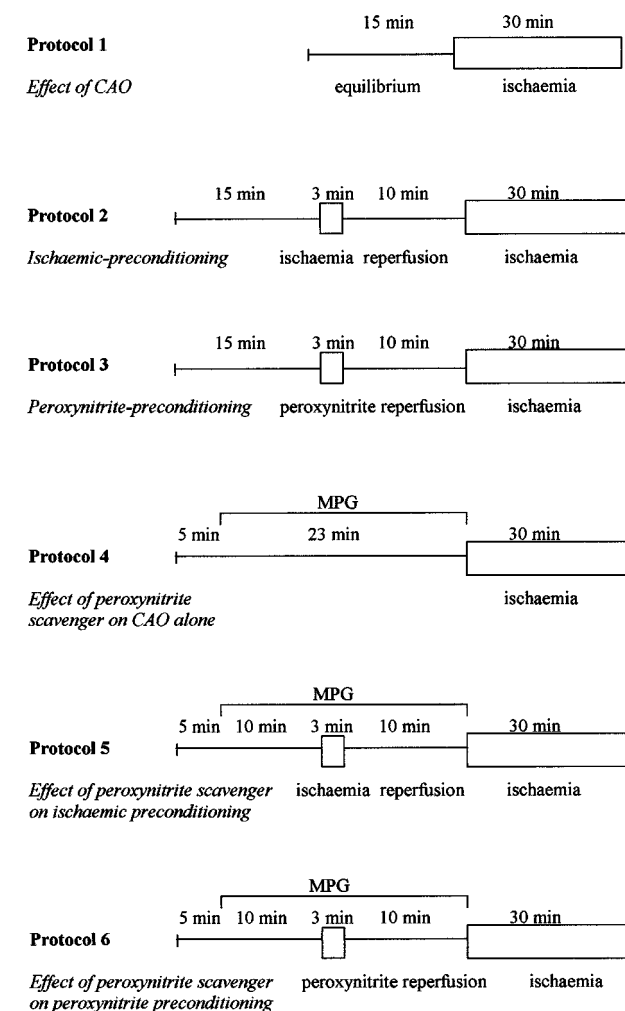


Figure 1 Experimental protocols for the study: Isolated rat hearts perfused with Krebs-Henseleit solution were subjected to 30 min ischaemia (protocol 1). Rat isolated hearts were preconditioned against 30 min ischaemia by a 3 min period of coronary occlusion and 10 min reperfusion (protocol 2). Three different concentrations of peroxynitrite, 0.1, 1 and 10 μM for 3 min were infused in order to precondition the heart (protocol 3). MPG, a peroxynitrite scavenger, (300 μM) 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).

occlusion. In the second group of experiments (protocol 2, preconditioned, $n=10$) hearts were preconditioned with single 3 min of occlusion of LAD followed by 10 min reperfusion and subsequent 30 min occlusion. In the third group of experiments (protocol 3, peroxynitrite-induced preconditioning) hearts were subjected to 3 min infusion of peroxynitrite at three different concentrations (0.1, 1, 10 μM) followed by 10 min washout and then 30 min occlusion. Peroxynitrite was infused into the perfusion solution through the rubber tubing placed just proximal to the heart at a rate of 1 ml min^{-1} to achieve concentrations of 0.1, 1 or 10 μM reaching 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 peroxynitrite scavenger MPG (300 μM) was infused either alone ($n=9$) or in combination with the ischaemic preconditioning protocol ($n=8$) respectively. For the last series of experiments (protocol 6), the concentration of 1 μM of peroxynitrite was chosen since it was shown that this concentration could precondition hearts. In this group of experiments (MPG + peroxynitrite-induced preconditioning, $n=10$), hearts were preconditioned with peroxynitrite as in protocol 3 but received MPG for 23 min starting 10 min prior to peroxynitrite infusion and continued until the onset of the 30 min occlusion period.

Measured parameters

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 (VT), ventricular fibrillation (VF), and ventricular ectopic beat (VEB). VEB is defined as a discrete and identifiable premature QRS complex. VT was diagnosed as four or more consecutive VEBs. VF was diagnosed when the ECG recording showed chaotic activity with an amplitude less than that of the normal ECG. Irreversible VF was defined as VF 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 LAD 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.

Materials

MPG, sodium nitrite, Evans blue, luminol and catalase (from bovine liver) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Hydrogen peroxide was purchased from Merck (Darmstadt, Germany).

Statistical analysis

Values were presented as mean \pm s.e.mean or the percentage incidence. A Fischer's extract Chi-square test was used to detect significant differences in the incidence of VT, VF and irreversible VF between control and drug-treated groups. In the chemiluminescence studies, results were calculated as peak CL or a percentage of the peak CL. Duplicate assays were performed in CL experiments. 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 was considered to be statistically significant.

Results

Effects of peroxynitrite on ischaemic preconditioning

Preconditioning the hearts with 3 min of ischaemia suppressed arrhythmias during the 30 min occlusion period. VEB number was significantly lower than controls. The incidences of VT and VF were also significantly lower than in the control hearts

Table 1 Effects of 3 min period of preconditioning by ischaemia or peroxynitrite infusion on the severity of arrhythmias induced by 30 min of coronary artery occlusion

	n	VEB	%VT	%VF	%irr. VF
Control	14	683 \pm 77 (14)	100 (14)	50 (7)	14 (2)
Preconditioning	10	197 \pm 37* (10)	50 (5)*	0 (0)*	0 (0)
Peroxynitrite (0.1 μM)	8	424 \pm 84* (8)	100 (8)	25 (2)	0 (0)
Peroxynitrite (1 μM)	13	217 \pm 37* (13)	62 (8)*	0 (0)*	0 (0)
Peroxynitrite (10 μM)	8	384 \pm 51* (8)	100 (8)	63 (5)	13 (1)

* $P < 0.05$ significantly different when compared to control. The numbers in parentheses are the numbers of hearts that experienced that particular type of arrhythmia.

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

	n	Baseline	Temporary occlusion or peroxynitrite		Reperfusion or washout		Permanent occlusion	
			1 min	3 min	1 min	10 min	1 min	30 min
Control	14	—	—	—	—	33 \pm 2	65 \pm 2*	66 \pm 2*
Preconditioning	10	35 \pm 2	68 \pm 2*	70 \pm 2*	37 \pm 2	36 \pm 2	63 \pm 1*	63 \pm 1*
Peroxynitrite	13	31 \pm 3	32 \pm 3	32 \pm 2	31 \pm 2	31 \pm 3	58 \pm 2*	61 \pm 2*
MPG	9	31 \pm 2	31 \pm 2	32 \pm 2	33 \pm 2	32 \pm 2	61 \pm 2*	60 \pm 2*
MPG + preconditioning	8	33 \pm 2	63 \pm 2*	62 \pm 2*	31 \pm 2	31 \pm 2	61 \pm 2*	60 \pm 2*
MPG + peroxynitrite	10	34 \pm 2	36 \pm 2	39 \pm 3	38 \pm 3	37 \pm 3	65 \pm 3*	69 \pm 4*

Rat isolated hearts were subjected to 3 min of coronary occlusion followed by 10 min of reperfusion in preconditioned group. The other three groups received peroxynitrite at 1 μM concentration for 3 min followed by 10 min washout period. All groups were then subjected to 30 min of coronary occlusion. Values were given as mean \pm s.e.mean. * $P < 0.05$ significantly different when compared to pre-occlusion values.

(Table 1). In order to study the effects of peroxynitrite infusion on ischaemic preconditioning, three different concentrations of peroxynitrite were studied (0.1, 1, 10 μM). All concentrations of peroxynitrite studied decreased VEB number, but the most pronounced cardioprotective effect was seen with 1 μM . At this concentration, the incidences of VT and VF were significantly reduced and none of the hearts had an irreversible VF (Table 1). Therefore this concentration was chosen for the further peroxynitrite-induced preconditioning experiments. Peroxynitrite administration did not cause any significant change in coronary perfusion pressure throughout the experimental protocol (Table 2). Similarly pressure rate index was not modified by peroxynitrite administration (data not shown).

Effects of MPG on peroxynitrite-induced chemiluminescence

In luminol CL, MPG inhibited the peroxynitrite-induced response in a concentration-dependent manner (Figure 2). No inhibition was evident at 1 μM MPG ($3 \pm 1\%$, $n = 10$) whereas the highest concentration tested (10 mM) caused $79 \pm 3\%$ ($n = 8$) inhibition.

Effects of MPG on ischaemic and peroxynitrite-induced preconditioning

Administration of the peroxynitrite scavenger MPG (300 μM) reversed beneficial effects of ischaemic preconditioning on ischaemia-induced arrhythmias (Table 3). Both VT and total VF times were increased from 21 ± 10 s ($n = 4$) to 101 ± 33 s

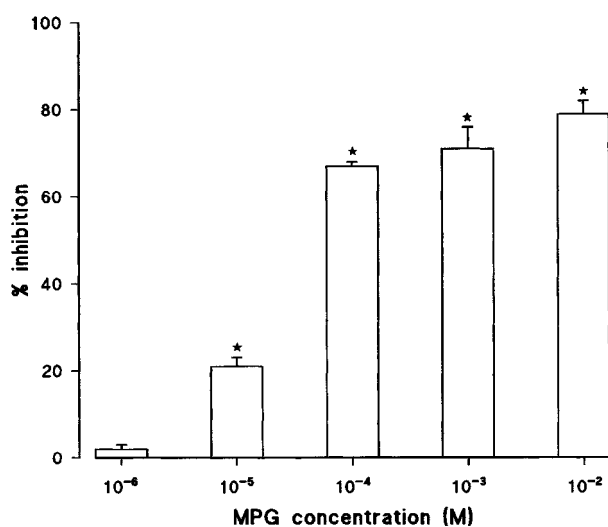


Figure 2 Concentration-dependent effects of MPG ($n = 6-9$) on peroxynitrite-induced luminol chemiluminescence. Data are shown as mean \pm s.e.mean. * $P < 0.05$ significantly less than its control value.

($n = 8$) and from none to 166 ± 83 s ($n = 5$) respectively. MPG (300 μM) also reversed the beneficial effects of peroxynitrite-induced preconditioning on ischaemia-induced arrhythmias. It increased the incidence of VT from 62% ($n = 8$) to 100% ($n = 10$) and total VF from 0% ($n = 0$) to 67% ($n = 6$) (Table 3). Also total VF time was increased from 0 to 270 ± 109 s ($n = 6$).

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

Area at risk measurements

No significant differences were noted in the left ventricular area at risk between control hearts ($32 \pm 4\%$) and those infused with 1 μM peroxynitrite for 3 min ($34 \pm 6\%$). The area at risk was similar in hearts from preconditioned ($33 \pm 5\%$), MPG alone ($31 \pm 2\%$), MPG + preconditioning ($34 \pm 7\%$) or MPG + peroxynitrite preconditioning groups ($33 \pm 9\%$).

Discussion

In this study we have shown that exposure to 1 μM concentration of peroxynitrite on its own was capable of mimicking the beneficial effects of ischaemic preconditioning. Peroxynitrite at this concentration markedly reduced the severity of ischaemia-induced arrhythmias. This effect was lost in the presence of the peroxynitrite scavenger, MPG. MPG treatment also attenuated the beneficial effects of ischaemic preconditioning. To our knowledge, this is the first evidence that peroxynitrite generated during brief initial ischaemia and reperfusion can initiate a protective response against arrhythmias and involve the signalling cascade of preconditioning.

It has been proposed that maximally achievable concentration of peroxynitrite formed in *in vivo* conditions is in the low micromolar range (i.e., 2–5 μM), and concentrations above these levels would probably not be formed *in vivo* (Nossuli *et al.*, 1998). Therefore, the concentrations of peroxynitrite (0.1 and 1 μM) used in the present experiment are highly likely to be reached in *in vivo* conditions. We have observed that 1 μM peroxynitrite afforded significant cardioprotective effects and reduced both the number of VEBs, the incidence of VT and VF during the 30 min occlusion period. The lower concentration of peroxynitrite (0.1 μM) was less effective than 1 μM only in reducing the ectopic activity.

The mechanism responsible for the beneficial effects of preconditioning is not well established and it seems that more than a single mechanism is involved. Many endogenous substances are released and/or operative during ischaemic preconditioning including adenosine, acetylcholine, catecholamines, angiotensin II, bradykinin, endothelin, nitric oxide and

Table 3 Effects of peroxynitrite scavenger MPG (300 μM) infusion on preconditioning induced by 3 min ischaemia and by 3 min peroxynitrite infusion at 1 μM concentration

	<i>n</i>	VEB	% VT	% VF	% irr. VF
Control	14	683 ± 77 (13)	100 (14)	50 (7)	14 (2)
Preconditioning	10	$197 \pm 37^*$ (10)	50* (5)	0 (0)*	0 (0)
Peroxynitrite	13	$217 \pm 37^*$ (13)	62* (8)	0 (0)*	0 (0)
MPG	9	758 ± 74 (9)	100 (9)	56 (5)	0 (0)
MPG + preconditioning	8	$309 \pm 41^*$ (8)	100‡ (8)	75‡ (6)	25 (2)
MPG + peroxynitrite	10	$493 \pm 63^*\ddagger$ (10)	100‡† (10)	67‡† (6)	22 (2)

$P < 0.05$ when compared to control (*), preconditioning (‡), or peroxynitrite group (†). Numbers in parentheses are the number of hearts that exhibited that particular type of arrhythmia.

opioids which have all been identified either as a trigger or a mediator (Parratt & Vegh, 1996; Yellon *et al.*, 1998). There is considerable evidence that bradykinin is released early in ischaemia and has been considered as a trigger for the release of NO and peroxynitrite (Kooy & Royall, 1994; Parratt & Vegh, 1996). A constitutive NOS is present in cardiac myocytes (Belhassen *et al.*, 1996; Depre *et al.*, 1997) and increased synthesis of NO by this enzyme may occur during preconditioning, since ischaemia increases intracellular calcium levels and elevates the availability of NADPH whereas reperfusion provides the oxygen required for NO generation. There is increasing evidence that NOS may produce peroxynitrite rather than NO under conditions in which the local tissue levels of L-arginine decline (Xia *et al.*, 1996). If this were the case during brief myocardial ischaemia/reperfusion, the trigger or mediator for preconditioning could be an increased generation of peroxynitrite by NOS.

It is known that endothelial production of superoxide radical is accelerated in the early phase of reperfusion (Wang & Zweier, 1996). There is also evidence for increased superoxide levels immediately after brief, intermittent anoxic preconditioning in rat isolated myocytes (Zhou *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). Furthermore, it has been shown that free radicals generated from hypoxanthine plus xanthine oxidase reaction induced protection against infarct size in rabbit hearts (Baines *et al.*, 1997). Additionally, NO formation is also markedly increased during ischaemia and/or reperfusion in rat isolated heart (Zweier *et al.*, 1995; Liu *et al.*, 1997) probably through activation of the constitutive heart NO synthase (Depre *et al.*, 1997). It has been demonstrated that the generation of NO contributes to the marked antiarrhythmic effects of preconditioning in canine myocardium (Vegh *et al.*, 1992b). Taken together, these studies suggest that both superoxide and NO are produced and peroxynitrite are formed in myocardial ischaemia and reperfusion.

It has been suggested that free radicals generated during the first ischaemic insult serve as a trigger for the development of late preconditioning against myocardial infarction (Sun *et al.*, 1996; Qiu *et al.*, 1997). Recently, Takano *et al.* (1998) showed that NO donors may induce late preconditioning and this phenomenon involves the generation of oxidant species, possibly peroxynitrite and/or hydroxyl radical. It has been proposed that peroxynitrite and/or hydroxyl radical formed after an ischaemic stimulus may activate a protein kinase C-mediated signal transduction cascade that culminates in the development of a protective effect 24 h later (Takano *et al.*, 1998). Thus, peroxynitrite released during brief periods of ischaemia-reperfusion appear to play a role in triggering both the early and the late phases of ischaemic preconditioning. The present study provides new experimental evidence that peroxynitrite is involved in the antiarrhythmic effects of early phase of ischaemic preconditioning in rats.

MPG is proposed to be a cell-permeant antioxidant that reacts rapidly with both peroxynitrite and hydroxyl radical by virtue of its thiol group (Takano *et al.*, 1998). Our data with luminol chemiluminescence showed that MPG is a direct peroxynitrite scavenger. Since peroxynitrite can decompose to form hydroxyl radical (Merenyi *et al.*, 1998), it can be considered that the administration of MPG is useful to scavenge intracellular and extracellular free radicals. Our results demonstrated that MPG completely abolished the preconditioning induced either by ischaemia/reperfusion or

peroxynitrite infusion. Therefore, it seems highly likely that peroxynitrite or its byproducts elicit preconditioning in the present study. This conclusion can be further supported by the observations that ischaemic preconditioning can be blocked both by inhibiting NOS (Vegh *et al.*, 1992b) and by scavenging superoxide (Tanaka *et al.*, 1994; Osada *et al.*, 1994). Our results are in agreement with the observations in both *in situ* and *in vitro* rabbit hearts that MPG abolished protection afforded by a single, but not multiple, cycle of preconditioning (Baines *et al.*, 1997). Richard *et al.* (1993) also used MPG in their study to test the role of free radicals in preconditioning, but they failed to block the infarct size limiting effect of ischaemic preconditioning in rats. These differences could be due to the three cycles of ischaemia-reperfusion that were used to induce protection. The multiple cycles may have caused multiple mediators to be released such that elimination of the free radical signal would not have brought the stimulation to a subthreshold level (Baines *et al.*, 1997).

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.

In recent years it has become apparent that relatively low concentrations of peroxynitrite are able to mediate beneficial effects in some physiological processes. Peroxynitrite relaxes various arteries including coronary arteries through stimulation of cyclic GMP (Liu *et al.*, 1994; Wu *et al.*, 1994; Tarpey *et al.*, 1995). Peroxynitrite produces S-nitrosothiols which stimulate guanylyl cyclase and release NO (Moro *et al.*, 1994; 1995; Wu *et al.*, 1994). Peroxynitrite has also been found to be cardioprotective in low micromolar concentrations both in *in vivo* and *in vitro* experiments (Lefer *et al.*, 1997; Nossuli *et al.*, 1997; 1998; Altuğ *et al.*, 1999). Although the exact mechanism is not known, there are several possible mechanisms whereby peroxynitrite could induce cardioprotective action. Firstly, peroxynitrite can S-nitrosylate glutathione or other thiol-containing substances in tissues causing the formation of S-nitrosothiols (Moro *et al.*, 1994; 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; Balazy *et al.*, 1998). 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 although there is evidence that these channels are not involved in preconditioning in rats (Parratt & Kane, 1994). Since peroxynitrite administration did not cause any significant change on coronary perfusion pressure and pressure rate index measured at certain time points throughout the experiment, the cardioprotective effect seen with peroxynitrite administration was not due to an effect on these variables.

In conclusion, the results of this study showed that exogenously administered peroxynitrite at 1 μ M concentration was able to mimic the beneficial effects of ischaemic preconditioning. Furthermore, we observed that MPG, a peroxynitrite scavenger, given prior to initial occlusion also

blocked 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 mediators of cardioprotection. The present study provides new insights into the mechanism of ischaemic preconditioning.

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