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RESEARCH PAPER

Peroxynitrite decreases arrhythmias induced by ischaemia reperfusion in anaesthetized dogs, without involving mitochondrial K_{ATP} channels

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Background and purpose: Exogenous peroxynitrite from nanomolar to micromolar concentrations exerts cardioprotection. Here, we have assessed its effects on ischaemia- and reperfusion-induced ventricular arrhythmias *in vivo* and a possible role for mitochondrial K_{ATP} channels in these effects, using the channel inhibitor 5-hydroxydecanoate (5-HD).
Experimental approach: Chloralose-urethane-anaesthetized dogs were treated twice for 5 min with peroxynitrite (100 nM, by intracoronary infusions) in both the absence and presence of 5-HD (150 $\mu g\,kg^{-1}\,min^{-1}$), and then subjected to 25 min occlusion of the left anterior descending coronary artery. The severity of ischaemia and of arrhythmias, as well as the levels of nitrotyrosine were assessed and compared with a group of control dogs, subjected only to a 25 min occlusion and reperfusion insult.
Key results: Compared with controls, infusion of peroxynitrite markedly suppressed the number of ventricular premature beats (388 ± 88 vs 133 ± 44), the incidence of ventricular fibrillation both during occlusion (50% vs 10%) and reperfusion (100% vs 44%), and increased survival (0% vs 50%; all $P<0.05$). The severity of ischaemia (epicardial ST-segment changes, inhomogeneity of electrical activation) during occlusion and nitrotyrosine levels on reperfusion were significantly less in the peroxynitrite-treated dogs than in the controls. 5-HD did not modify the cardioprotective effects of peroxynitrite.
Conclusion and implications: Exogenous peroxynitrite provided antiarrhythmic protection *in vivo,* which might have been on account of a reduction in endogenous peroxynitrite formation. This protection seemed not to be mediated through mitoK_{ATP} channels.
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Keywords: peroxynitrite; arrhythmias; mitochondrial K_{ATP} channels; nitric oxide; free radicals

Abbreviations: 5-HD, 5-hydroxydecanoate; HR, heart rate; LAD, left anterior descending coronary artery; L-NAME, L^{ω}-nitro-arginine-methyl-ester; LVEDP, left ventricular end-diastolic pressure; PC, preconditioning; PN, peroxynitrite; NT, nitrotyrosine; ROS, reactive oxygen species; VF, ventricular fibrillation; VPBs, ventricular premature beats; VT, ventricular tachycardia

Introduction

There is growing evidence that both reactive nitrogen species, particularly nitric oxide (NO) and reactive oxygen species (ROS), especially superoxide, are involved in the cardioprotective effects of ischaemic preconditioning (recently reviewed by Ferdinándy and Schulz, 2003; Downey *et al.*, 2007; Rastaldo *et al.*, 2007). It seems to be well established as well that their actions can, somehow, be connected with mitochondrial K_{ATP} (mitoK_{ATP}) channels Downey and Cohen, 1997; Pain *et al.*, 2000; Quin *et al.*, 2004). Indeed, the first evidence that NO has a function in the protection associated with ischaemic preconditioning came from our studies performed on anaesthetized dogs, where inhibition of the L-arginine NO pathway with L-NAME, administered both before preconditioning and the prolonged occlusion, markedly attenuated the antiarrhythmic effect of preconditioning (Végh *et al.*, 1992c). Although then the involvement of NO in both the delayed (Végh *et al.*, 1994; Qiu *et al.*, 1997; Kis *et al.*, 1999a, b) and, more recently, the early phases (Csonka *et al.*, 1999; Lochner *et al.*, 2000) of the preconditioning-induced protection has been confirmed.

Although the involvement of ROS in cardioprotection associated with preconditioning seems to also be established, the situation regarding their importance in eliciting protection is rather controversial. Although some studies have

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shown that ROS generation is necessary for inducing cardioprotection (e.g. Tanaka *et al*., 1994; Baines *et al*., 1997; Das *et al*., 1999), others have failed to demonstrate such a significant effect (Iwamoto *et al*., 1991; Richard *et al*., 1993; Hajnal *et al*., 2005). These differences may be related to the model used; that is, as to whether the experiments are performed under *in vitro* or *in vivo* conditions, the dose and route of administration of the antioxidant applied, as well as the intensity of the preconditioning stimulus (that is, the duration and number of the preconditioning occlusions), which may substantially determine the signalling pathways that lead to cardioprotection (Liem *et al*., 2005). Furthermore, the situation regarding the possible trigger or mediator role of ROS and their relation to the mitoK$_{ATP}$ channel, is also not fully understood (Gross and Peart, 2003; Kevin *et al*., 2003; Lebuffe *et al*., 2003).

To make the situation even more confused these nitrogen- and oxygen-derived radicals, generated during ischaemia and reperfusion, are chemically unstable and form other highly reactive species, such as peroxynitrite (Beckman and Crow, 1992; Squadrito and Pryor, 1995). There is still ongoing debate on whether peroxynitrite has a protective or detrimental function in ischaemia- and reperfusion-induced myocardial injury (recently reviewed by Ferdinándy and Schulz, 2001, 2003, Ferdinándy *et al*., 2007; Uppu *et al*., 2007). A number of studies support the major contribution of peroxynitrite to myocardial and endothelial dysfunction (Szabó, 1996; Yasmin *et al*., 1997), cell necrosis (Wang and Zweier, 1996), and to arrhythmias (Tecder-Ünal and Kanzik, 2004; Danson and Paterson, 2006) and that a reduction in peroxynitrite formation underlies the cardioprotective effect of ischaemic preconditioning (Csonka *et al*., 2001). In contrast, there is growing evidence that peroxynitrite may actually be beneficial rather than cytotoxic. For example, it was shown that exogenously administered peroxynitrite, at high nanomolar-to-low micromolar concentrations, preserved myocardial and coronary endothelial function (Lefer *et al*., 1997; Nossuli *et al*., 1998) and reduced infarct size (Nossuli *et al*., 1997). Similarly, in rat-isolated hearts, the administration of peroxynitrite reduced ischaemia-induced arrhythmias (Altug *et al*., 2001), whereas in the same model, peroxynitrite, generated during the brief ischaemia and reperfusion insults (preconditioning), was involved in the antiarrhythmic effect of preconditioning (Altug *et al*., 2000). Although the precise mechanism by which peroxynitrite provides cardioprotection is still unclear, one possible explanation might be that peroxynitrite, by oxidizing thiols, may serve as a NO donor (Mayer *et al*., 1995). The release of NO from *S*-nitrosothiols and the subsequent stimulation of soluble guanylate cyclase (Nossuli *et al*., 1998) or the activation of tyrosine kinase pathway (Söylemez *et al*., 2003) were proposed to have functions in the cardioprotective effect of peroxynitrite; both mechanisms are also known to be implicated in the antiarrhythmic effect of ischaemic preconditioning (Végh *et al*., 1992b; Fatehi-Hassanabad and Parratt, 1997).

Two questions stimulated the present study. First, the characterization of the potential antiarrhythmic effect of peroxynitrite in an *in vivo* model is lacking. Second, although there is substantial evidence that in the cardioprotective effects of NO, superoxide, or in the antiarrhythmic effect of preconditioning (Végh and Parratt, 2002) mitoK$_{ATP}$ channels are involved, little is known as to whether the effects of peroxynitrite are also mediated through the activation of these channels. Therefore, we gave peroxynitrite during brief (5 min) periods of intracoronary infusion in both the absence and presence of the mitoK$_{ATP}$ channel inhibitor 5-hydroxydecanoate (5-HD) to anaesthetized dogs that were subjected, 5 min later, to a 25 min occlusion and reperfusion of the left anterior descending (LAD) coronary artery. The severity of ischaemia and of ventricular arrhythmias, occurring during ischaemia and reperfusion and levels of nitrotyrosine, formed following such a period of ischaemia and reperfusion were evaluated, and compared with a group of control dogs, subjected simply to a 25 min occlusion and reperfusion insult. The results show that administration of peroxynitrite significantly reduced the severity of ventricular arrhythmias both during occlusion and reperfusion, and attenuated the formation of peroxynitrite that was generated during a combined ischaemia–reperfusion insult in the anaesthetized dogs. As the administration of 5-HD did not modify the protective effects of peroxynitrite, we concluded that activation of mitoK$_{ATP}$ channels was unlikely to have a mandatory function in the peroxynitrite-induced protection against arrhythmias.

A preliminary account of these results was presented at the World Congress of the International Society for Heart Research, in Bologna in June 2007 (Juhász *et al*., 2007; Kiss *et al*., 2007).

Materials and methods

Animals and surgical preparation

All animal procedures were in accordance with Hungarian law (XXVIII, chapter IV, paragraph 31) with regard to large experimental animals, which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We used adult, mongrel dogs of either sex, with a mean body weight of 21 ± 3 kg. Under light anaesthesia (20 mg kg^{-1} intravenous sodium pentobarbitone) polyethylene catheters were inserted into the right femoral artery for monitoring systolic, diastolic and mean arterial blood pressures, and into the right femoral vein for anaesthetic (combination of chloralose 60 mg kg^{-1} and urethane 200 mg kg^{-1}) administration. Another catheter was introduced into the cavity of the left ventricle, through the left carotid artery, for the measurement of left ventricular systolic and end-diastolic pressures (Statham P23XL transducers connected to SYTEM6, Triton Technology, USA). Changes in positive and negative LVdP/dt$_{max}$ were also assessed. The dogs were ventilated with room air and blood gases were monitored and kept within the normal range (Végh *et al*., 1992a). Temperature was recorded from the rectum or mid-oesophagus and maintained at 37 ± 0.5°C.

Thoracotomy was performed at the left fourth and fifth intercostal space and the pericardium was excised. The anterior branch of the left coronary artery was prepared for occlusion just proximal to the first main diagonal branch

and a side branch of this artery was catheterized for the intracoronary administration of saline or of peroxynitrite. A composite electrode (Végh *et al*., 1987, 1992a) was sutured on the surface of the potentially ischaemic area to record the degree of inhomogeneity of electrical activation. This electrode gives a summarized recording of R-waves from 24 epicardial measuring points. In the normal, adequately perfused myocardium all sites are activated almost simultaneously, resulting in a single large spike. However, after occlusion widening and fractionation of this summarized R-wave occur, indicating that the adjacent fibres are not simultaneously activated because of the inhomogeneous conduction. We expressed this as the greatest delay in activation (in ms) within the ischaemic area. The composite electrode also contains four unipolar leads by which epicardial ST-segment changes were measured. All the data, together with a limb lead electrocardiogram, were recorded on an eight channel Medicor R81 recorder.

Evaluation of ventricular arrhythmias and area at risk

Ventricular arrhythmias during a 25 min (LAD) coronary artery occlusion and following reperfusion were assessed as outlined in the 'Lambeth Conventions' (Walker *et al*., 1988, and modified as previously described Végh *et al*., 1992a). During occlusion, we assessed the total number of ventricular premature beats (VPBs) as single ectopics and the incidence and number of episodes of ventricular tachycardia (VT; defined as a run of four or more VPBs at a rate faster than the resting sinus rate). We also determined the incidence of ventricular fibrillation (VF) during both occlusion and reperfusion. Survival from the combined ischaemia–reperfusion insult was defined as dogs that were still alive 1–2 min after reperfusion. These survivals were killed with an excess of anaesthetic before sample taking and the evaluation of the risk area. This latter was assessed in some dogs of each group by injecting patent blue V dye into the LAD at a pressure not greater than the systolic arterial pressure in that animal. It was defined as the percentage area of the left ventricular wall together with the septum served by the occluded artery (Végh *et al*., 1992a).

Synthesis of peroxynitrite

Peroxynitrite was synthesized from acidified nitrite and hydrogen peroxide (H_2O_2) according to the method of Beckman *et al*. (1994). Briefly, an aqueous solution of $NaNO_2$ (0.83 g) was mixed with a solution containing of H_2O (3.8 mL), HNO_3 (11.1 M) and H_2O_2 (8.2 M), and immediately quenched with NaOH (4.2 M). All reactions were performed on ice. Powdered MnO_2 was added to remove the excess of H_2O_2, and then the solution was kept in the dark for approximately 5 min. The mixture was filtered and the final concentration of the aliquot peroxynitrite was measured spectrophotometrically (peak absorbance at 302 nm wave length). The stock solutions (150-200 mM) were stored in aliquots at −80 °C away from light. Before each experiment the absorbance of peroxynitrite was again measured and the concentration was adjusted to 100 nM with pH 8.4 saline. The pH of saline solution was adjusted by an appropriate volume of 0.1 M NaOH added directly to the normal saline (Nossuli *et al*., 1997).

Determination of peroxynitrite formation

This was determined by the measurement of nitrotyrosine formation using western immunoblot. Tissue samples, taken from the ischaemic area 1–2 min after reperfusion, were frozen, powdered and homogenized in ice-cold buffer (HEPES 10 mM, sucrose 0.32 mM, EDTA 0.1 mM, DTT 1.0 mM, trypsin inhibitor 10 $\mu g\,mL^{-1}$, leupeptin 10 $\mu g\,mL^{-1}$, aprotinin 2.1 $\mu g\,mL^{-1}$, PMSF 10 $mg\,L^{-1}$; pH 7.2). The homogenates were then centrifuged for 10 min at 4 °C at 10 000 *g*. The protein concentrations of the supernatants were determined by a bicinchoninic acid protein assay kit (Sigma, St Louis, MO, USA). To assess tyrosine nitrosylation, 20 μg of total protein was loaded onto 8% polyacrylamide gel. The electrophoresis was performed at 100 V, 400 mA, for 120 min, the proteins were then transferred into nitrocellulose membrane by electroblotting (40 V, 400 mA, 120 min). To block non-specific binding sites, the membrane was incubated overnight with TBS Tween-20 solution (the volume fraction is 0.05) containing 5% non-fat milk powder at 4 °C. After this, the membrane was incubated at room temperature for a period of 90 min with monoclonal anti-nitrotyrosine antibody (diluted to 1:1000; MAB5404, Chemicon, Millipore Corp., Billerica, MA, USA). A horseradish peroxidase-conjugated rabbit anti-mouse IgG (P0161, Dakocytomation A/S, Glostrup, Denmark) diluted to 1:1000 was used as secondary antibody at room temperature, for 50 min. The membrane was then developed with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare, Little Chalfont, Buckinghamshire, UK), exposed to X-ray film and scanned. Density of nitrotyrosine bands were calculated by integrating the area (in pixels) and expressed in arbitrary units using the Quantity One (Bio-Rad Laboratories, Hercules, CA, USA) programme.

Experimental protocol

This is shown in Figure 1. Six groups of randomly selected dogs were used. In all groups a 30 min recovery period was allowed to stabilize after surgery, and then pH 8.4 saline (solvent for peroxynitrite; Nossuli *et al*., 1997) was infused at a rate of 0.5 $mL\,min^{-1}$ locally, into a side branch of the LAD over a period of 10 min to evaluate the effects of alkaline saline solution on the haemodynamic parameters. Four dogs served as sham-operated controls. These dogs were instrumented, infused locally with normal saline for 30 min and, without subjecting them to ischaemia, they were killed and samples were taken for the determination of nitrotyrosine. In four additional dogs 100 nM peroxynitrite (chosen as one-tenth of the intravenous concentration used previously *in vivo*; Nossuli *et al*., 1997) was administered in intracoronary infusion at a rate of 0.5 $mL\,min^{-1}$ two times for 5 min, after which the hearts were stopped and tissue samples were harvested. In the other groups, each animal was subjected to a 25 min LAD occlusion followed by rapid reperfusion. Control dogs ($n = 10$) were infused, with normal saline (pH 7.4), locally into a small side branch of the LAD over a period of 30 min, just before the 25 min occlusion of the LAD. Three

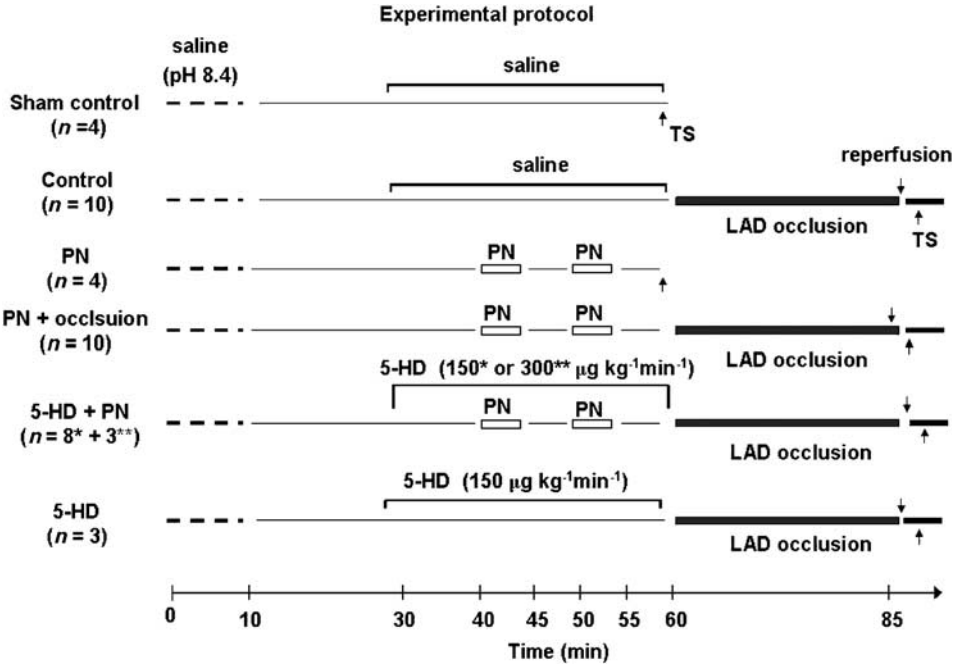

Figure 1 Experimental protocol. Before occlusion every group was given saline (pH: 8.4 at a rate of 0.5 mL min^{-1}) by intracoronary infusion over 10 min. Twenty minutes later control dogs ($n=10$) were infused with normal saline for a period of 30 min, and then subjected to 25 min LAD occlusion followed by rapid reperfusion. Twenty-five dogs were infused with peroxynitrite (PN) by intracoronary route at a rate of 0.5 mL min^{-1} two times for 5 min. In four out of these dogs, samples were taken immediately after the cessation of the infusion, whereas 21 dogs were subjected to coronary artery occlusion. In eleven out of the peroxynitrite-treated dogs, 5-hydroxydecanoate (5-HD) was also infused over a period of 30 min by the intracoronary route, starting the infusion 10 min before the first administration of PN. In eight out of these eleven dogs, 5-HD was administered in a dose of 150 μg kg^{-1} min^{-1}*, whereas in the remaining three dogs 300 μg kg^{-1} min^{-1}** 5-HD was infused. In additional three dogs the effects of 150 μg kg^{-1} min^{-1} dose of 5-HD on ischaemia and reperfusion changes were assessed. Tissue samples (TS) were taken from the ischaemic area for the determination of nitrotyrosine levels 1–2 min after reperfusion.

other groups of dogs ($n=21$) were infused with peroxynitrite by the same route and concentration as described above, 5 min before the commencement of the coronary artery occlusion. In eight out of these peroxynitrite-treated dogs, 5-hydroxydecanoate (5-HD) was also given in intracoronary infusion in a dose of 150 μg kg^{-1} min^{-1} (Végh and Parratt, 2002), whereas in the remaining three dogs the dose of 5-HD was 300 μg kg^{-1} min^{-1}. In another three dogs the effects of 5-HD (150 μg kg^{-1} min^{-1}) alone, on the ischaemia and reperfusion-induced changes, were examined.

The drug and molecular target nomenclature, used in this study, complies with proposals outlined in the *British Journal of Pharmacology* (Alexander *et al*., 2008).

Statistical evaluation

All data are expressed as means ± s.e.mean and differences between means compared by ANOVA for repeated measures or by the one-way ANOVA as appropriate, using the Fisher *post hoc* test. Ventricular premature beats and episodes of ventricular tachycardia were compared using the Kruskal–Wallis test. The incidences of arrhythmias (such as VT and VF) and survival from the combined ischaemia and reperfusion insult were compared by the Fisher Exact test and also by the χ^2 test as appropriate. Differences between groups were considered significant when $^*P<0.05$.

Materials

5-Hydroxydecanoate (5-HD), anaesthetics and all other chemical were purchased from Sigma-Aldrich Corp (St Louis, MO, USA).

Results

Haemodynamic effects of peroxynitrite, 5-hydroxydecanoate and coronary artery occlusion

Neither the intracoronary infusion of the alkaline saline (pH 8.4), nor the local administration of peroxynitrite, 5-HD or the combination of peroxynitrite and 5-HD resulted in significant changes in the haemodynamic parameters (Table 1). The occlusion-induced changes were similar in all groups, except that in dogs given peroxynitrite, the elevation of left ventricular end-diastolic pressure and the decrease of negative dP/dt were significantly less than in the controls (Table 2).

Ventricular arrhythmias during coronary artery occlusion

These are illustrated in Figures 2 and 3. Compared with the controls the total number of VPBs during the 25 min occlusion was reduced by about 60% ($P<0.05$) in the peroxynitrite-treated dogs, and this was not substantially

Table 1 Haemodynamic changes during the infusions of pH 8.4 saline, PN, 5-HD and 5-HD + PN

	Saline (pH 8.4) (n = *10*)		*PN* (n = *10*)		*5-HD* (*150 μg kg^{-1} min^{-1}*; n = *3*)		*5-HD + PN* (*150 μg kg^{-1} min^{-1}*; n = *8*)		*5-HD + PN* (*300 μg kg^{-1} min^{-1}*; n = *3*)	
	Baseline	*Change*	*Baseline*	*Change*	*Baseline*	*Change*	*Baseline*	*Change*	*Baseline*	*Change*
SABP (mm Hg)	131 ± 7	4 ± 3	135 ± 5	−4 ± 1	139 ± 6	1 ± 2	127 ± 6	6 ± 2	137 ± 3	3 ± 3
DABP (mm Hg)	88 ± 6	1 ± 2	90 ± 4	−4 ± 1	83 ± 6	1 ± 1	84 ± 6	4 ± 1	90 ± 5	1 ± 1
MABP (mm Hg)	103 ± 6	3 ± 2	105 ± 3	−4 ± 1	98 ± 5	1 ± 1	98 ± 6	4 ± 1	100 ± 3	2 ± 2
LVSP (mm Hg)	134 ± 6	4 ± 3	136 ± 4	−5 ± 2	139 ± 5	1 ± 2	133 ± 5	3 ± 1	137 ± 2	3 ± 3
LVEDP (mm Hg)	2.8 ± 0.4	0 ± 0	3.2 ± 0.3	0.1 ± 0.1	2.4 ± 0.3	0.2 ± 0.1	2.8 ± 0.4	0.1 ± 0.2	3.0 ± 1.0	0.5 ± 0.5
+ dP/dt (mm Hg s^{-1})	2912 ± 12	37 ± 48	2591 ± 82	−150 ± 40	3211 ± 212	19 ± 81	3061 ± 208	80 ± 73	2975 ± 75	75 ± 75
−dP/dt (mm Hg s^{-1})	2241 ± 70	24 ± 12	1814 ± 63	−85 ± 26	2438 ± 260	19 ± 36	2659 ± 219	19 ± 36	2385 ± 240	62 ± 18
HR (beats min^{-1})	153 ± 6	2 ± 2	153 ± 4	0 ± 1	167 ± 7	0 ± 2	166 ± 8	−1 ± 1	163 ± 3	3 ± 1

Abbreviations: DABP = diastolic arterial blood pressure, HR = heart rate, LVEDP = left ventricular end-diastolic pressure, LVSP = left ventricular systolic pressure, MABP = mean arterial blood pressure, SABP = systolic arterial blood pressure. Data are means ± s.e.mean. Data presented as changes were determined 5 min after starting the infusion of saline, PN, 5-HD and 5-HD + PN.

Table 2 Haemodynamic changes during a 25 min occlusion of the LAD

	Control		*PN*		*5-HD + PN*	
	Baseline	*Max. change*	*Baseline*	*Max. change*	*Baseline*	*Max. change*
SABP (mm Hg)	126 ± 5	−12 ± 2	131 ± 6	−12 ± 5	133 ± 7	−9 ± 2
DABP (mm Hg)	88 ± 4	−13 ± 1	86 ± 5	−8 ± 2	88 ± 6	−8 ± 2
MABP (mm Hg)	100 ± 4	−12 ± 1	101 ± 5	−9 ± 2	103 ± 6	−8 ± 2
LVSP (mm Hg)	133 ± 5	−17 ± 3	129 ± 6	−9 ± 2	137 ± 6	−11 ± 2
LVEDP (mm Hg)	2.5 ± 0.6	12.0 ± 0.7	3.6 ± 0.3	7.5 ± 0.7*	3.1 ± 0.4	6.5 ± 0.57*
+ dP/dt (mm Hg s^{-1})	2846 ± 323	−600 ± 139	2794 ± 94	−560 ± 37	3195 ± 236	−370 ± 46*
−dP/dt (mm Hg s^{-1})	2586 ± 211	−538 ± 92	1950 ± 76	−315 ± 47*	2445 ± 130	−285 ± 34*
HR (beats min^{-1})	157 ± 5	−2 ± 2	148 ± 5	8 ± 3	164 ± 9	2 ± 2

Abbreviations: DABP = diastolic arterial blood pressure, HR = heart rate, LVEDP = left ventricular end-diastolic pressure, LVSP = left ventricular systolic pressure, MABP = mean arterial blood pressure, SABP = systolic arterial blood pressure. Data are means ± s.e.mean calculated from $n = 8$–12 experiments, *$P < 0.05$ compared to the control.

modified by the earlier administration of 5-HD ($P < 0.05$ vs controls; Figure 2). Similarly, the incidence and the number of episodes of VT were less in the peroxynitrite-treated animals than in the controls (both $P < 0.05$; Figure 2). This reduction in arrhythmia severity was also not influenced by 5-HD (both $P < 0.05$ vs controls). The effects on occlusion and reperfusion-induced VF, and on survival from the combined ischaemia–reperfusion insult are shown in Figure 3. In the control dogs there was 50% incidence of VF during occlusion and all the remaining dogs fibrillated following reperfusion, thus no control dog survived the combined ischaemia–reperfusion insult. In contrast, in dogs given peroxynitrite, only one dog out of 18 dogs fibrillated during the occlusion period, irrespective of whether they had been given 5-HD. These results indicate that the 150 μg kg^{-1} min^{-1} dose of 5-HD, which by itself does not modify arrhythmia severity during coronary artery occlusion (VPBs: 325 ± 22, VT episodes: 5–7 per dog. VF: 1 out of 3; data obtained from 3 observations), failed to modify the antiarrhythmic effect of peroxynitrite. Similarly, the higher dose of 5-HD (300 μg kg^{-1} min^{-1}) given to another three peroxynitrite-treated dogs was unable to abolish or even attenuate the antiarrhythmic protection (VPBs, 98 ± 6, no VT or VF occurred in these three dogs).

Changes in the severity of ischaemia following coronary artery occlusion

This was examined using both epicardial ST-segment mapping and the degree of inhomogeneity of electrical activation. In control dogs, the epicardial ST-segment increased rapidly, especially during the initial 5 min period of the occlusion (reaching the maximum at 5 min of the occlusion), and this was maintained throughout the whole ischaemic period (Figure 4). The infusion of peroxynitrite significantly reduced these ST-segment changes (measured at 5 min of the occlusion; $P < 0.05$ vs controls), which were not reversed by the administration of 5-HD. Changes in electrical activation are shown in Figure 5; this index of conduction delay, which is around 50–60 ms in the normal non-ischaemic heart, was markedly increased during ischaemia (by around 170 ms), and again, peroxynitrite infusion reduced this throughout the entire occlusion period. Administration of 5-HD did not modify this peroxynitrite-induced reduction in the degree of inhomogeneity.

The effect of peroxynitrite infusion on nitrotyrosine formation

Nitrotyrosine formation was used to assess peroxynitrite production resulting from ischaemia and reperfusion. The results are shown in Figure 6. Compared with the

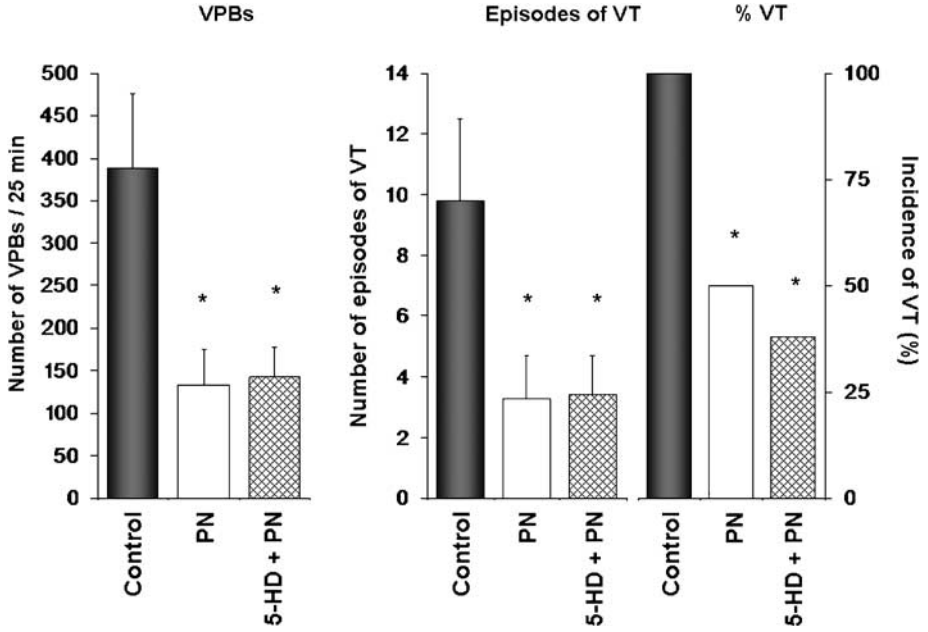

Figure 2 The total number of ventricular premature beats (VPBs), and the incidence (%) and number of episodes of ventricular tachycardia (VT) in control dogs, in PN-treated dogs and in dogs given 5-HD (150 μg kg^{-1} min^{-1}) and PN (5-HD + PN) during a 25 min occlusion of the LAD. Compared with the controls peroxynitrite markedly suppressed these arrhythmias. This antiarrhythmic effect was not modified by 5-HD. Values are means ± s.e.m. *$P<0.05$ vs control.

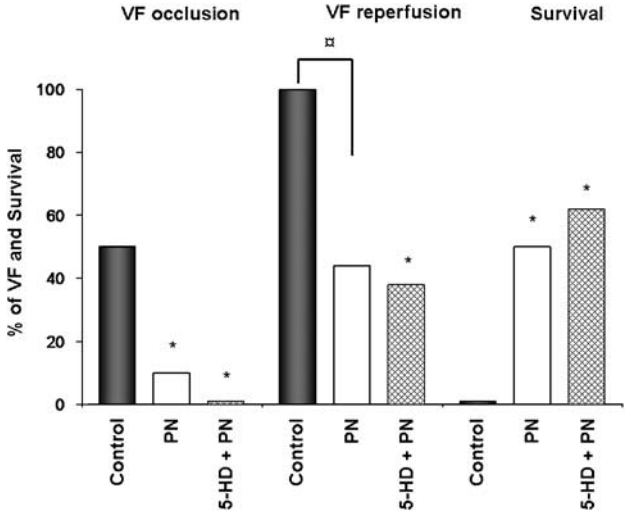

Figure 3 Ventricular fibrillation (VF, %) during a 25 min coronary artery occlusion (VF occlusion), following reperfusion (VF reperfusion) and survival following the combined ischaemia–reperfusion insult. The reduction in VF and the increase in survival following the administration of peroxynitrite is not modified by 5-HD. *$P<0.05$ compared to the control, ¤$P=0.06$ using the Fisher exact test and ¤$P=0.03$ using the χ^2 test.

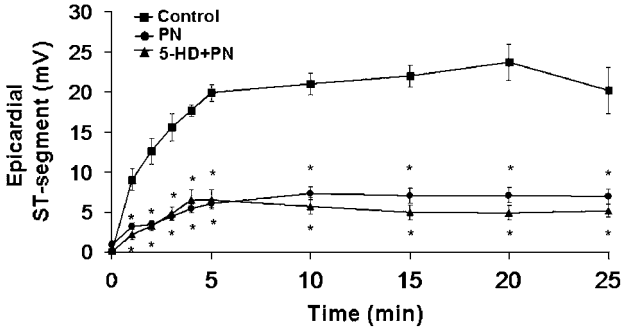

Figure 4 Changes in epicardial ST-segment during a 25 min coronary artery occlusion. The maximal elevation had appeared by the first 5 min of occlusion and was maintained over the entire ischaemic period. Peroxynitrite significantly reduced these changes that were not modified by 5-HD. Values are means ± s.e.m. *$P<0.05$ compared to the control.

sham-operated non-ischaemic animals ($n=4$), nitrotyrosine formation was significantly increased in dogs subjected to a 25 min coronary artery occlusion and subsequent reperfusion. This formation was, however, markedly reduced in dogs in which peroxynitrite was infused before occlusion. This suppression in nitrotyrosine formation was not influenced by the 150 μg kg^{-1} min^{-1} infusion of 5-HD.

Area at risk

There were no significant differences in the area at risk between those five groups where this was measured. These were 34.3 ± 1.4% ($n=5$) in the controls, 35.2 ± 2.2% ($n=3$) in the controls given 5-HD, 34.6 ± 1.2% ($n=3$) in the peroxynitrite group, 34.2 ± 1.3% ($n=3$) in the 5-HD (150 μg kg^{-1} min^{-1}) + peroxynitrite group, 33.8 ± 2.6% ($n=3$) in the peroxynitrite-treated dogs also given 5-HD in a dose of 300 μg kg^{-1} min^{-1}.

Discussion

As we have previously described, in anaesthetized dogs the severity of ventricular arrhythmias that arise from the

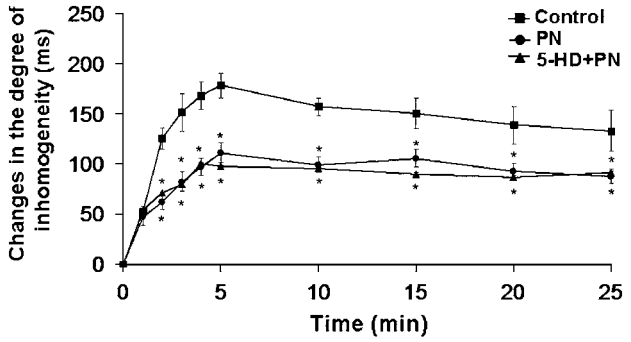

Figure 5 Changes in the degree of inhomogeneity of electrical activation during a 25 min period of LAD occlusion. Peroxynitrite markedly reduced these changes whether or not 5-HD had been administered. Values are means ± s.e.m. *$P<0.05$ compared to the control.

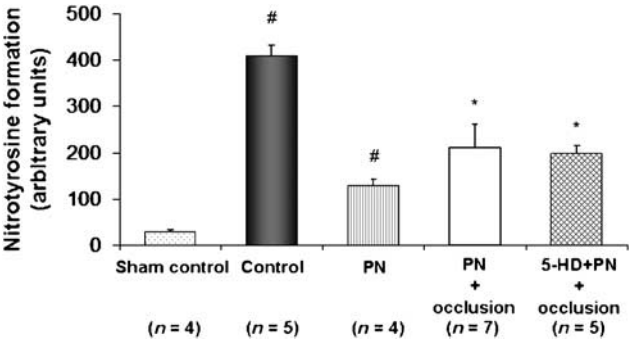

Figure 6 Nitrotyrosine (NT) formation (in arbitrary units) determined 1–2 min after the reperfusion of the myocardium from a 25 min ischaemia. Compared with the sham-operated controls, NT formation was significantly increased in dogs that were simply subjected to coronary artery occlusion and following reperfusion. Two brief periods of peroxynitrite (PN) infusion markedly suppressed the occlusion and reperfusion-induced NT production, which was not modified by 5-HD. Values are means ± s.e.m. *$P<0.05$ compared to the control, $^{\#}P<0.05$ compared to the sham control.

occlusion and subsequent reperfusion of a main coronary artery is much reduced when this is preceded by one or two brief occlusions (preconditioning) of that same coronary artery (Végh *et al*., 1992a). We have now demonstrated that, in this model, a similar protection against ischaemia- and reperfusion-induced arrhythmias was achieved by brief periods of a low concentration (100 nM) of peroxynitrite infusion, administered directly into the coronary circulation. This result is in agreement with the previous findings of Altug *et al*. (2000); a short (3 min) period peroxynitrite administration in rat-isolated heart results in reductions in occlusion-induced arrhythmias similar to that observed with the same period of preconditioning ischaemia. Nevertheless, there are good reasons for believing that our results provide the first evidence that peroxynitrite protects against ischaemia and reperfusion-induced arrhythmias *in vivo*. Furthermore, our results clearly show that the severity of ischaemia that we usually assess from changes in epicardial ST-segment and in the degree of inhomogeneity of electrical activation within the ischaemic area (Végh *et al*., 1987, 1992a), was also much reduced by peroxynitrite. It has been argued that the less pronounced reduction in ischaemia severity following peroxynitrite administration is the cause of the less severe arrhythmias during the ischaemic period. As we have described previously (Parratt *et al*., 1996), the degree of ischaemia is only one of several factors that influence arrhythmia severity after coronary artery occlusion, and the precise relationship between ischaemia severity, as recorded in these ways and ventricular arrhythmias during occlusion is not well defined. Other possibilities include the size of the ischaemic area, the degree of collateral circulation (which is variable in this species), heart rate and the activity of the autonomic nervous system (reviewed, for example, by Wit and Janse, 1993).

One other finding of interest of the present study is that nitrotyrosine formation, a marker of peroxynitrite generation (Wang and Zweier, 1996; Csonka *et al*., 2001; Ferdinándy and Schulz, 2003), was significantly reduced in dogs given peroxynitrite, compared with the untreated controls. This is in accordance with the findings of Csonka *et al*. (2001) who showed, albeit in a different model, that the generation of peroxynitrite following a prolonged period of ischaemia and reperfusion was attenuated by ischaemic preconditioning. Although we do not know the precise mechanisms by which both the exogenously administered peroxynitrite and the preconditioning ischaemia are able to reduce the formation of peroxynitrite following prolonged ischaemia and reperfusion, it is almost certain that this should result from a reduced production of either NO or superoxide. Although in the present study neither ROS nor NO generation was measured, our preliminary results suggest (unpublished data) that the administration of peroxynitrite attenuates superoxide production that results from a prolonged period of ischaemia and reperfusion. Recently, cross talk between NO and superoxide generating systems has been proposed to be important in the regulation of NO and ROS formation under both physiological and pathophysiological conditions (Saavedra *et al*., 2002; Kinugawa *et al*., 2005; Ichinose *et al*., 2007). For example, Saavedra *et al*. (2002) found that, under certain circumstances, NO, derived from NOS (eNOS, nNOS), inhibits xanthine oxidase, reduces ROS generation and, thereby, improves myocardial function and survival. Similarly, peroxynitrite formed in biological systems can feed back and downregulate xanthine oxidase activity and superoxide production, thus limiting further peroxynitrite formation (Lee *et al*., 2000). Presumably, the activation of such feedback mechanisms might explain the reduced peroxynitrite production observed in the present study following peroxynitrite administration.

One other potential mechanism that may account for the protective effect of peroxynitrite is the formation of *S*-nitrosothiols (Wu *et al*., 1994). Peroxynitrite *S*-nitrosylates glutathione and other thiol-containing substances and forms *S*-nitrosothiols (Wu *et al*., 1994), which may then serve as NO reservoirs and release NO over a prolonged period of time (Wu *et al*., 1994; Moro *et al*., 1995; Hallström *et al*., 2008). More recent evidence suggests that similar *S*-nitrosylation may occur after ischaemic preconditioning and the administration of NO donors (Sun *et al*., 2007). In our previous studies NO donors, such as nicorandil (Végh *et al*., 1996) and isosorbide mononitrate (György *et al*., 2000), similar to

preconditioning (Végh *et al*., 1992a), were protective against ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs. Thus, it is likely, that there is a common pathway in the cardioprotective (antiarrhythmic) effects of peroxynitrite, preconditioning and NO donors, which can somehow be connected to *S*-nitrosylation and the subsequent sustained release of NO. This NO may regulate several pathways that can be connected to cardioprotection. For example, NO through the activation of protein kinase G opens mitoK_{ATP} channels that are thought to have a major function in the preconditioning-induced protection (Vanden Hoek *et al*., 1998; Pain *et al*., 2000; Oldenburg *et al*., 2002). Indeed, in our own studies closing these channels by 5-hydroxydecanote before the preconditioning procedure completely abolished the antiarrhythmic effect of preconditioning (Végh and Parratt, 2002). Thus, the other purpose of the present study was to examine whether mitoK_{ATP} channels have any part in the antiarrhythmic effects of peroxynitrite. The results clearly show that 5-HD given in a dose of $150\,\mu g\,kg^{-1}\,min^{-1}$, which had previously abolished the antiarrhythmic effect of preconditioning, failed to modify the antiarrhythmic effect of peroxynitrite. The incidences of VT and VF and the distribution of VPBs were similar in the peroxynitrite-treated dogs, irrespective of whether 5-HD had been given or not. Similarly, the marked reductions in ischaemia severity and in nitrotyrosine formation following peroxynitrite administration were not influenced by 5-HD. Thus, we concluded that the activation of mitoK_{ATP} channels are most probably not involved in the peroxynitrite-induced protection. This conclusion was drawn, bearing in mind the fact, that the pharmacological modulators of these channels, such as diazoxide and 5-HD, are not specific and selective for these channels; they may have other targets in the heart (Hanley *et al*., 2002; Lim *et al*., 2002). Whether these other effects of 5-HD would explain the failure of this drug to modify the protective effects of peroxynitrite we do not know; some earlier studies showed that 5-HD, acting on sarcolemmal K_{ATP} channels, may even reduce arrhythmias (Niho *et al*., 1987).

An alternative pathway by which NO, derived from peroxynitrite, may result in antiarrhythmic protection is the direct inhibitory effect of NO and peroxynitrite on mitochondrial electron transport chain (ETC; Poderoso *et al*., 1996; Xu *et al*., 2004; Guidarelli *et al*., 2007). Furthermore, as we have proposed previously for the antiarrhythmic effects of ischaemic preconditioning, NO may also activate guanylyl cyclase and thus elevate cGMP levels (Végh *et al*., 1992b). This cGMP could influence arrhythmogenesis and ischaemia severity in a number of ways, including inhibition of calcium influx through L-type calcium channels (Sun *et al*., 2007), stimulation of a cGMP-dependent cAMP phosphodiesterase and the subsequent decrease in myocardial cAMP levels, etc. (Végh and Parratt, 1996). There is also evidence that NO inhibits noradrenaline release from the nerve endings (Addicks *et al*., 1994: Schwartz *et al*., 1995) and enhances, acting on presynaptic muscarinic receptors and through a cGMP-dependent pathway, the effect of vagal nerve stimulation (Schwartz *et al*., 1995; Sears *et al*., 1999). More recently Brack *et al*. (2007) showed that the antifibrillatory effect of vagus nerve stimulation is mediated by NO, involving mechanisms that modify the restitution of action potential duration. This result suggests that NO, by stimulating signalling pathways, would affect ionic channels (Brack *et al*., 2007) and also on gap junctions (Gönczi *et al*., 2008), thus influencing directly the arrhythmogenesis. Thus, the explanation for the antiarrhythmic effect of peroxynitrite might be as simple as the stimulation of the guanylyl cyclase-cGMP pathway by NO derived from *S*-nitrosothiols, although a direct stimulatory effects of both *S*-nitrosothiols and peroxynitrite on guanylyl cyclase cannot be ruled out (Tarpey *et al*., 1995).

The present studies suggest that the administration of peroxynitrite in anaesthetized dogs markedly suppresses arrhythmia severity during a prolonged period of ischaemia and reperfusion. This antiarrhythmic effect is most probably associated with a reduced generation of peroxynitrite resulting from a prolonged period of ischaemia and reperfusion. Furthermore, we think that the protective effects of peroxynitrite are mainly on account of the effects of NO, derived from peroxynitrite, and it seems unlikely that mitoK_{ATP} channels are involved.

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Conflict of interest

The authors state no conflict of interest.

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