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RESEARCH PAPER

Modulation of gap junctions by nitric oxide contributes to the anti-arrhythmic effect of sodium nitroprusside?

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Background and purpose: Nitric oxide (NO) donors provide a preconditioning-like anti-arrhythmic protection in the anaesthetized dog. As NO may modulate gap junction (GJ) function, the present study investigated whether this anti-arrhythmic effect is due to a modification of GJs by NO, derived from the NO donor sodium nitroprusside (SNP).
Experimental approach: In chloralose-urethane-anaesthetized, open-chest dogs, either saline (controls; $n = 11$) or SNP (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$; $n = 10$) was infused at a rate of 0.5 $mL \cdot min^{-1}$ by the intracoronary route. The infusions were started 20 min prior to and maintained throughout the entire 60 min occlusion period of the left anterior descending coronary artery. The severity of ischaemia and of arrhythmias, tissue electrical impedance and permeability, as well as the phosphorylation of connexin43, were assessed.
Key results: Compared with the controls, SNP infusion markedly suppressed the total number of ventricular premature beats (666 ± 202 vs. 49 ± 18; $P < 0.05$), and the number of ventricular tachycardiac episodes (8.1 ± 2.3 vs. 0.2 ± 0.1; $P < 0.05$) without significantly modifying the incidence of ventricular tachycardia or ventricular fibrillation. The severity of ischaemia (epicardial ST-segment changes, inhomogeneity of electrical activation) and tissue electrical impedance changes were significantly less in the SNP-treated dogs. SNP improved GJ permeability and preserved the phosphorylated form of connexin43.
Conclusion and implications: The anti-arrhythmic protection resulting from SNP infusion in the anaesthethized dog may, in part, be associated with the modification of gap junctional function by NO.
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Keywords: NO donors; sodium nitroprusside; arrhythmias; gap junction; myocardial ischaemia

Abbreviations: CBF, coronary blood flow; Cx43, connexin43; GJ, gap junction; HR, heart rate; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; LY, lucifer yellow; PC, preconditioning; SNP, sodium nitroprusside; TD, TRITC-dextran; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia

Introduction

There is increasing evidence that gap junctions (GJs), which provide a tight intercellular connection and thereby a rapid electrical and metabolic communication between the adjacent cells, play an important role in the generation of the early ventricular arrhythmias that result from acute myocardial ischaemia (Smith *et al.*, 1995; Kléber and Rudy, 2004). In particular the 1b phase of these arrhythmias, which often terminates in ventricular fibrillation (VF) and thus is responsible for sudden cardiac death, is thought to result, in part, from the uncoupling of GJs (Smith *et al.*, 1995). We have previous evidence that these severe ventricular arrhythmias are markedly suppressed by ischaemic preconditioning (PC) (Végh *et al.*, 1992a) and, more recently, we also demonstrated that this anti-arrhythmic effect can be associated with the modification of GJs by the PC ischaemia (Papp *et al.*, 2007).

Several factors have been proposed to modulate the opening and closing of GJs (see Dhein, 1998). Thus, metabolic and ionic changes, such as the loss of ATP, a rise in intracellular calcium and protons, are considered as the main physiological stimuli to result in closure of GJs during ischaemia (White *et al.*, 1990). Similarly, pharmacological interventions, via the activation of various intracellular signalling pathways, are able to modify GJ function (Dhein, 1998). For example, we showed in a recent study that carbenoxolone, a relatively selective uncoupler of GJs, given prior to ischaemia in anaesthetized dogs, resulted in a PC-like anti-arrhythmic protection

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(Papp *et al.*, 2007). This protection was, however, markedly attenuated when carbenoxolone was infused during the PC ischaemia, suggesting that the closure of GJs with carbenoxolone perhaps inhibits the transfer of those endogenous protective mediators that are necessary for triggering the protection.

A number of endogenous substances have been suggested as being involved in the cardioprotective effects of ischaemic PC (Parratt, 1993) and one of these is nitric oxide (NO). The evidence that NO modulated arrhythmia severity came from those canine studies in which we demonstrated: (i) that L-NAME, an inhibitor of the L-arginine-nitric oxide pathway, given either prior to PC or the prolonged occlusion, markedly attenuated the anti-arrhythmic protection (Végh *et al.*, 1992c); and (ii) that molecules that are able to donate NO, such as nicorandil (Végh *et al.*, 1996) or isosorbide-2-mononitrate (György *et al.*, 2000), induced a PC-like early protection against the ischaemia and reperfusion-induced ventricular arrhythmias.

There is some evidence, derived mainly from studies in non-cardiac tissues, that NO may modulate GJ function either by directly modifying the expression of connexin isoforms (Hoffmann *et al.*, 2003) or by stimulating the soluble guanylyl cyclase-cGMP pathway and activating protein kinase G (Patel *et al.*, 2006). This then phosphorylates connexins and thus influences the coupling of GJs, although it is still controversial whether the phosphorylation of these proteins results in opening or closing of these channels (Lampe and Lau, 2004).

The activation of the guanylyl cyclase-cGMP pathway by NO has been proposed to be involved in the anti-arrhythmic effect of PC (Végh *et al.*, 1992b) and of NO donors (Rakhit *et al.*, 2000). However, it is not known whether this anti-arrhythmic effect can be attributed to the modulation of GJs by NO. Thus, in the present study we infused sodium nitroprusside (SNP) locally into a small branch of the left anterior descending coronary artery (LAD) just prior to and throughout a 60 min occlusion of that same artery in anaesthetized dogs. The number and incidence of ventricular arrhythmias were assessed and compared with changes in tissue impedance, an indirect measure of the intercellular electrical coupling. Ischaemia severity was evaluated by the measurement of changes in epicardial ST-segment and in the degree of inhomogeneity of electrical activation. At the end of the experiments, tissue samples were taken for the determination of gap junctional permeability and of the phosphorylation status of connexin43 (Cx43), a major GJ protein. The results show that SNP protects against ischaemia-induced ventricular arrhythmias and this protection can be associated, at least in part, with a preserved electrical and metabolic coupling of cells within the ischaemic area.

A preliminary account of these results was presented at the European Congress of the International Society for Heart Research (ISHR), in Athens in May 2008 (Gönczi *et al.*, 2008).

Methods

In vivo *studies in anaesthetized dogs*

All animal procedures were in accord with the Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals, which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

The studies here were similar to those already described in detail elsewhere (Végh *et al.*, 1992a; Papp *et al.*, 2007). In brief, adult mongrel dogs of both sexes with a mean body weight of 20 ± 3 kg were used. Under light pentobarbitone anaesthesia (30 $mg \cdot kg^{-1}$ i.v.), the right femoral vein was catheterized through which a mixture of α-cloralose and urethane (60 and 200 $mg \cdot kg^{-1}$ i.v. respectively) was administered to maintain anaesthesia. After intubation, the dogs were ventilated with room air using a Harvard Respirator (USA) at a rate and volume sufficient to maintain arterial blood gases and pH within normal limits (Végh *et al.*, 1992a). Temperature was recorded from the mid-oesophagus and maintained at 37 ± 0.5°C by means of a heating pad.

Polyethylene catheters were inserted into the left femoral artery for monitoring blood pressure, and into the cavity of the left ventricle, through the left carotid artery, for the measurement of left ventricular systolic pressure (LVSP) and left ventricular end-diastolic (LVEDP) pressure, as well as changes in positive and negative dP/dt_{max}.

The animals were thoracotomized at the fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. Distal to the proposed occlusion site, a small side branch of the LAD was catheterized for the local intracoronary administration of SNP or saline. Epicardial ST-segment changes and the degree of inhomogeneity of electrical activation (expressed as the greatest delay in activation in ms) were measured from the left ventricular wall distal to the occlusion site using a composite electrode described previously (Végh *et al.*, 1987; 1992a). In some dogs, coronary blood flow (CBF) changes were also determined. In these animals, a Doppler flow probe (diameter 2.0 mm; Triton Technologies, USA) was positioned around the coronary artery, proximal to the first main diagonal branch of the LAD, to measure CBF velocity ($cm \cdot s^{-1}$). The circumflex branch of the left coronary artery (LCX) was also dissected free to allow the measurement of CBF ($mL \cdot min^{-1}$) by means of a 2.0 mm electromagnetic flow probe attached to a Statham SP2202 flowmeter. All these parameters, together with a chest II lead electrocardiogram were monitored with a Plugsys Haemodynamic Apparatus (Hugo Sachs Electronics, Germany), and recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany).

Ventricular arrhythmias were assessed as previously described (Végh *et al.*, 1992a). Thus, the number of ventricular premature beats (VPBs), the incidence and number of episodes of ventricular tachycardia [VT; defined as a run of four or more consecutive VPBs at a rate faster than the resting heart rate (HR)], and the incidence of VF during coronary artery occlusion were evaluated.

Measurement of gap junctional electrical coupling

This was similar to that described in detail previously (Papp *et al.*, 2007). In brief, four stainless steel electrodes, mounted on a non-conductive panel, were calibrated in saline (0.9%, resistivity: 71 Ω-cm), and were inserted into the left ventricu-

lar wall within the proposed ischaemic myocardial region. A subthreshold alternating current (10 μA, 8 kHz) was applied through the outer pair of electrodes, and voltage was measured between the inner electrode pair using a lock-in amplifier (SR830 DSP; Stanford Research Systems, CA, USA). A current frequency of 8 kHz was selected in order to detect maximal changes in phase angle without impairing the assessment of resistivity (Padilla *et al.*, 2003). Changes in resistivity (in Ohm-cm) and in phase angle (in °) were recorded by a computer with an acquisition time of 4 s and plotted at 1 min intervals. To eliminate small oscillations, resulting from ventilation, five consecutive 4 s measures were averaged at each minute.

Determination of gap junctional metabolic coupling

This was evaluated by the measurement of tissue permeability using the 'double-dye loading' method as described previously (Papp *et al.*, 2007). Freshly excised transmural tissue blocks from both the ischaemic and non-ischaemic ventricular walls were submerged in a mixture of lucifer yellow (LY, 1.5 mg·mL^{-1}) and TRITC-dextran (TD, 3.5 mg·mL^{-1}; both dyes purchased from Sigma, St. Louis, MO, USA) for 15 min. Following fixation in paraformaldehyde (4%; at pH 7.4), cryosections (25 μm) were prepared at –20°C from the midmyocardial layer of the tissue blocks and 10 pairs of fluorescent images were taken from each sample with a CCD camera connected to an Olympus IX71 fluorescent microscope (Olympus, Tokyo, Japan). The ratio of LY and TD stained areas was calculated using the ImageJ software. GJ permeability within the ischaemic area was expressed as a percentage of permeability measured within the non-ischaemic wall region.

Determination of Cx43 phosphorylation by Western blot

This was also described in detail elsewhere (Papp *et al.*, 2007). In brief, freshly excised tissue samples were immersed in liquid nitrogen and stored at –70°C. Membrane protein fraction was prepared, and the protein concentration was determined by the method of Lowry. From each sample 30 μg protein was separated on 12% polyacrylamide gels, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blots were blocked with 5% non-fat milk, dissolved in TTBS for 1 h, and labelled overnight with a rabbit polyclonal anti-Cx43 antibody (Zymed Laboratories INC, San Francisco, CA, USA) diluted to 1:2000, and then incubated for 1 h with HRP-conjugated anti-rabbit goat secondary antibody (Santa Cruz Biotech Inc., Santa Cruz, CA, USA) diluted to 1:8000 at room temperature. Blots were developed with the ECL Plus kit (Amersham Biosciences, Piscataway, NJ, USA) and scanned with a Typhoon laser scanner (Amersham Biosciences, Piscataway, NJ, USA). Band intensities were determined by the Image Quant software (version 5.2, Molecular Dynamics), and the relative amount of phosphorylated and dephosphorylated Cx43 isoforms were expressed as a percentage of the total sarcolemmal connexin content.

Experimental protocol

Two groups of dogs were used. The animals were randomly assigned to control or treated group by providing an equal distribution of men and women in each group. In all groups a 30 min recovery period was allowed to stabilize after surgery. Eleven dogs served as controls. In these dogs saline was infused into the side branch of the LAD distal to the occlusion site at a rate of 0.5 mL·min^{-1} 20 min prior to and throughout the 60 min occlusion period. In other 10 dogs, the NO donor SNP (Sigma, St. Louis, MO, USA) was infused in a dose of 0.2 μg·kg^{-1}·min^{-1} by the same intracoronary route both prior to and over the entire occlusion period. For dose finding we performed some additional experiments. We have also considered our previous studies with nicorandil and isosorbide-2-mononitrate and tried to select a dose of SNP, which produced a similar reduction (less than 10 mm Hg) in arterial blood pressure. The syringes containing SNP solution were covered with foil to avoid photodegradation of SNP. In additional eight dogs in which we determined blood flow changes, four dogs were infused with SNP and the other four with saline. At the end of the experiments the animals were given an overdose of the anaesthetic, and the hearts were rapidly excised and placed in ice cold saline. Tissue samples were taken from both the ischaemic and non-ischaemic regions of the left ventricular wall for further analyses. 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 values are expressed as means ± SEM and the differences between means were compared by Student's *t*-test and with ANOVA for repeated measures using Fisher's *post hoc* test as appropriate. VPBs and the number of tachycardiac episodes were compared using the Mann–Whitney *U*-test and were presented as mean ± SEM for the sake of simplicity. For comparison of incidences of VT and VF, the Fisher exact probability test was used. Differences between groups were considered significant at $P < 0.05$.

Results

Haemodynamic effects of intracoronary SNP and of the coronary artery occlusion

These are summarized in Table 1. There were no significant differences between groups in the haemodynamic parameters at baseline. This dose of SNP given in local intracoronary infusion produced slight decreases in arterial blood pressure, LVEDP and in positive dP/dt_{max}. When the LAD was occluded in the control dogs, there was a significant decrease in arterial blood pressure, an increase in LVEDP and reductions in both positive and negative dP/dt_{max}. These changes were not significantly modified by the administration of SNP. The HR remained unchanged in both groups. The infusion of SNP did not substantially affect the diastolic CBFs, measured in both the LAD and the LCX, and as the diastolic perfusion pressure was only slightly decreased, these represent no significant changes in coronary vascular resistance. The compensatory blood flow changes that occur on the LCX when the adjacent LAD branch is occluded were also not modified by the infusion of SNP (Table 1).

Table 1 Haemodynamic changes following saline and SNP infusions as well as coronary artery occlusion

Haemodynamic parameters	*Control*				*SNP*			
	Baseline values	*Saline max. change*	*Pre-occlusion values*	*Occlusion max. change*	*Baseline values*	*SNP max. change*	*Pre-occlusion values*	*Occlusion max. change*
SABP	146 ± 8	4 ± 2	146 ± 8	–15 ± 2*	149 ± 6	–4 ± 1*	147 ± 6	–10 ± 2*
DABP	103 ± 6	3 ± 2	96 ± 6	–10 ± 2*	105 ± 6	–4 ± 1*	104 ± 5	–7 ± 2*
MABP	118 ± 7	3 ± 2	112 ± 6	–10 ± 2*	120 ± 6	–5 ± 1*	119 ± 6	–9 ± 2*
LVSP	137 ± 9	0 ± 4	128 ± 8	–12 ± 4*	136 ± 7	–3 ± 1	136 ± 7	–13 ± 3*
LVEDP	9 ± 1	–1 ± 0	8 ± 0	6 ± 1*	9 ± 0	–1 ± 0*	8 ± 0	6 ± 1*
$+dP/dt_{max}$	2719 ± 258	–11 ± 82	3084 ± 263	–416 ± 177*	2700 ± 290	–192 ± 95*	2673 ± 253	–394 ± 200*
$-dP/dt_{max}$	2894 ± 271	111 ± 153	2630 ± 210	–436 ± 94*	2904 ± 183	–158 ± 69	2956 ± 207	–695 ± 143*
HR	167 ± 6	1 ± 2	158 ± 6	4 ± 2	165 ± 6	3 ± 4	165 ± 5	–1 ± 4
LAD flow[a]	21 ± 1	1 ± 2	22 ± 1	0	18 ± 0	2 ± 1	19 ± 1	0
LAD resistance[a]	4.07 ± 0.51	–0.02 ± 0.05	4.08 ± 0.49	0	4.47 ± 0.12	–0.56 ± 0.15	4.18 ± 0.23	0
LCX flow[a]	70 ± 3	0	70 ± 4	19 ± 6	71 ± 6	3 ± 2	71 ± 6	16 ± 4
LCX resistance[a]	1.22 ± 0.16	0	1.24 ± 0.13	–0.34 ± 0.10	1.15 ± 0.14	–0.09 ± 0.02	1.14 ± 0.14	–0.27 ± 0.17

Values are means ± SEM.
[a]$n = 4$ per group.
*$P < 0.05$ compared with baseline values or with pre-occlusion values.
DABP, diastolic arterial blood pressure (mm Hg); HR, heart rate (beat·min^{-1}); $+dP/dt_{max}$ (mm Hg·s^{-1}); $-dP/dt_{max}$ (mm Hg·s^{-1}); LAD, left anterior descending coronary artery; LAD flow (cm·s^{-1}); LAD resistance (mm Hg cm·s^{-1}); LCX, left circumflex coronary artery; LCX flow (mL·min^{-1}); LCX resistance (mm Hg mL·min^{-1}); LVEDP, left ventricular end-diastolic pressure (mm Hg); LVSP, left ventricular systolic pressure (mm Hg); MABP, mean arterial blood pressure (mm Hg); SABP, systolic arterial blood pressure (mm Hg).

Distribution of VPBs and changes in tissue impedance during a 60 min occlusion of the LAD

In control dogs subjected to a 60 min period of coronary artery occlusion, there was an immediate rise in tissue resistivity and a decline in phase angle accompanied by an increased number of VPBs (Figure 1). These early rapid changes lasted up to around 8 min of the ischaemia (phase 1a), after which the ectopic activity spontaneously decreased and changes in tissue impedance substantially slowed. Then, around 15 min of the occlusion a second steeper rise in resistivity and decline in phase angle occurred and these changes just preceded the appearance of phase 1b arrhythmias. This arrhythmia peak lasted up to 25 min of the occlusion; thereafter, the number of VPBs was markedly reduced, but the tissue impedance changes (increase of resistivity and decrease of phase angle) were continued over the rest of the ischaemia. Although the intracoronary infusion of SNP itself did not produce any significant change in tissue impedance (data not shown), it markedly attenuated the rise of resistivity and almost completely abolished the ectopic activity during coronary artery occlusion. Although the phase angle changes were not significantly modified by SNP (there was even a somewhat more marked reduction in this parameter during the first 10 min of the occlusion than in the controls; a phenomenon that deserves further consideration), it attenuated those rapid phase angle changes that occurred in the controls during the critical phase of the ischaemia. Thus, in the presence of SNP, the phase angle changes remained virtually constant between 15 and 20 min of the occlusion. After this period, the phase angle again declined and did not differ significantly from the controls.

Effects of SNP on the severity of ischaemia-induced ventricular arrhythmias

These are summarized in Figure 2. In control dogs the total number of VPBs was 550 ± 207 during the first 25 min of the occlusion, and this was only slightly increased by the end of the 60 min occlusion (666 ± 202). Local intracoronary infusion of SNP markedly suppressed the ectopic activity both during the first 25 min (30 ± 15) and also the rest (49 ± 18) of the occlusion. Similarly, the high number of VT episodes that occurred in 55% of control dogs was substantially reduced in the SNP-treated dogs; only two animals in this group exhibited short episodes of VT. The infusion of SNP, however, failed to modify the incidence of VF. In both groups, two dogs fibrillated during occlusion.

Changes in epicardial ST-segment and in the degree of inhomogeneity of electrical activation during a 60 min occlusion of the LAD

The changes observed in these two indices of ischaemia severity are shown in Figure 3. In control dogs coronary artery occlusion resulted in immediate and significant elevation in epicardial ST-segment (A) and a marked increase in the degree of inhomogeneity of electrical activation (B) within the myocardial region supplied by the occluded vessel. The local intracoronary infusion of SNP significantly reduced these parameters over the entire occlusion period.

Changes in GJ permeability

Occlusion of the LAD for 60 min in control dogs reduced GJ permeability within the ischaemic area to 65 ± 3% of the non-ischaemic value (100 ± 7%). In dogs infused with SNP, this reduction was much less (only 5%); the permeability within the occluded area was 95 ± 5% of the non-ischaemic value, indicating a preserved metabolic coupling of GJs (65 ± 3% vs. 95 ± 5%; $P < 0.05$).

Changes in the phosphorylation status of Cx43 following coronary artery occlusion

These are illustrated on a representative Western blot (Figure 4A) and summarized in Figure 4B. In samples taken

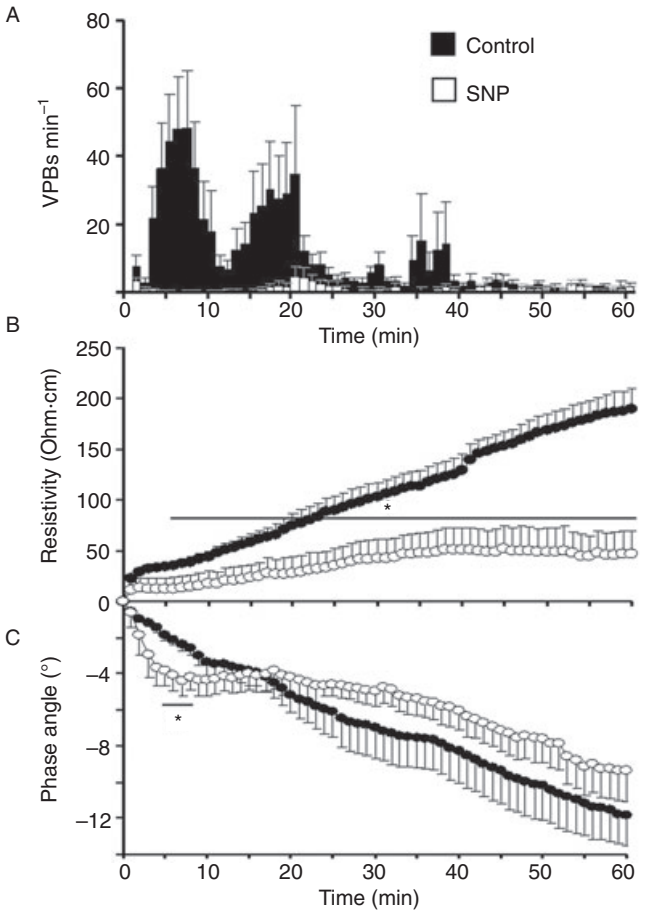

Figure 1 Distribution of VPBs and relative changes in tissue impedance (resistivity and phase angle) at 1 min intervals during a 60 min LAD occlusion in control dogs and in dogs infused with SNP. Compared with the controls, the infusion of SNP markedly reduced the number of VPBs (A) and attenuated the rise in tissue resistivity (B) over the entire occlusion period. SNP, however, did not substantially modify the decline in phase angle, although during the critical period of ischaemia (between 15 and 25 min), the change of phase angle remained virtually constant. Values are means ± SEM obtained from 5 to 10 dogs. *$P < 0.05$ compared with the controls. LAD, left anterior descending coronary artery; SNP, sodium nitroprusside; VPB, ventricular premature beat.

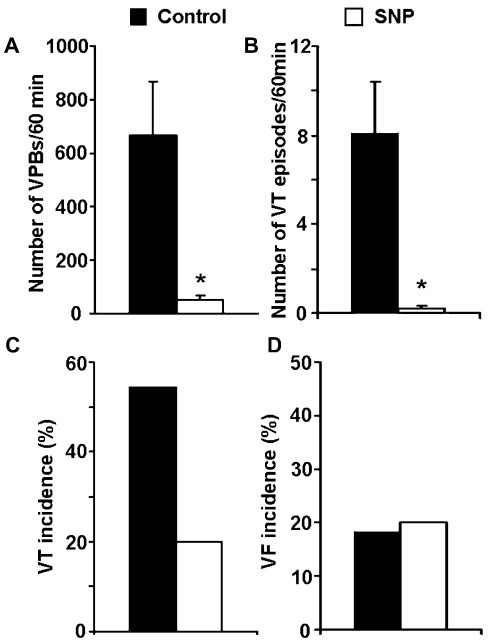

Figure 2 Arrhythmia events (numbers of VPBs and episodes of VT, incidences of VT and VF) occurring during a 60 min occlusion of the LAD. Compared with the controls, the infusion of SNP markedly reduced the total number of VPBs (A), the number of VT episodes (B), but it failed to significantly modify the incidence of VT (C) or of VF (D). Values are means ± SEM. *$P < 0.05$ compared with the controls. LAD, left anterior descending coronary artery; SNP, sodium nitroprusside; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia.

from the non-ischaemic area, the amount of the phosphorylated and dephosphorylated forms of Cx43 are almost equal, as represented by two distinct bands at ~41 kDa (dephosphorylated Cx43) and at 45 kDa (phosphorylated Cx43) on the Western blot (Figure 4A). This phospho/dephospho Cx43 ratio (53%/47%) was shifted to around 28%/72% in control dogs by the end of the coronary artery occlusion, indicating that the membrane fraction of Cx43 protein was mostly dephosphorylated (Figure 4B). Infusion of SNP preserved the phosphorylated form of Cx43 even after the 60 min of ischaemia both within the normal and the ischaemic region; thus, the phospho/dephospho ratio was 58%/42% and 62%/38% respectively (Figure 4B).

Discussion

We have recently demonstrated that in anaesthetized dogs, ischaemic PC markedly reduced the severity of ventricular arrhythmias induced by a 60 min coronary artery occlusion, and this effect was associated with a preserved GJ function (Papp *et al*., 2007). This latter was assessed by the measurement of tissue electrical impedance *in situ*, and also by the *in vitro* determination of Cx43 phosphorylation and GJ permeability in tissue samples taken from the hearts. As we have previous evidence that NO plays an important role in the PC-induced anti-arrhythmic protection (Végh *et al*., 1992c), further NO donors may have a similar effect against the ischaemia and reperfusion-induced arrhythmias (Végh *et al*., 1996; György *et al*., 2000), we aimed to investigate whether these anti-arrhythmic effects can be attributed to the modification of GJ function by NO. One possible way to examine this is to provide NO using an NO donor, and assess arrhythmia severity in relation to changes in GJ function during coronary artery occlusion. Thus, in the present study we gave SNP in intracoronary infusion to anaesthetized dogs, commencing the infusion both prior to and over the entire occlusion period. The results confirm our previous findings that NO donors may provide protection against ischaemia-induced ventricular arrhythmias, but the anti-arrhythmic effect of SNP was not as marked as that following the intracoronary administration of isosorbide-2-mononitrate (György *et al*., 2000) or nicorandil (Végh *et al*., 1996). Although SNP virtually abolished ectopic activity and reduced the number of VT episodes during the entire occlusion period, it failed to inhibit the occurrence of severe arrhythmias, such as VT or VF. The effect of NO donors on ischaemia-induced arrhythmias is rather controversial. There are studies in pigs (Wain-

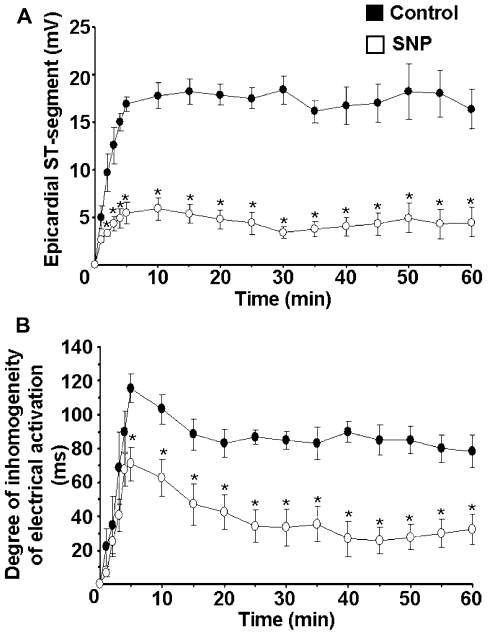

Figure 3 Changes in the epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 60 min occlusion of the LAD. In control dogs, both indices of ischaemia severity were markedly increased, especially during the initial 5 min of the occlusion. These ischaemic changes were markedly attenuated following the intracoronary infusion of SNP. Values are means ± SEM. **P* < 0.05 compared with the controls. LAD, left anterior descending coronary artery; SNP, sodium nitroprusside.

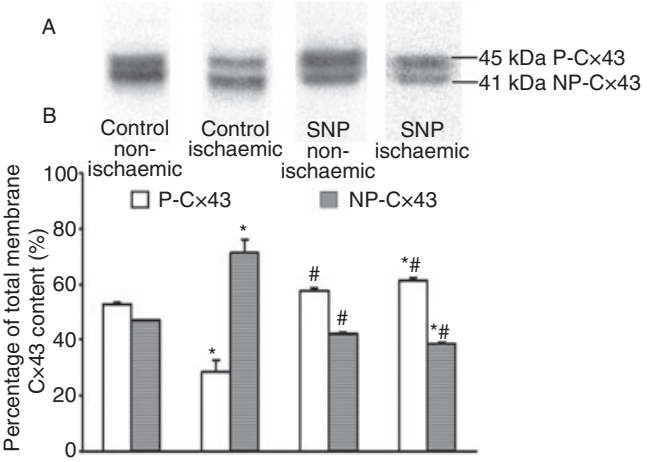

Figure 4 A representative Western blot (A), and changes in the phosphorylated (P-Cx43) and dephosphorylated Cx43 (NP-Cx43) isoforms as a percentage of the total sarcolemmal Cx43 content, following a 60 min LAD occlusion (B). The phospho/dephospho ratio within the normal area is around 53%/47%. This shifted to 28%/72% in hearts of the control dogs following a 60 min coronary artery occlusion. SNP infusion prevented this shift and preserved the phosphorylated form of this protein both within the normal non-ischaemic (58%/42%) and the ischaemic myocardial region (62%/38%). Values are means ± SEM. **P* < 0.05 compared with the non-ischaemic samples, #*P* < 0.05 compared with the control group. LAD, left anterior descending coronary artery; SNP, sodium nitroprusside.

wright and Martorana, 1993; Wainwright *et al.*, 2002) and in humans (Mihalick *et al.*, 1974; Margonato *et al.*, 1991) that support a potential anti-arrhythmic effect of NO donors during myocardial ischaemia. For example, the i.v. administration of pirsidomine (Wainwright and Martorana, 1993) and the oral administration of NO-aspirin (Wainwright *et al.*, 2002) significantly reduced the number of VPBs, but did not influence the occurrence of VF. In contrast, NO donors failed to modify arrhythmia severity in anaesthetized rats (Kane *et al.*, 1984; Barnes and Coker, 1995), although in Langendorff-perfused rat hearts both glyceryl trinitrate and SIN-1 was able to mimic the effect of ischaemic PC on reperfusion-induced ventricular arrhythmias (Bilinska *et al.*, 1996). These differences may be related to the model used, the dose and route of administration of the NO donor applied, and perhaps the ability of the molecule to donate NO (Miller and Megson, 2007). We suspect, however, that the failure to obtain a significant protection against ischaemia-induced VF can be explained, in part, by the smaller body weight (or heart) of dogs included in the present study. This, as we have pointed out previously (Végh *et al.*, 1992c; Parratt *et al.*, 1996), is an important contributory factor to arrhythmia severity. Thus, in the present study where the weight of dogs varied from 15 to 25 kg (mean body weight around 20 kg), the incidence of VF, particularly in the control group, was very low; only two dogs out of eleven dogs fibrillated during the first 25 min of the occlusion. As in the SNP-treated group also two dogs out of 10 dogs fibrillated during the same occlusion period, this index of arrhythmia severity is unlikely to be informative under these conditions. In contrast, in our previous studies where we were able to use larger dogs, usually with a mean body weight of 25 kg, the control animals exhibited a much higher incidence of VF (e.g. 82% of the controls fibrillated during coronary artery occlusion; György *et al.*, 2000). Thus, compared with those controls, the administration of isosorbide-2-mononitrate significantly reduced the incidence of VF (82% vs. 25%).

Although it remains unclear whether certain NO donors are indeed able to protect against VF, it seems likely that they markedly suppress ectopic activity during acute myocardial ischaemia. The observed anti-arrhythmic effect was mainly attributed to the favourable haemodynamic, as well as the anti-ischaemic and platelet aggregation inhibitory effects (Wainwright and Martorana, 1993; Wainwright *et al.*, 2002). The coronary vasodilator effect may also account for the beneficial anti-ischaemic and, perhaps anti-arrhythmic effects of NO donors. As neither CBFs, measured in both main branches of the left coronary artery, nor the compensatory blood flow changes, assessed in the LCX branch when the LAD was occluded, were substantially modified by the infusion of SNP, it seems unlikely that coronary vasodilatation explains the marked arrhythmia suppression. However, the reduction in ischaemia severity, as shown by a significant reduction in both epicardial ST-segment elevation and the degree of inhomogeneity of electrical activation in the SNP-treated animals (Figure 3), may be responsible for the anti-arrhytmic effect.

There is increasing evidence, coming mainly from non-cardiac tissues, that NO may modulate GJ function by influencing the permeability of GJ channels and the expression of connexin isoforms (Bolanos and Medina, 1996; Kamerisch *et al.*, 2003; Yao *et al.*, 2005). The direct modulatory role of

NO on GJs has also been developing in coronary vessel physiology; it is suggested that NO by attenuating the conduction of signals through GJs that are responsible for eliciting vasoconstriction would provide vasodilatation (Rodenwaldt *et al*., 2007). In contrast, there is much less evidence for the regulation of gap junctional function by NO in the myocardium; the signalling pathways, which regulate the level and phosphorylation status of Cx43 and thus modulate the GJ channel properties, are even less understood. It is proposed that stimulation of both the β and α_1-adrenoceptors, although through the activation of different pathways and protein kinases (PKA and PKC respectively), leads to connexin phoshorylation and to the opening of GJs (Saez *et al*., 1997; Dhein, 1998). In contrast, the activation of the guanylyl cyclase-cGMP pathway and the subsequent stimulation of PKG would result in closing of these channels (Dhein, 1998). A more recent study, however, showed that in H9c2 cells, isolated from the rat myocardium, the hypoxia-induced decrease in total Cx43 protein level was restored by acetylcholine and also by the NO donor SNAP (Zhang *et al*., 2006). As the protective effect of acetylcholine was inhibited by L-NAME, it was suggested that acetylcholine, through a NO-mediated pathway, prevents the loss of Cx43 protein during hypoxia, and thereby improves intercellular communication.

In the present study, GJ function and its modification by NO was assessed under both *in vivo* and *in vitro* conditions by measuring tissue electrical impedance and changes in GJ permeability and in connexin phosphorylation respectively. These results showed that compared with the controls, the infusion of SNP significantly attenuated the increase in tissue resistivity, but it did not substantially influence the decrease in phase angle that occurred during coronary artery occlusion. In the presence of SNP, there was indeed a more marked reduction in phase angle during the first 10 min period of the occlusion. Interestingly, this pronounced early change in impedance was not accompanied by an increase in arrhythmia severity during phase 1a (Figure 1): a phenomenon that deserves further investigation. Furthermore, in the presence of SNP infusion, the steep decline in phase angle that occurs in the controls just prior to the appearance of phase 1b arrhythmias was absent; the changes of this parameter during the critical period of ischaemia (i.e. between 15 and 20 min) remained virtually constant. These results may suggest a preserved GJ function during ischaemia following SNP administration and explain, in part, the marked reduction in arrhythmia severity. The present findings also confirm our previous supposition that the rate of uncoupling prior to phase 1b is of particular importance in the generation of arrhythmias (Papp *et al*., 2007). In addition, this is a period of ischaemia during which catecholamines are released. These are also known to contribute to the electrical instability of the heart (Schwartz and Stone, 1982). As NO may inhibit noradrenaline release (Schwartz *et al*., 1995) and facilitate the release of acetylcholine from nerve endings (Addicks *et al*., 1994; Sears *et al*., 1999), these mechanisms may also account for the anti-arrhythmic effect of SNP. Although we do not know whether opening or closing of GJs by NO leads to the anti-arrhythmic protection, the electrical impedance measurements suggest that in the presence of SNP the rapid impedance changes that precede the occurrence of phase 1b arrhythmias in control dogs were markedly attenuated. Preservation of GJ function was observed even after 60 min of ischaemia in the SNP-treated dogs, as shown by data obtained from the Cx43 phosphorylation and GJ permeability measurements. Both parameters clearly indicated that compared with the controls, in the presence of SNP, the membrane fraction of Cx43 remained in phosphorylated form and the metabolic coupling of the adjacent cells was substantially improved.

The present study provides further evidence that NO, derived from NO donors, protects the heart against the ischaemia-induced early, in particular, ectopic-type rhythm disturbances. This anti-arrhythmic protection, at least in part, can be associated with the effect of NO, or of the NO-stimulated pathways on GJs, as their function is largely preserved in the presence of SNP. Whether this protection is due to an increase in myocardial cGMP and a resultant decrease in the intracellular calcium level (Méry *et al*., 1993; Sun *et al*., 2007), which directly modulates GJs during ischaemia (White *et al*., 1990), or to some other ability of NO donors, including the preservation of endothelial function (Lefer *et al*., 1993) and the favourable haemodynamic and anti-aggregatory effects, which may also account for the anti-arrhythmic effect, warrants further investigations.

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Conflict of interest

The authors state no conflict of interest.

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