The role of gap junctions in cardioprotection against the ischaemia and reperfusion-induced severe ventricular arrhythmias

PhD Thesis

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LIST OF PUBLICATIONS

Full papers

1. Végh Á, Gönczi M, Miskolczi G, Kovács M. Regulation of gap junction by nitric oxide influence the generation of arrhythmias resulting from acute ischemia and reperfusion in vivo, *Frontiers in PHARMACOLOGY*, 4, **2013**.06. 4:76, eCollection.

2. Kovács M, Kiss A, Gönczi M, Miskolczi G, Seprényi Gy, Kaszaki J, J. Khor M, Murphy E, Végh Á. Effect of sodium nitrite on ischaemia and reperfusion-induced arrhythmias in anaesthetized: Is protein S-nitrosylation involved?, *PLOS One*, **2015**. 24**:** 10:0122243

3. Miskolczi G, Gönczi M, Kovács M, Seprényi Gy, Végh Á. Further evidence for the role of gap junctions in the delayed antiarrhythmic effect of cardiac pacing, *Canadian Journal of Physiology and Pharmacology* **2015.** 93: 545-553

4. Miskolczi G, Gönczi M, Kovács M, Végh Á. Examination of the effect of sodium nitrite on gap junction function during ischaemia and reperfusion in anaesthetized dogs, *Acta Biologica Hungarica,* **2017** 68: 34-49

Abstract for oral presentation

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Abstract for poster presentation

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Cx43	Connexin 43
DABP	Diastolic arterial blood pressure
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
MABP	Mean arterial blood pressure
$NaNO_2$	Sodium nitrite
NO	Nitric oxide
NOS	Nitric oxide synthase
PC	Preconditioning
PVDF	Polyvinylidene-fluoride
SABP	Systolic arterial blood pressure
SDS	Sodium dodecyl-sulphate
TAT	Total activation time
TRITC	Tetramethyl-rhodamine isothiocyanate
VF	Ventricular fibrillation
VPB	Ventricular premature beats
VT	Ventricular tachycardia

SUMMARY

Ventricular tachyarrhythmias are one of the main causes responsible for sudden cardiac death. The prevention and treatment of these severe arrhythmias are still remained a challenge of cardiology in the industrialized countries. Since the generation of these life-threatening ventricular arrhythmias is associated with uncoupling of gap junctions (GJs), we aimed to examine, whether the GJ channels may have a role in the delayed antiarrhythmic effect of cardiac pacing (I), and also in the early cardioprotective effect of sodium nitrite (II).

I. We have previous evidence that rapid cardiac pacing results in changes in the expression of the GJ forming protein connexion 43 (Cx43), and leads to functional and structural preservation of GJs. These effects may play a role in the delayed antiarrhythmic protection. The present study aimed to further investigate the involvement of GJs in the pacing-induced delayed antiarrhythmic effect, raising the question; whether the pharmacological inhibition of GJs prior to ischaemia would modify the pacing-induced cardioprotection. For this purpose we used carbenoxolone (CBX), a selective uncoupler of GJs, administered in two concentrations (50 and 100 μmol L^{-1}) prior to and during a 25 min occlusion of left anterior descending (LAD) coronary artery in anaesthetized dogs, subjected to cardiac pacing, 24h previously.

We have found that CBX both of the doses, attenuated the antiarrhythmic and the anti-ischaemic effects of cardiac pacing, and, in parallel, the pacing-induced reductions in tissue electrical impedance. These results confirm the role of GJs in the delayed antiarrhythmic effect of rapid cardiac pacing.

II. There is evidence that nitric oxide (NO) regulates GJ function, and that this effect may have a role in the protection induced by preconditioning or by the administration of drugs, such as NO donors. Since we have previous evidence that the inorganic sodium nitrite provides marked protection against the ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs, we wondered, whether GJs play a role in this protection.

We have found that sodium nitrite infused in 0.2 μmol kg^{-1} min^{-1} before and during occlusion or 10 min just prior to reperfusion in anaesthetized dogs, significantly reduced the severity of ventricular arrhythmias and attenuated the marked impedance changes that resulted from coronary artery occlusion. These results suggest that in the antiarrhythmic effect of sodium nitrite the modulation of GJs may play a role.

1. Introduction

One of the most serious consequences of myocardial ischaemia, resulting from sudden coronary artery occlusion is the occurrence of those severe ventricular arrhythmias that are often leading to sudden cardiac death. Protecting the myocardium from ischaemic injury is thus a major aim in the reduction of morbidity and mortality from ischaemic heart disease. During the past decades many experimental and clinical efforts had been made on to understand the pathomechaisms of these life-threatening ventricular arrhythmias, and develop new antiarrhythmic drugs and treatment strategies.

1.1. The occurrence of ventricular arrhythmias following coronary artery occlusion

Following a sudden complete occlusion of one of the main branches of a coronary artery ventricular arrhythmias occur in two distinct phases, termed as phase 1a and phase 1b [1]. These acute arrhythmias are difficult to assess under clinical conditions, since they appear soon after the commencement of the coronary artery occlusion. However, in experimental animals these arrhythmias can be easily assessed and the underlying mechanisms established. Regarding the distribution of the acute arrhythmias it has been proposed that the phase 1a arrhythmias can be detected between 2 and 8 min of the occlusion, whereas the phase 1b arrhythmias occur between the 15 and 30 min of the ischaemia [1]. These are usually more severe than the arrhythmias during phase 1a, and often terminating in sudden ventricular fibrillation [2, 3].

The underlying mechanisms of the two arrhythmia phases are also different. The rapid metabolic changes (shift of metabolism from aerobic to anaerobic mode, fall in ATP generation and pH) following the abrupt occlusion of a coronary artery, directly affect the function of ion channels and exchangers leading to dramatic alterations in impulse generation and conduction [4]. The metabolic and ionic changes (increase in intracellular Na^+ level, enhanced K^+ efflux) cause alterations in action potential morphology and duration [5,6,7,8], providing conditions for re-entry, which is thought to be the main underlying mechanism of the phase 1a arrhythmias [6,7].

The processes underlying generation of phase 1b arrhythmias are less well understood, but there is strong evidence that uncoupling of gap junctions (GJs) play an important role. These channels that provide tight metabolic and electrical coupling between cells will be closed, when a loss in ATP [9,10], a decrease in intracellular pH [11], and an increase in calcium

[12], as well as the release of catecholamines [13] due to the progression of ischaemia, occur. The increased uncoupling of GJs results in an increased tissue resistance [14] and a decreased conduction velocity in the normal, longitudinal direction [3,15]. As a result of it non-uniform anisotropy develops [16], where conduction through the cardiac tissue becomes inhomogeneous. This asynchronous conduction may initiate and maintain re-entry during phase 1b [17]. On the other hand, the increased resistance that results from the interruption of cell-to-cell connections decreases the injury current. However, at moderate levels of uncoupling this current would still be sufficient to induce delayed afterdepolarizations and trigger focal activity [18].

1.2. The structure and function of gap junctions in myocardial tissue

The GJs are specific channels, which directly connect the cytoplasm of the adjacent cells thus providing fast electrical and metabolic coupling between the cells. The GJ channels can transport molecules smaller than 1kDa, such as ATP and Ca^{2+} (metabolic coupling) between the cells, most probably by a twisting motion resulting in an opened and closed state of the channel [19,20]. In myocardial tissues, these channels are accumulated in clusters, located in the intercalated disks, and they represent low resistance pathways between the neighbouring cells, allowing fast spread of impulse from the one cell to the other (electrical coupling) [20,21]. GJs are formed by connexin protein subunits. Six connexin proteins form a connexon or half-channel, and two half-channels form a GJ (**Figure 1,** [22]).

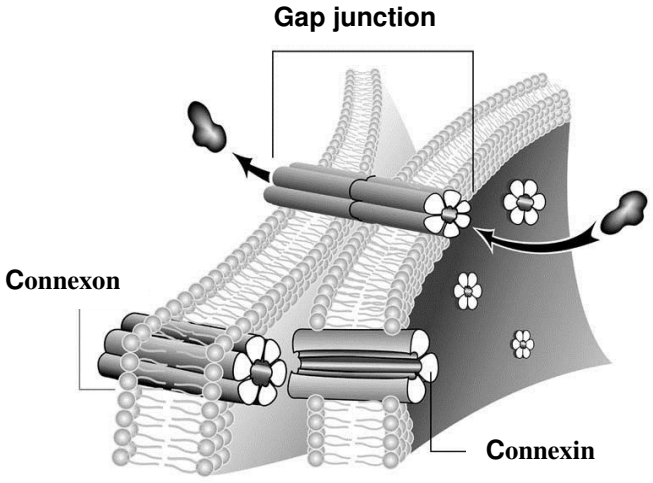

Figure 1.
The structure of gap junction [22]

In mammals, there are 21 connexin isoforms (Cx26-50); their numbering reflects the molecular weight (kDa) [23]. The variable size of connexins is due to the length of the C-terminal variable. In the myocardium, Cx40, 43 and 45 are the most frequent connexin types; Cx40 is mainly expressed in the atrium, while Cx43 is primarily found in the atrial and ventricular working (contractile) myocytes. The primary occurrence of Cx45 is in the pacemaker and cardiac conduction system (e.g. Purkinje fibre, [24]).

Since the shape of the ventricular cardiomyocytes is elongated and the GJs are preferably located in the longitudinal end of the cell, under normal conditions, the action potential is propagated in longitudinal direction [25-28]. This uniform anisotropy that mainly results from the structural arrangement (longitudinal vs. transversal) and the electrical properties of GJs (low resistance), makes possible that the heart behaves as an electrical syncytium [29,30].

Under certain pathological conditions such as heart failure [24,31,32] or ischaemic heart disease [33,34], there is a decrease in the expression of Cx43, and the GJs are re-located from the intercalated disc to the lateral cell membrane (lateralization; [35]; all these processes may contribute to increased arrhythmia generation. The opened/closed state of these channels is influenced by number of processes. For example, the Ca^{2+}-calmodulin interaction can regulate the coupling of GJs [36], or the phosphorylation state of connexin proteins is one of the main determinants of the gap junctional opening/closing [37]. A number of drugs have been described and developed in order to facilitate (e.g. rotigaptide) or inhibit (e.g. carbenoxolone) the coupling of GJ [38-40], and thereby modifying gap junctional function and the generation of arrhythmias [41].

1.3. The role of gap junctions in arrhythmia generation

The first proposal that GJs uncouple during ischaemia comes from studies of McCallister and his colleagues in 1979 [16]. Since then, several studies have proved the role of GJs in normal impulse propagation [e.g. 25], and also in arrhythmia generation associated with diseased heart, such as heart failure or ischaemic heart disease [42]. For example, under ischaemic conditions the rapid metabolic changes (loss of ATP, decrease of pH, increase in intracellular Ca^{2+} and release of catecholamines) promote the uncoupling of GJs [9-13]. These factors produce reduction in electrical conductance which prompts the development of sever ventricular arrhythmias during phase 1b [43]. In addition, ischaemia causes lateralization of Cx43 [34]. This mechanism is not yet fully understood; it has been suggested

that the ischaemia-induced dephosphorylation of Cx43 may play a role in Cx43 lateralization [44,45] and in GJ uncoupling [46,47], resulting in substrate for arrhythmia generation [45].

Although there is no doubt that GJs are implicated in both normal and diseased heart function, their exact role and importance in arrhythmia generation is difficult to assess, especially under *in vivo* conditions. One reason for this is that there are several other factors which may also influence excitability and conduction, and their contribution to the generation of phase 1b arrhythmias is difficult to separate from that of GJs. One of these factors, which inevitably influence arrhythmogenesis, is the local release of catecholamines, noradrenaline in particular, from the ischaemic nerve endings [48]. The time course of catecholamine accumulation and GJ uncoupling is similar; both start at around 12 min of the ischaemia [48], and together with the acidification [11,49], and the intracellular calcium overload [50], which have also established by this time of the occlusion, are such factors which either by themselves or via the closure of GJs [51] contribute to the generation of phase 1b arrhythmias.

The other problem for assessing the GJ function in arrhythmogenesis is the lack of the precise and direct measuring of the coupling status of GJs. The measurement of conduction velocity [52,53], GJ permeability [54,55], and the phosphorylation status of connexins [56,57] are frequently used methods to evaluate gap junctional coupling. These *in vitro* techniques, however, allow assessing the coupling status of GJs only at a certain time point.

The measurement of tissue electrical impedance represents a further approach in the assessment of GJ function. The first convincing evidence for the relationship between the increase in intracellular resistance and the uncoupling of GJs came from the study of Kléber and colleagues [14], using tissue impedance measurements. They showed in the rabbit isolated papillary muscle that during myocardial ischaemia tissue resistivity increases in a biphasic manner; it rises immediately after the onset of ischaemia that can be associated with cell swelling and oedema resulting from the collapse of the vasculature, and it rises again more steeply around the 15 min of ischaemia, which is thought to relate to the uncoupling of GJs [14]. The advantage of measuring tissue impedance is that changes in gap junctional function can be followed continuously, even under *in vivo* conditions, and allows relating GJ coupling to arrhythmia occurrence. The evidence for such a relationship came from two studies, both performed in anaesthetized pigs [2, 58]. In the first study Smith and colleagues [2] showed that the appearance of the phase 1b arrhythmias was preceded by a steep rise in resistivity, whereas in the second study Cinca and colleagues [58] reported that ischaemic PC

delayed the uncoupling of GJs and, in parallel, shifted the occurrence of the 1b phase of arrhythmias to a later period of the occlusion.

We have previous evidence that GJs plays a mandatory role in the generation of ventricular arrhythmias resulting from a 60 min coronary artery occlusion in anaesthetized dogs, and that PC not only shifts the appearance of the phase 1b arrhythmias to a later period of ischaemia, but it results in an absolute reduction in the number and severity of these acute arrhythmias [41,59]. In this study changes in tissue impedance were assessed in parallel with the distribution of arrhythmias. The results clearly showed that a steep rise in resistivity around the 15 min of ischaemia was immediately followed by an increased ectopic activity in the control ischaemic dogs, and that PC markedly reduced these resistivity changes, and, in parallel, suppressed the phase 1b arrhythmias [59]. Furthermore, the *in vitro* measurements showed that in PC dogs both GJ permeability and Cx43 phosphorylation were largely preserved even at the end of the 60 min occlusion period, indicating the involvement of GJs in the protective effect of PC [59]. Studies performed in our laboratory have also revealed that opening or closing GJs prior to ischaemia, using the GJ opener rotigaptide or the GJ inhibitor carbenoxolone, substantially influenced the generation of arrhythmias during the subsequent ischaemic insult [41]. Interestingly, in our experiments, both rotigaptide and carbenoxolone proved to be antiarrhythmic [41], providing an *in vivo* experimental support for the hypothesis, proposed first by the Amsterdam group for the role GJs in arrhythmogenesis [60]. They hypothesized that both an uncoupler and an opener may result in an antiarrhythmic effect, since the „*local, specific modulation of gap junctional uncoupling either by reducing or increasing it, may suppress severe, often lethal arrhythmias during 1b phase of myocardial ischaemia*" [61]. It was assumed that a moderate degree of uncoupling is associated with arrhythmias, whereas a more advanced uncoupling is antiarrhythmic [3,60]. Therefore, theoretically an uncoupler, selective to the ischaemic tissue, would preferentially uncouple the already poorly coupled cells thus resulting in a more marked reduction in conduction velocity in the poorly coupled than in the normally coupled tissue. On the other hand, keeping GJs coupled during ischaemia would result in an antiarrhythmic effect by maintaining conduction velocity [60].

We have also evidence that GJs also an important role in the delayed antiarrhythmic protection, induced, for example, by rapid cardiac pacing [61]. Pacing the dog hearts 24h prior to a 25 min period of I/R insult, markedly reduced the acute ischaemia-induced ventricular arrhythmias and changes in tissue impedance, preserved the structure and function

of GJs, which manifested in better metabolic and electrical coupling of cells during ischaemia, most probably through the maintenance of the phosphorylated form of Cx43 [61]. This study also showed that pacing not only results in changes in Cx43 phosphorylation, but it interferes with the connexin turnover by modifying the Cx43 gene and protein expression [61-63].

These experiments strongly suggested that cardiac pacing affect on GJ structure and function by causing a time-dependent alteration in the turnover of Cx43, the most abundant structural protein of GJs, which in turn results in preserved GJ function during the subsequent ischaemia, 24h later. A more direct evidence for the role of GJs in the delayed antiarrhythmic effect of pacing would be the use of pharmacological modulators of GJs. Hypothetically, if pacing induces delayed protection against arrhythmias through the modulation of GJs, then closing of GJs by the administration of a GJ uncoupler prior to and during ischaemia would attenuate these protective effects of pacing. Therefore, **we designed studies in which we examined whether the GJ uncoupler carbenoxolone (CBX) modifies the antiarrhythmic effect of cardiac pacing.**

In our laboratory, there is a long experience with the exploration of mechanisms, involved in the cardioprotective (antiarrhythmic) effect of PC. During the past three decades, a number of experiments showed that NO plays a key trigger and mediator role, both in the early and delayed antiarrhythmic effect of PC in anaesthetized dogs, induced either by brief coronary artery occlusions, cardiac pacing or heavy physical exercise [e.g.64-67]. We have also reported that drugs, which are able to release NO (NO donors), provide similar protection to the PC stimuli [68,69]. Furthermore, in a more recent study we have reported that sodium nitroprusside results in a marked protection against the acute ischaemia-induced ventricular arrhythmias through the modulation of GJs [70,71].

The regulatory role of NO on GJ function comes mainly from studies performed in non-cardiac tissues [72,73], in particular from those that are dealing with vessel physiology, where NO is one of the most important physiological mediators. These studies showed that NO is able to modify GJ permeability [74,75], and the expression of connexin isoforms [73,76]. Since we have evidence that changes in Cx43 expression play an important role in the delayed phase of cardioprotection induced by rapid cardiac pacing [61], and that NO has a key role in the PC-induced protection [64-67], it was obvious to hypothesize that NO might be one of the mediators, which modifies GJ function, and thereby influence arrhythmogenesis [71].

One possible ways to test this hypothesis is to increase NO bioavailability prior to or during ischaemia by giving drugs, which are able to release or donate NO. For this purpose organic nitrites and nitrates have been used for a long time to donate NO in various heart diseases [77]. However, in the past decade it has become evident that inorganic nitrite and nitrate, the natural metabolites of NO, which have been considered for a long time as inert molecules without further biological activity, may play an important physiological role in mediating the biological effects of NO [78,79]. It has been recognized that these oxidative metabolites of NO are readily reduced back to NO, especially under hypoxic conditions, when a drop in pH and oxygen tension promotes reductive processes [80]. This enzyme-independent NO formation might be of particular importance during ischaemia, when in the absence of oxygen, the NO production by (NOS) enzymes becomes limited. Thus nitrite can serve as a fundamental natural store of NO that serves to maintain cardiac function under ischaemic conditions [81,82].

We have just recently shown that the inorganic sodium, infused prior to and during ischaemia, or just prior to reperfusion in a concentration, which does not significantly modify arterial blood pressure and coronary blood flow, profoundly reduces the severity of ventricular arrhythmias that result from a 25 min coronary artery occlusion and reperfusion in anaesthetized dogs [83]. This protection was as especially marked (i.e. more than 90% of the dogs survived the combined ischaemia and reperfusion insult), when $NaNO_2$ had been administered 10 min prior to reperfusion, indicating that administration of inorganic nitrites may serve as useful therapeutic tool for protecting the heart against those severe reperfusion arrhythmias which occur during a reopening manoeuvre of a coronary vessel [84]. We proposed that in this acute effect of nitrite, the increased NO bioavailability may play a role, which is able to reduce the effect of oxidative stress by those NO-mediated mechanisms, such as protein S-nitrosylation and/or S-glutathionylation, which reversibly protect proteins from oxidation during the early ‘critical’ phase of reperfusion [85,86].

Beyond reducing the harmful consequences of oxidative stress, there are several other mechanisms by which nitrite may influence arrhythmia generation. These include, for example, the preservation of endothelial function [87] or the favourable haemodynamic (e.g. the reduction in preload [LVEDP], and consequently in myocardial stretch) and anti-aggregator abilities of NO donors. Furthermore, there is substantial evidence that NO may inhibit noradrenaline release [88], and facilitate the release of acetylcholine from nerve endings [89,90]; these mechanisms may also account for the antiarrhythmic effect of nitrates.

Since we had previous evidence that the antiarrhythmic effect of $NaNO_2$, at least in part, could be attributed to an NO-mediated direct effect on GJs [70], **we have now designed studies, in which the effects of sodium nitrite on GJ function were examined.**

2. AIMS

The purpose of this thesis was to answer the following questions, raised in two separate studies according to the enclosed publications:

I. **Are gap junctions involved in the delayed antiarrhythmic effect of cardiac pacing?**

To evaluate the possible involvement of GJs in the delayed antiarrhythmic effect of rapid cardiac pacing, we have used a pharmacological approach; i.e. whether closing of GJs prior to ischaemia with the relatively selective GJ uncoupler carbenoxolone, would modify the delayed antiarrhythmic effect of cardiac pacing.

II. **Is there a role of gap junctions in the antiarrhythmic effect of sodium nitrite?**

This part of the thesis contains the results of studies, which aimed to explore the antiarrhythmic effect and the underlying mechanisms of inorganic sodium nitrite. A part of the study has examined the role of reactive oxygen species (ROS) in the $NaNO_2$-induced protection [80], whereas, in parallel, the effect of nitrite on GJ function was also determined.

3. MATERIALS AND METHODS

3.1. Ethic statement

Adult mongrel dogs of both sexes with a mean body weight of 20 ± 1 kg were used. The origin and upkeep of the dogs 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' (ILAR1996), The protocols were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-5-2012) and by the department of Animal Health and Food Control of the Ministry of Agriculture and rural Development (authority approval number XIII/1211/2012).

3.2. Surgical preparations

3.2.1. Cardiac pacing protocol

Under light pentobarbitone anaesthesia (0.5 mg kg^{-1} of body weight, intravenously; Euthanyl® Bimeda-MTC Animal Health Inc.), which allowed the dogs to breathe spontaneously, a Millar tip catheter (F5, Millar Instruments) was inserted into the left carotid artery for measuring arterial blood pressure. Through the jugular vein, a Cordis F4 bipolar pacing electrode (Johnson & Johnson Company, USA) was introduced into the right ventricle through which the heart was paced four times for 5 min at a rate of 240 beats min^{-1}. Sham-operated dogs served as controls (SP); in these animals the electrode was introduced into the right ventricle but the dogs were not paced.

3.2.2. Coronary artery occlusion

The anaesthesia was introduced with intravenous pentobarbital (0.5 mg kg^{-1} of body weight; Euthanyl® Bimeda-MTC Animal Health Inc.). The right femoral vein was prepared and cannulated for the further administration of the anaesthetic (mixture of chloralose and urethane [60 and 200 mg kg^{-1} of body weight respectively; Sigma]). The dogs were intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus,Natick, MA, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits [64]. Body temperature was measured from the rectum, and maintained by means of a heating pad between 36.5 and 37.5°C

The right femoral artery was prepared and a polyethylene catheter was introduced to measure arterial blood pressure (systolic: SABP, diastolic: DABP, and mean: MABP). The

Millar tip catheter, which had been inserted previously into the carotid artery, was now pushed into the left ventricle to detect changes in LVSP and end LVEDP pressures, as well as in changes in positive and negative dP/dt_{max}. The depth of anaesthesia was monitored by the examination of the cornea and pain reflexes, as well as by the measurement of blood pressure, and when it was necessary, a further bolus injection of the anaesthetic was given.

A thoracotomy was performed at the left fifth intercostal space and the anterior descending branch of the LAD was prepared for occlusion just proximal to the first main diagonal branch. Myocardial ischaemia was induced by a 25 min period LAD occlusion, followed by rapid reperfusion.

3.3. Measurement of ischaemia severity

The severity of myocardial ischaemia was assessed by the measurement of changes in the degree of inhomogeneity of electrical activation and in the epicardial ST-segment, using a mapping electrode positioned within the proposed ischaemic myocardial region. Signals were collected from 31 unipolar electrodes (inter-electrode distance; 2 mm) at a frequency of 1 kHz, stored on a computer and analysed offline by creating activation and ST maps. Changes in epicardial activation were assessed as a time delay between the first and the last point activated under the electrode and expressed as the TAT (total activation time) in milliseconds. Elevations of epicardial ST-segment, recorded in each minute from the unipolar electrodes, were averaged and expressed in mV.

3.4. Measurement of gap junction electrical coupling

Assessment of GJ electrical coupling was similar to that described in detail previously [59,61]. 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 ventricular wall within the proposed ischaemic myocardial area. A sub-threshold 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). A current frequency of 8 kHz was selected in order to detect maximal changes in phase angle without impairing the assessment of resistivity. Changes in resistivity (in Ω·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, 5 consecutive 4 s measurements were averaged at each minute.

3.5. Assessment of ventricular arrhythmias

Ventricular arrhythmias during a 25 min coronary artery occlusion and following reperfusion were assessed according to the Lambeth Conventions [91] with modification as has been outlined previously [64]. In brief, the total number of VPBs, the incidence and the number of episodes of VT (defined as a run of four or more consecutive VPB at a rate faster than the resting HR), and the incidence of VF were assessed during the occlusion period. During the reperfusion only the incidence of VF, which is a fatal event in this species, was determined. Dogs that were alive 5 min after reperfusion were considered to be survivors. At the end of the experiments the animals were euthanized with an overdose of the anaesthetic. In those dogs from which myocardial tissue samples were taken for the *in vitro* measurements, the animals were euthanized at the end of the occlusion period. The hearts were rapidly excised and placed in ice-cold saline. Tissue samples were taken from the area supplied by the occluded LAD and from the non-ischaemic area supplied by the LCX.

3.6. Determination of changes in the cellular distribution of Cx43

The cellular distribution of Cx43 was determined using immunofluorescence analysis by measuring and calculating the intensity and area fraction of Cx43. Small transmural tissue blocks (0.5cm×0.5cm×2.0cm), cut from the ischaemic area supplied by the occluded LAD, were embedded in OCT compound, frozen in liquid nitrogen and stored at −80°C until processing. Longitudinal sections (8μm thick) were cut from the mid-myocardial layer in a cryostat (Leica) at −20°C, and the slices were mounted on gelatine-coated slides. After blocking in 5% BSA, the samples were labelled overnight with a polyclonal rabbit anti-Cx43 antibody (1:800 dilution; Sigma-Aldrich) at 4°C, and also with FITC conjugated secondary antibody for 1 h at room temperature, and then with WGA-Texas Red (1: 500 dilution; Invitrogen) for 30 min, at room temperature (25°C). Images were captured by a laser scanning confocal microscope (FV1000; Olympus) using a 40-fold magnifying objective. Well-preserved areas of myocytes were selected and analysed with ImageJ (Molecular Dynamics). Intensity of Cx43 was measured by costume made software. The area fraction of Cx43 was defined as a region occupied by Cx43 fluorescence within the intercalated discs.

3.7. Determination of changes in Cx43 protein expression and phosphorylation

Immunoblot analysis was used to determine Cx43 protein expression and phosphorylation as described in detail elsewhere [59,61]. 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 Bredford [92]. From each sample 10 μg protein was separated on 10% polyacrylamide gels, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blots were blocked with 5% non-fat milk, dissolved in TBST for 1 h, and labelled overnight with a rabbit polyclonal anti-Cx43 antibody (Sigma-Aldrich) diluted to 1:500000, and then incubated for 1 h with HRP-conjugated anti-rabbit goat secondary antibody (Santa Cruz Biotech Inc., Santa Cruz, CA, USA) diluted to 1:10000 at room temperature. Blots were visualized with the ECL Plus kit (GE, Healthcare) using x-ray film (GE Healthcare). Band intensities were determined by the ImageJ 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.

3.8. Determination of gap junctional metabolic coupling

Gap junctional metabolic coupling was evaluated by measuring tissue permeability using the 'double-dye loading' method, described previously [59,61]. In brief, freshly excised transmural tissue blocks from both the ischaemic and non-ischaemic ventricular walls were submerged in a mixture of LY (Lucifer Yellow; 1.5 mg/ml) and TRITC–dextran (TD, 3.5 mg ml-1; both dyes purchased from Sigma], fixed in paraformaldehyde (PFA) and cryosections (25 μm) prepared. Ten pairs of fluorescent images were taken from each sample with a CCD camera connected to an Olympus IX71 fluorescent microscope (Olympus). The ratio of LY and TD stained areas was calculated using the ImageJ software. GJ permeability within the ischaemic (LAD) area was expressed as a percentage of permeability measured within the non-ischaemic (LCX) wall region.

3.9. Experimental protocols

3.9.1. Protocol I. The role of gap junctions in the delayed cardioprotective effect of cardiac pacing

This is illustrated in **Figure 2**. Under light pentobarbital anaesthesia 24 dogs were subjected to rapid right ventricular pacing (4 x 5 min, at a rate of 240 beats min^{-1}) and, 24 hours later, to a 25 min occlusion of the LAD. Ten paced dogs remained without treatment (PO group), whereas in 14 paced dogs CBX, an inhibitor of GJ opening, was infused locally into a small branch of the LAD in doses of 50 μmol L^{-1} (PO+CBX50, n = 7) and in 100 μmol L^{-1} (PO+CBX100, n = 7), starting the infusion 20 min prior to, and maintained over the entire occlusion period. Another 10 dogs were instrumented but not paced, only subjected to a 25

min occlusion (SPO group). These dogs served as controls. At the end of the occlusion period the hearts were stopped by an excess of anaesthetic and myocardial tissue samples were taken from both the LAD and LCX areas for further biochemical analyses.

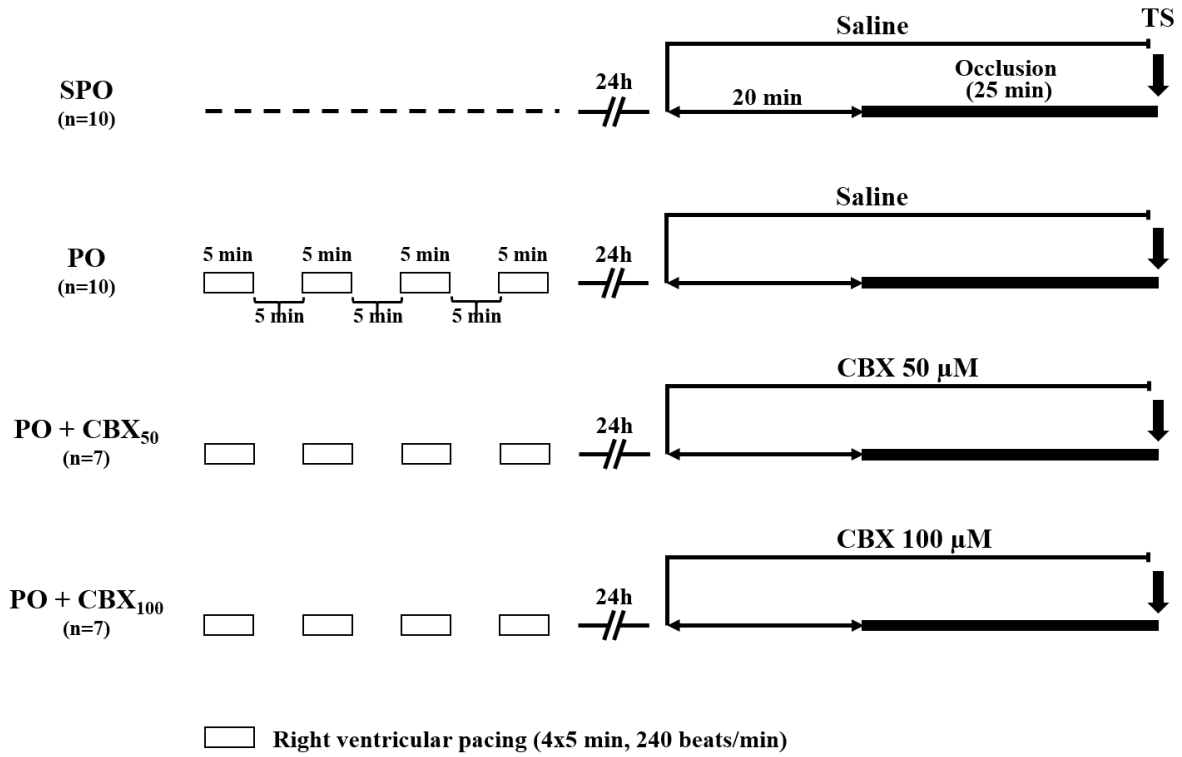

Figure 2.

Experimental protocol for the evaluation of the effect of gap junction uncoupling on the delayed antiarrhythmic effect of cardiac pacing. (TS= tissue sample)

3.9.2. Protocol II. The role of gap junctions in the antiarrhythmic effect of sodium nitrite

This is illustrated in **Figure 3.** In this series of experiments a total of 42 dogs were used and randomly divided into three groups. Each animal was subjected to a 25 min LAD occlusion followed by rapid reperfusion. Control dogs (n = 15) were infused with saline, commencing 10 min prior to the end of the coronary artery occlusion. In the other two groups $NaNO_2$ was administered by intravenous infusion at a concentration of 0.2 µmol kg^{-1} min^{-1} either 10 min prior to and throughout the occlusion ($NaNO_2$-PO; n = 14) or 10 min prior to reperfusion ($NaNO_2$-PR; n = 13), as has been described previously [83]. In dogs that survived the combined ischaemia and reperfusion insult, the hearts were stopped with an overdose of the anaesthetic either within 2 min (where myocardial tissue samples were taken) or 5 min (where the area at risk was determined). In dogs that suddenly fibrillated on reperfusion, the heart was quickly excised when the fibrillation had been observed. In 7 control and 7 nitrite treated dogs, after excising the heart, the risk area was assessed by injecting Patent Blue V

dye into the re-occluded artery using the same method that has been described in detail elsewhere [64].

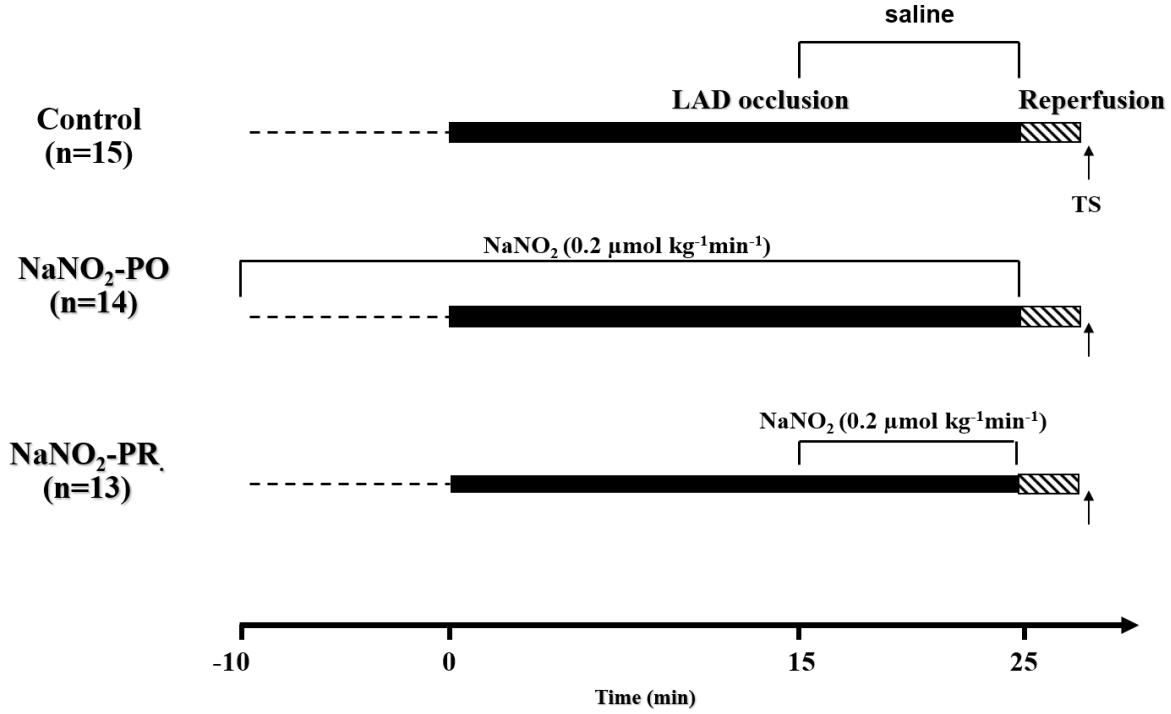

Figure 3.

Experimental protocol for the evaluation of the cardioprotective effect of sodium-nitrite. (TS= tissue sample)

3.10. Statistical analysis

All values are expressed as means ± S.E.M. Differences between groups were compared with Student's *t* test or ANOVA for repeated measures with Fisher post-hoc test, as appropriate. The data with non-normal distribution, e.g., the number of VPB and of VT episodes and incidences of VT and VF as well as survival was compared between groups with Mann–Whitney *U* test and Fisher's exact test respectively. Differences between groups were considered significant at $P < 0.05$.

4. RESULTS

4.1. The evidence for the involvement of gap junctions in the delayed antiarrhythmic effect of cardiac pacing

4.1.1. Haemodynamic effects of carbenoxolone and of coronary artery occlusion

These are illustrated in **Table 1** and in **Table 2**. Compared with the saline treated dogs, the local intracoronary infusion of CBX in both doses resulted in no significant alterations in the haemodynamic parameters. The occlusion of LAD significantly decreased arterial blood pressure, LVSP, the positive and negative dP/dt_{max}, and increased LVEDP in all groups, but these changes were somewhat less pronounced in the paced dogs, irrespective whether they were treated with CBX or not. In anaesthetized dogs none of the interventions modified substantially the heart rate.

Table 1. Haemodynamic effects of carbenoxolone and saline

	SPO; PO		$PO+CBX_{50}$		$PO+CBX_{100}$	
	Baseline	Change	Baseline	Change	Baseline	Change
SABP (mm Hg)	159 ± 5	-2 ± 2	144 ± 11	4 ± 2	145 ± 4	-2 ± 2
DABP (mm Hg)	104 ± 8	0 ± 1	97 ± 7	3 ± 2	100 ± 2	-2 ± 2
MABP (mm Hg)	119 ± 7	-1 ± 2	113 ± 8	3 ± 2	115 ± 2	-4 ± 2
LVSP (mm Hg)	158 ± 9	-3 ± 1	131 ± 11	3 ± 2	124 ± 3	-1 ± 2
LVEDP (mm Hg)	5 ± 1	0 ± 1	8 ± 1	1 ± 1	9 ± 2	2 ± 0
+dP/dt (mm Hg s^{-1})	2980 ± 50	-9 ± 2	2834 ± 202	145 ± 86	2374 ± 273	39 ± 72
-dP/dt (mm Hg s^{-1})	2640 ± 134	8 ± 44	2477 ± 127	203 ± 113	1952 ± 333	-120 ± 100
HR (beats min^{-1})	156 ± 9	1 ± 2	153 ± 5	0 ± 2	155 ± 9	-2 ± 2

Data are means ± s.e.m.

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

	SPO		PO		$PO+CBX_{50}$		$PO+CBX_{100}$	
	Baseline	Max.change	Baseline	Max. change	Baseline	Max.change	Baseline	Max.change
SABP (mm Hg)	156 ± 5	-11* ± 4	160 ± 5	-9* ± 4	148 ± 14	-9* ± 1	150 ± 5	-10* ± 4
DABP (mm Hg)	108 ± 4	-12* ± 4	107 ± 3	-7* ± 3	100 ± 9	-7* ± 2	104 ± 5	-8* ± 2
MABP (mm Hg)	124 ± 4	-14* ± 2	125 ± 4	-6* ± 3	116 ± 10	-8* ± 1	119 ± 4	-8 ± 3
LVSP (mm Hg)	160 ± 6	-13* ± 4	163 ± 4	-4* ± 3	157 ± 15	-7* ± 1	158 ± 5	-9* ± 2
LVEDP (mm Hg)	8 ± 1	8* ± 1	8 ± 1	8* ± 0	9 ± 1	7* ± 1	10 ± 1	10* ± 1
+dP/dt (mm Hg s^{-1})	3039 ± 199	-678* ± 68	2943 ± 140	-362* ± 163	3120 ± 299	-387* ± 97	2483 ± 247	-487* ± 110
-dP/dt (mm Hg s^{-1})	2586 ± 153	-351* ± 164	2708 ± 153	-446* ± 57	2633 ± 181	-346 ± 104	1394 ± 247	-499* ± 94
HR (beats min^{-1})	167 ± 6	6 ± 3	149 ± 5	6 ± 3	150 ± 6	-1 ± 3	154 ± 9	3 ± 6

*Data are means ± s.e.m. * $p<0,05$ compared to the baseline value.*

4.1.2. The severity of ventricular arrhythmias during a 25 min occlusion of the LAD

The number and severity of ventricular arrhythmias during a 25 min occlusion of the LAD are illustrated in **Figure 4**. Compared with the sham-paced dogs, in dogs subjected to cardiac pacing 24h previously, the number of VPBs (267 ± 31 vs. 36 ± 8), the number of episodes of VT (8.7 ± 2.8 vs. 0.3 ± 0.3), and the incidences of VT (90% vs.10%) and VF (50% vs. 10%) were markedly reduced during coronary artery occlusion. The infusion of CBX in both concentrations (50 and 100 μmol L^{-1}) attenuated or even abolished the protective effect of pacing against arrhythmias (VPBs: 76 ± 11 and 236±101; VT episodes: 1.3 ± 0.8 and 5.3 ± 4.2; VT incidence: 43% and 57%; VF incidence: 57% and 29%).

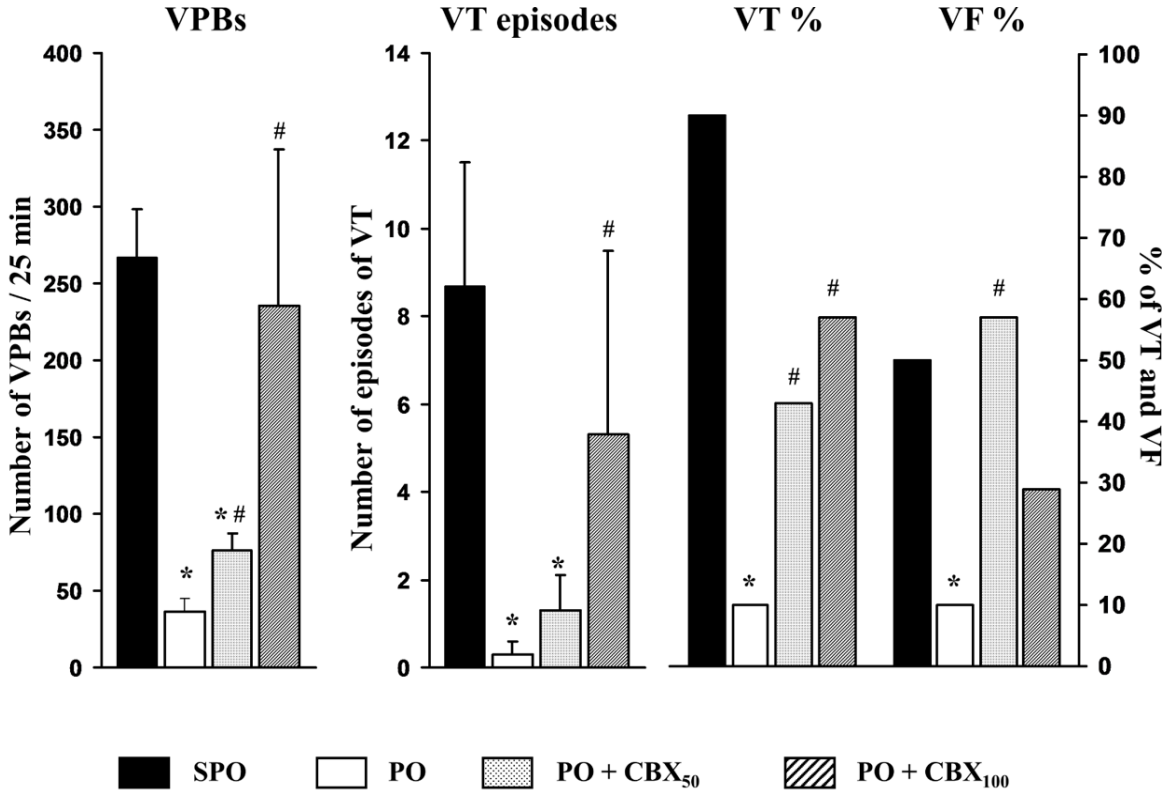

Figure 4.

*The severity of ventricular arrhythmias during a 25 min occlusion of the LAD. Values are the mean ± s.e.m. * $p<0.05$ compared with the SPO group, # $p<0.05$ compared with the PO group*

4.1.3. Changes in tissue resistivity in relation to the distribution of VPBs during a 25 min occlusion of the LAD

These are illustrated in **Figure 5.** In the non-paced control dogs occlusion of the LAD resulted in marked changes in tissue electrical resistance that occurred in two phases. There was a marked increase in resistivity immediately after the commencement of the coronary

artery occlusion, and then, a second steep rise could be observed around the 15 min of the occlusion. In parallel, the ectopic activity occurred also in two phases; there was an increase in the number of VPBs between 3 and 10 min (peaked around 5 min) of the occlusion that is called as phase 1a arrhythmias, which was followed by a second arrhythmia burst, termed as phase 1b, occurring between 16 and 25 min (peaked around 18 min) of the ischaemia. Both the resistivity changes and the number of VPBs over the entire occlusion period were significantly less in dogs that had been subjected to cardiac pacing 24h before the coronary artery occlusion. In the presence of both doses of CBX the resistivity changes were similar to that occurred in the non-paced controls and the number of VPBs was also significantly increased. Interestingly, CBX in 50 μmol L^{-1} concentration (PO+CBX_{50} group) increased the number of VPBs during phase 1b, whereas in 100 μmol L^{-1} concentration (PO+CBX_{100} group) the number of ectopic beats was significantly increased during both phase 1a and phase 1b.

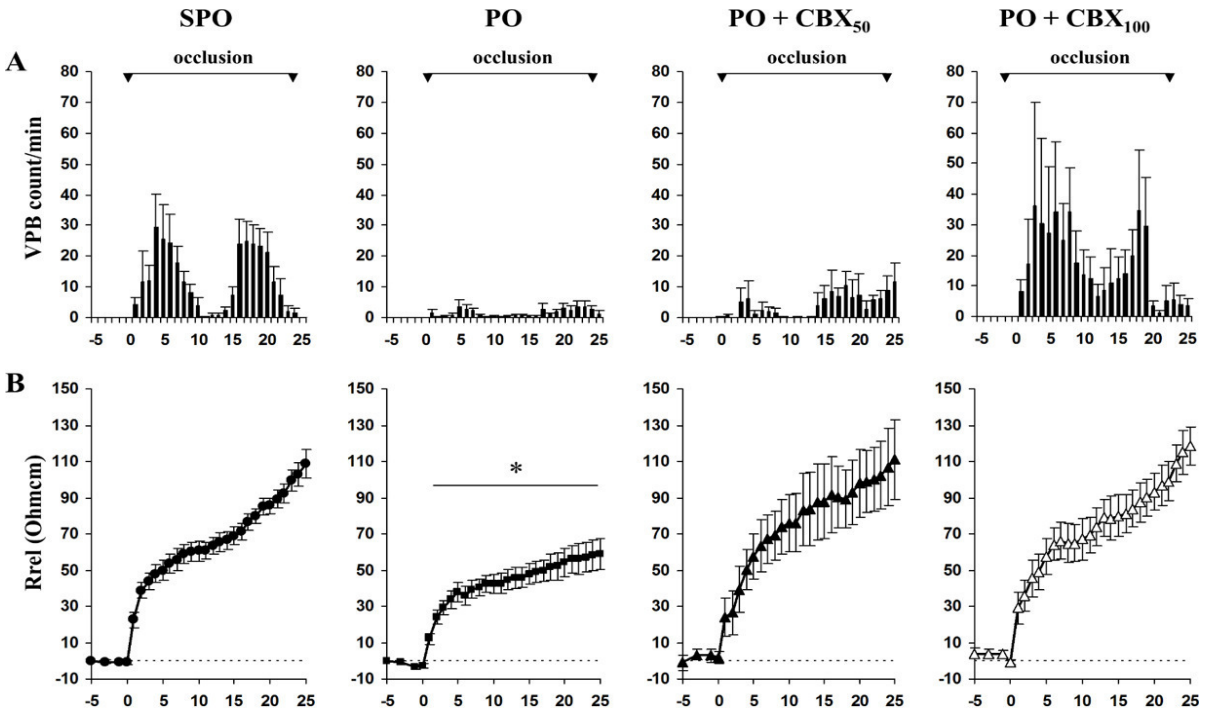

Figure 5.

*Distribution of VPBs and changes in tissue resistivity during a 25 min occlusion of the LAD. Values are the mean ± s.e.m. * $p<0.05$ compared with the SPO group*

4.1.4. Changes in ischaemia severity during coronary artery occlusion

The severity of ischaemia was assessed by two indices; i.e. changes in the epicardial ST-segment and in the TAT, recorded from the epicardial surface of the left ventricular wall, supplied by the occluded LAD. In the non-paced dogs, occlusion of the LAD resulted in

immediate and marked increase in both epicardial ST-segment (**Figure 6A**) and TAT (**Figure 6B**). These ischaemia-induced electrophysiological changes were significantly less marked in dogs subjected to cardiac pacing, 24h previously. In the presence of CBX these indices of ischaemia severity were again increased.

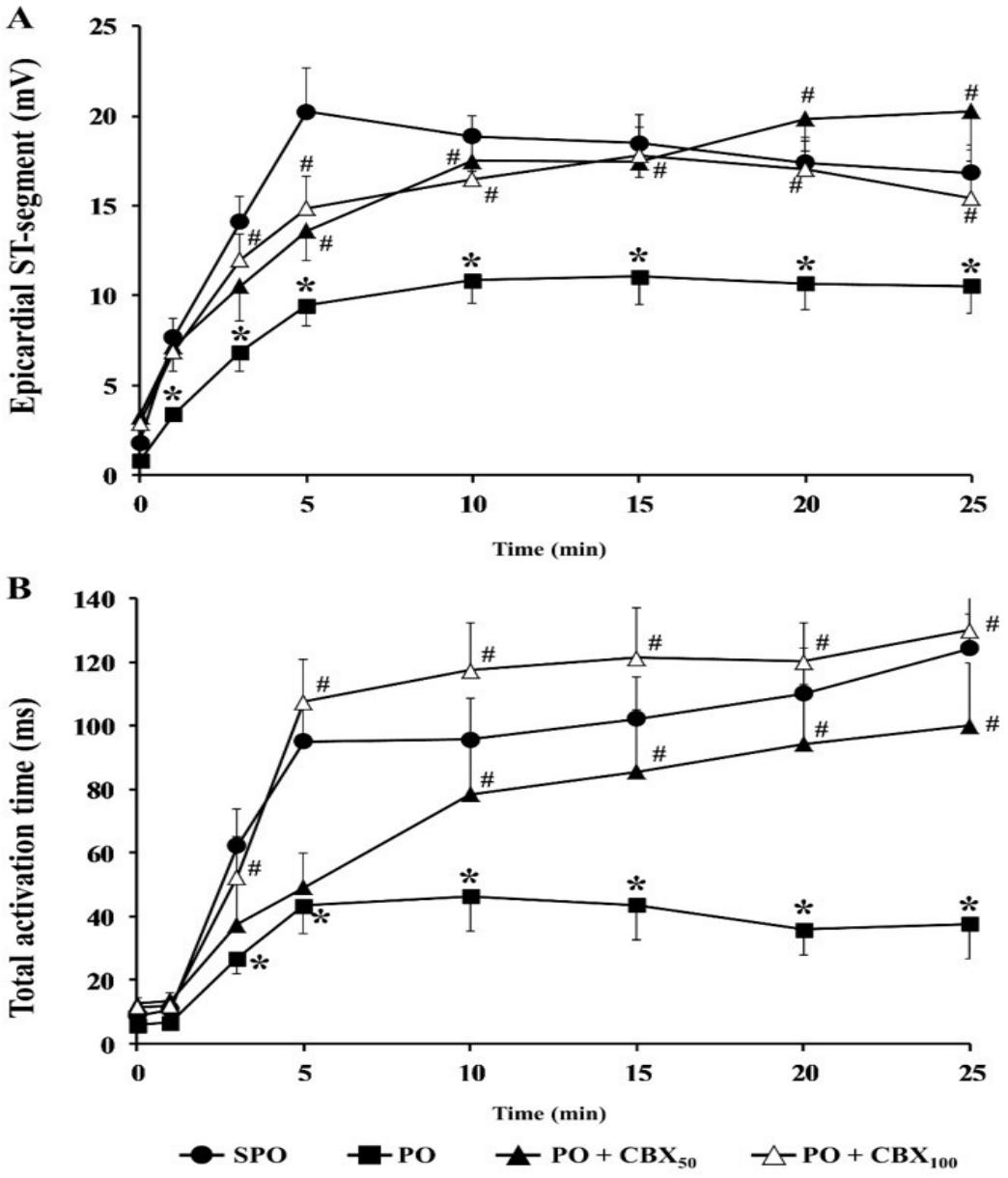

Figure 6.

*Changes in ischaemia severity during a 25 min coronary artery occlusion. Values are the mean ± s.e.m. * $p<0.05$ compared with the SPO group, # $p<0.05$ compared with the PO group.*

4.1.5. Changes in connexin 43 phosphorylation by cardiac pacing and following CBX administration

The phosphorylation of Cx43 following a 25 min ischaemia is illustrated in **Figure 7** In sham control hearts the phosphorylated form of Cx43 was somewhat higher than dephosphorylated form. This distribution was significantly altered in the heart of dogs that had been subjected to a 25 min LAD occlusion; i.e. the dephosphorylated form of Cx43 was increased and the phosphorylated form decreased. This ischaemia-induced shift in the phospho/dephospho ratio was prevented by pacing. Both doses of CBX increased again the dephosphorylation of Cx43.

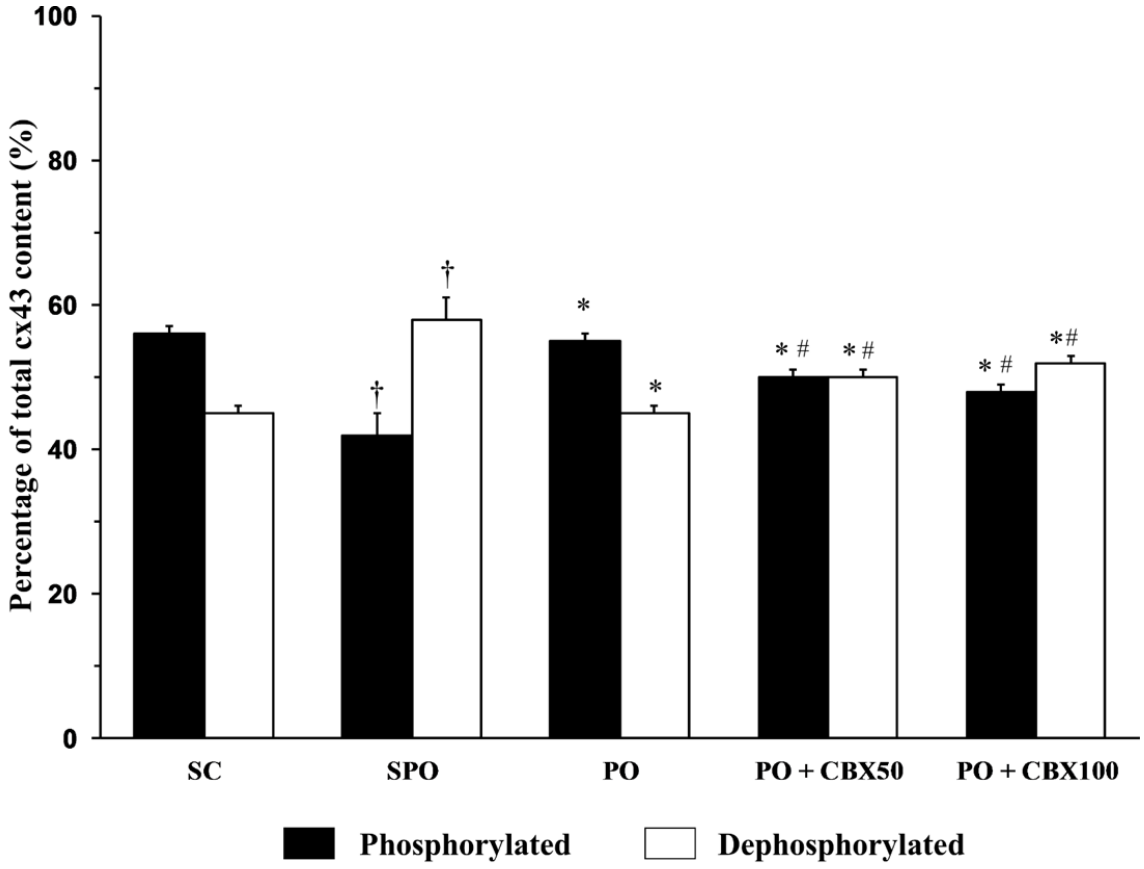

Figure 7.

*Changes in the phosphorylated and dephosphorylated isoforms of Cx43 after 25 min LAD occlusion. Values are the mean ± s.e.m. * $p < 0.05$ compared with the SPO group, # $p<0.05$ compared with the PO group, † $p<0,05$ compared with the SC group.*

4.1.6. Changes in gap junctional permeability following cardiac pacing and CBX

GJ permeability within the ischaemic area was expressed as a percentage of permeability measured within the non-ischaemic area, and the results are summarized in **Figure 8.** In the

non-paced dogs, occlusion of the LAD reduced GJ permeability by 32% within the ischaemic area, compared to the 100% of non-ischaemic area. Cardiac pacing preserved the metabolic coupling of GJs, but this was again significantly decreased, when pacing was performed in the presence of CBX.

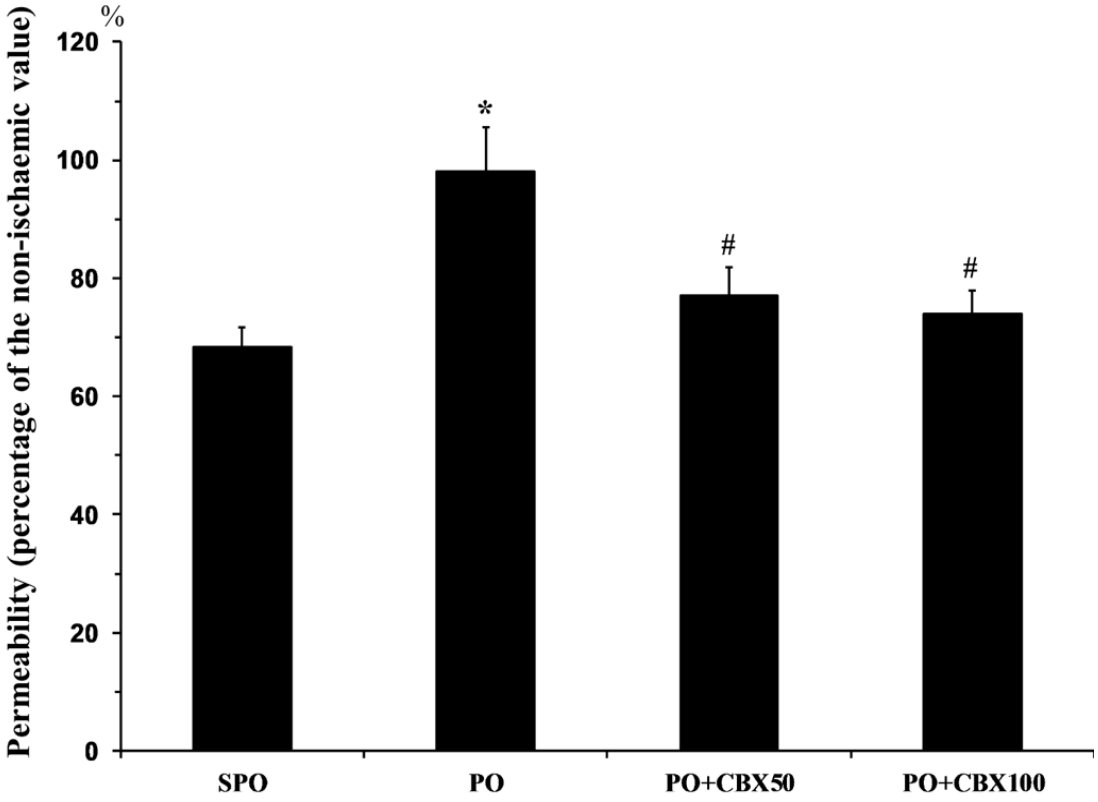

Figure 8.

*Gap junction permeability after 25 min of ischaemia. Permeability of the ischaemic tissue samples are expressed as percentage of the non-ischaemic self-controls. Values are the mean ± s.e.m. * $p < 0.05$ compared with the SPO group, # $p < 0.05$ compared with the PO group.*

4.1.7. Structural alterations of the intercalated discs following cardiac pacing and CBX administration

The structural alterations occurring at the end of a 25 min occlusion of the LAD in non-paced and paced dogs, with and without CBX administration are shown in **Figure 9,** using a series of representative confocal immunofluorescence images obtained from tissue samples collected from both the ischaemic (supplied by the LAD) and the non-ischaemic (supplied by the LCX) myocardial wall. In the non-paced controls (SPO), occlusion of the LAD resulted in, within its supplied region, signs of structural impairment of the intercalated discs (indicated by arrows); i.e. the contour of the end-to-end connections became "blurred" compared with those images which were obtained in samples taken from the non-ischaemic

(LCX) myocardial region (Figure 9A). Cardiac pacing, 24h previously, prevented these ischaemia-induced structural changes, and there were no visible differences between the ischaemic and the normal areas (Figure 9B). Carbenoxolone, given in 50 and 100 μmol L^{-1} concentrations to paced dogs prior to and during the occlusion, resulted in again perceivable alterations (dim membrane density, 'blurred' contour) around the disc areas, but, interestingly, these changes occurred in both areas, supplied either by the occluded LAD or the patent LCX arteries (Figure 9C and D), suggesting that CBX itself may cause re-arrangements in the structure of GJs.

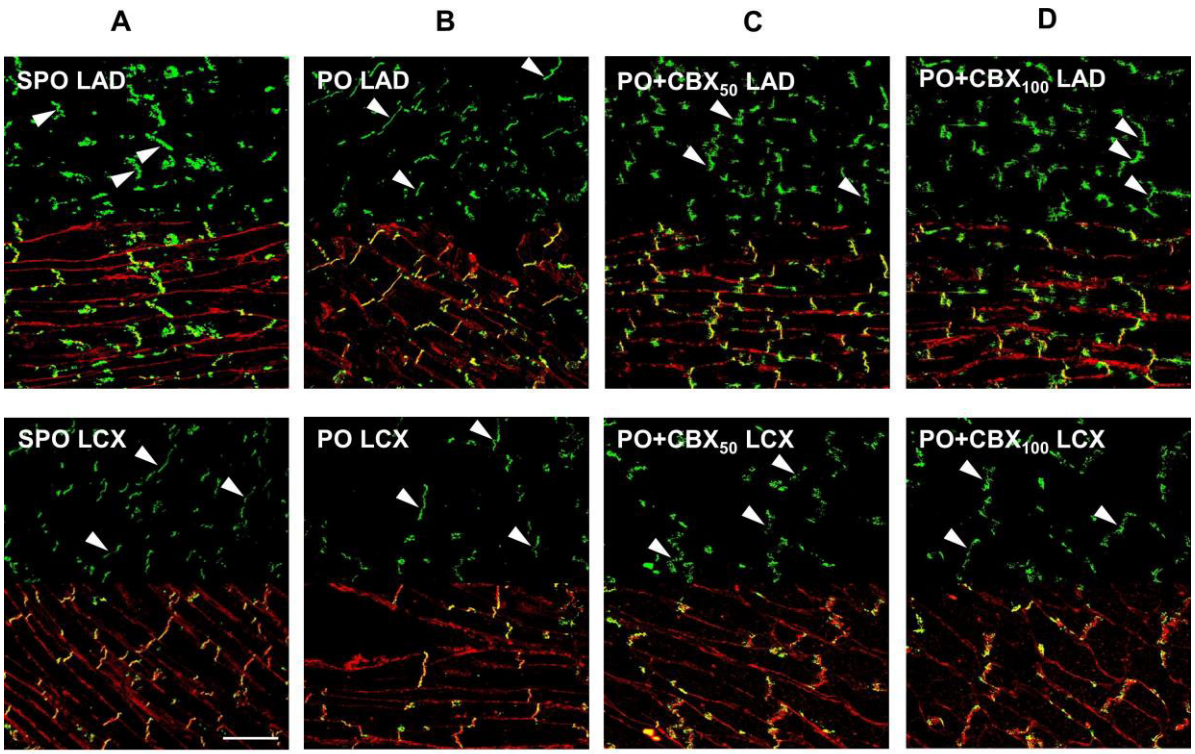

Figure 9.

Structural alteration in gap junction distribution by representative immunofluorescence images. Arrow heads point to intercalated discs. Scale bar= 50um.

4.2. The role of gap junctions in the antiarrhythmic effect of sodium nitrite

4.2.1. Haemodynamic effects of sodium nitrite and coronary artery occlusion

These are summarized in **Table 3**. The administration of $NaNO_2$ resulted in a slight reduction in arterial blood pressure, LVEDP and in positive and negative LV dP/dt_{max} without substantial changes in heart rate. Occlusion of the LAD resulted in significant reduction in arterial blood pressure, left ventricular systolic pressure, positive and negative dP/dt_{max} and an increase in LVEDP in both groups during the occlusion. Intravenous infusion of sodium nitrite did not modify the haemodynamic parameters, except for the LVEDP.

Table 3. The haemodynamic effects of sodium nitrite infusion and of coronary artery occlusion

	$NaNO_2$-PO		Control			$NaNO_2$-PO			$NaNO_2$-PR		
				0-15 min	15-25 min		0-15 min	15-25 min		0-15 min	15-25 min
	Baseline	Max.change	Baseline	Max.change occlusion	Max.change occlusion	Baseline	Max.change occlusion	Max.change occlusion	Baseline	Max.change occlusion	Max.change occlusion
SABP (mm Hg)	132 ± 7	-8 ± 3*	143 ± 4	-12 ± 3*	-14 ± 3*	124 ± 6	-12 ± 2*	-13 ± 1*	152 ± 3	-13 ± 1*	-15 ± 2*
DABP (mm Hg)	89 ± 6	-6 ± 2*	101 ± 4	-9 ± 1*	-11 ± 2*	85 ± 5	-10 ± 2*	-9 ± 1*	104 ± 4	-13 ± 2*	-14 ± 3*
MABP (mm Hg)	103 ± 6	-6 ± 2*	115 ± 3	-10 ± 1*	-12 ± 2*	98 ± 5	-12 ± 4*	-11 ± 1*	121 ± 9	-13 ± 2*	-14 ± 2*
LVSP (mm Hg)	135 ± 6	-6 ± 2*	144 ± 4	-14 ± 1*	-15 ± 1*	124 ± 6	-12 ± 3*	-12 ± 4*	150 ± 5	-14 ± 2*	-16 ± 2*
LVEDP (mm Hg)	4,6 ± 0.3	-1,1 ± 0,3*	3.7 ± 0.3	11.4 ± 1.4*	12 ± 1.3*	4,8 ± 0,3	5,9 ± 0,5*#	6,4 ± 0,3*#	4 ± 0.4	8,8 ± 0.4*	6.2 ± 0.4*#
+dP/dt (mm Hg s^{-1})	2924 ± 77	-98 ± 48*	3161 ± 118	-635 ± 92*	-654 ± 87*	2846 ± 106	570 ± 54*	525 ± 44*	2972 ± 134	-411 ± 62*	-423 ± 60*
-dP/dt (mm Hg s^{-1})	2257 ± 59	-112 ± 32*	2635 ± 83	-596 ± 95*	-609 ± 91*	2157 ± 77	439 ± 64*	456 ± 48*	2333 ± 79	-423 ± 63*	-444 ± 56*
HR (beats min^{-1})	162 ± 4	4 ± 3	164 ± 4	-3 ± 2	-2 ± 1	164 ± 4	2 ± 2	1 ± 1	170 ± 3	1 ± 2	2 ± 2

*Values are the mean ± s.e.m. * $p<0.05$ compared with baseline value, # $p<0,05$ compared with control group*

4.2.2 The severity of ventricular arrhythmias during coronary artery occlusion and reperfusion

This is shown in **Figure 10.** In control dogs, occlusion of the LAD resulted in a high number of VPBs (525 ± 90) and episodes of VT (19.8 ± 5.6) that occurred in 92% of these dogs. In this group 38% of the dogs fibrillated during occlusion and no control dog survived reperfusion. Compared to these controls, the intravenous administration of $NaNO_2$, prior to and during the occlusion ($NaNO_2$-PO group) significantly reduced these arrhythmias (VPBs: 157 ± 78, episodes of VT: 3.4 ± 2.4, incidence of VT: 29% and VF: 0%) during occlusion, and increased survival from 0% to 50%. Similarly, when $NaNO_2$ was administered 10 min prior to reperfusion ($NaNO_2$-PR), the number and incidence of arrhythmias were significantly reduced. Thus in this group the number of VPBs was 236 ± 38, the number of VT episodes

was 4.27 ± 1.2 that occurred in 54% of the dogs, furthermore only 1 dog out of 13 dogs fibrillated during the occlusion and 85% of the animals survived the reperfusion.

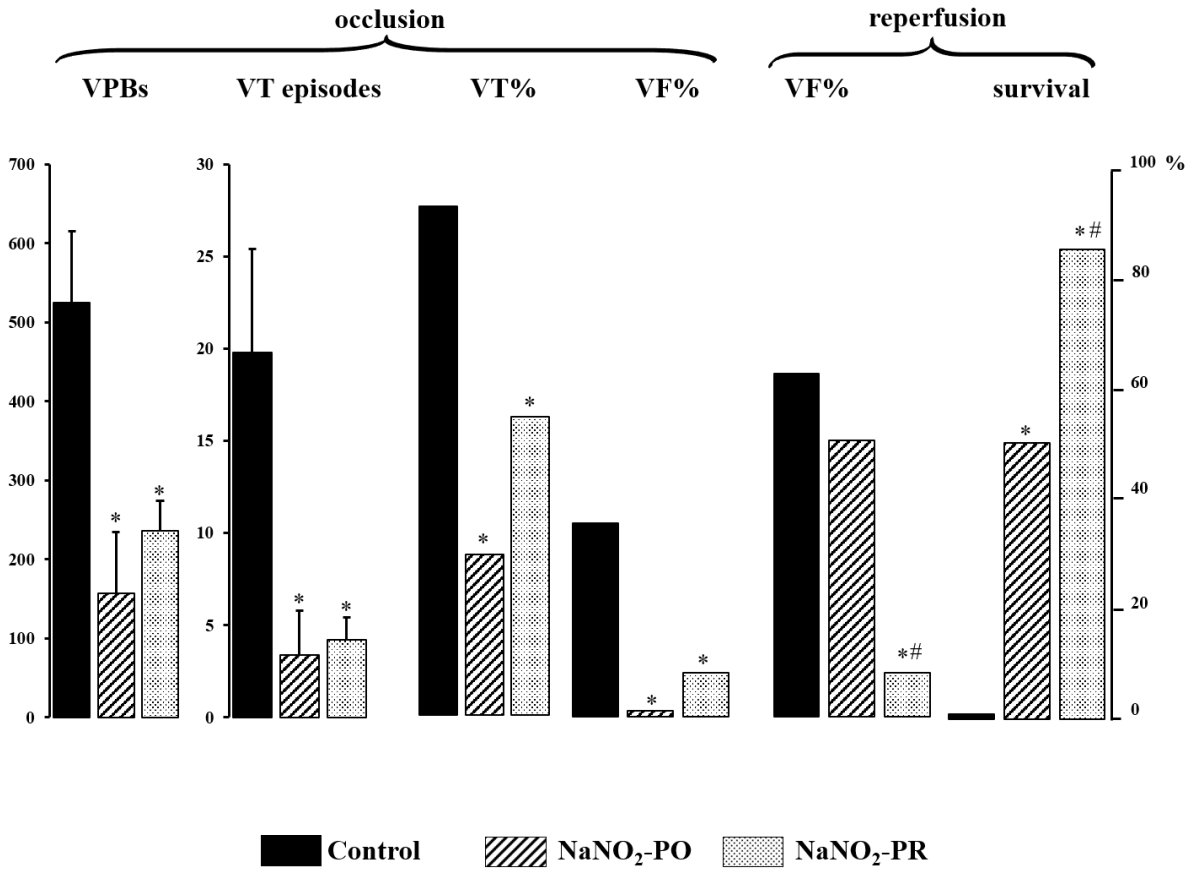

Figure 10.

*Total number of VPBs, the number of episodes and the incidence of VT, the incidence of VF during a 25 min of ischaemia. Values are means ± s.e.m. * $p<0.05$ compared to the controls, # $p<$ compared to the $NaNO_2$-PO group*

4.2.3. Changes in tissue electrical impedance and in the distribution of VPBs during a 25 min LAD occlusion

These are illustrated in **Figure 11.** In control dogs occlusion of the LAD resulted in an immediate increase in tissue resistivity and a decrease in phase angle accompanied by the occurrence of many ectopic beats. These changes were apparent during the first 10 min of ischaemia (phase 1A), after which the impedance changes became slower and the number of VPBs was significantly decreased. A second steeper rise in resistivity and decline in phase angle started around 13 min of the occlusion, followed immediately by the re-appearance of the phase 1B arrhythmias. In dogs that had received $NaNO_2$ prior to and during the occlusion, the steep impedance changes were markedly reduced, and in parallel, the ectopic activity over

the entire occlusion period was attenuated. In dogs, in which $NaNO_2$ was infused 10 min prior to reperfusion, the ischaemia-induced impedance changes, as well as the ectopic activity, were reduced only during the second phase of the occlusion (i.e. almost immediately after the commencement of the nitrite infusion), indicating a rapid effect of $NaNO_2$ on GJs.

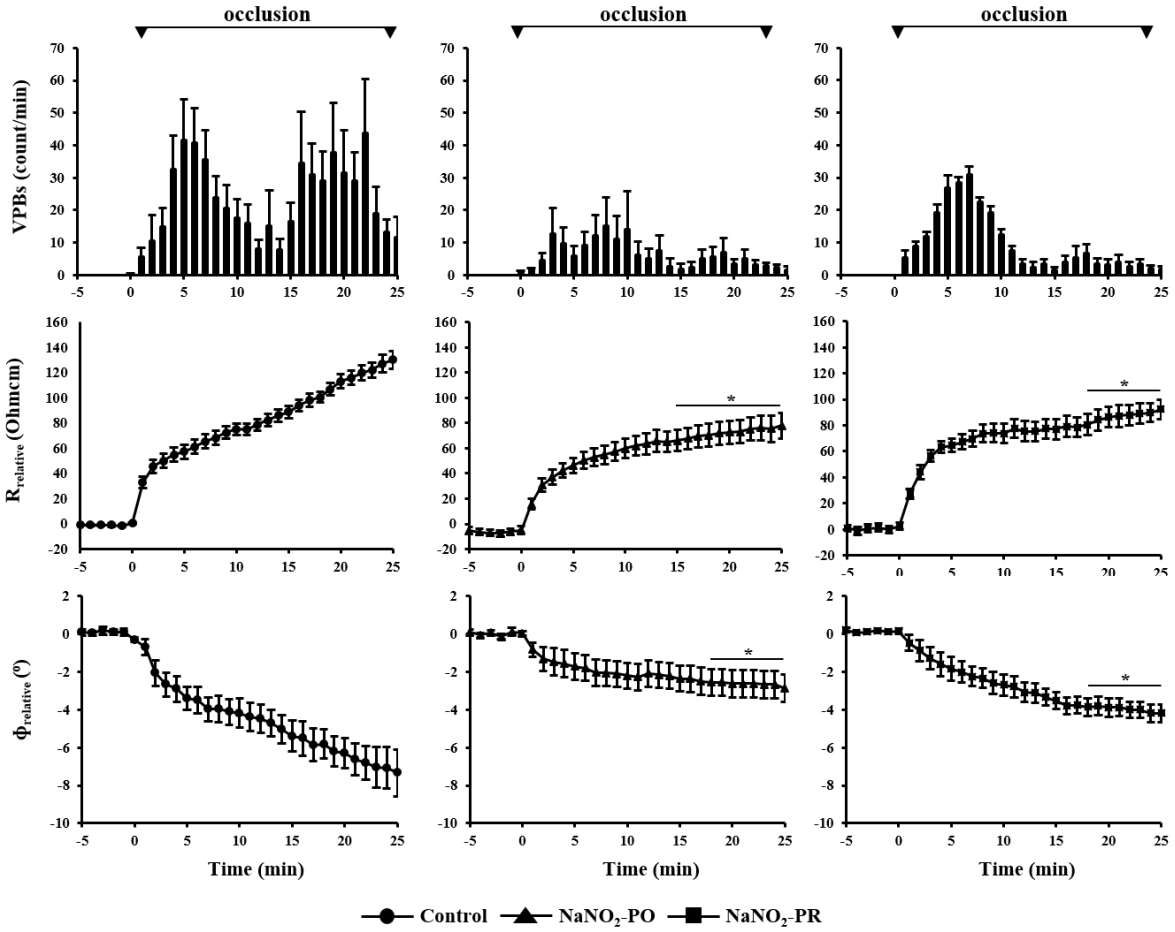

Figure 11.

*Distribution of VPBs and changes in tissue impedance during a 25 min occlusion. Values are means ± s.e.m. * p<0,05 compared to the controls.*

4.2.4. Changes in epicardial ST-segment and total activation time during occlusion of the LAD

These were assessed by two parameters; i.e. changes in epicardial ST-segment (**Figure 12A**) and TAT (**Figure 12B**) during a 25 min occlusion of the LAD. In control dogs occlusion of the LAD resulted in immediate and marked increase in both parameters, reaching the maximum values within the first 5 min of the occlusion. In dogs, given $NaNO_2$ prior to and during occlusion ($NaNO_2$-PO group) both the absolute values and the development of these ischaemia parameters were significantly less pronounced over the entire occlusion period. In contrast, in dogs in which nitrite was infused over the last 10 min

of the occlusion ($NaNO_2$-PR group), the reduction in ischaemia severity occurred almost immediately after starting the infusion, and maintained by the end of the occlusion period.

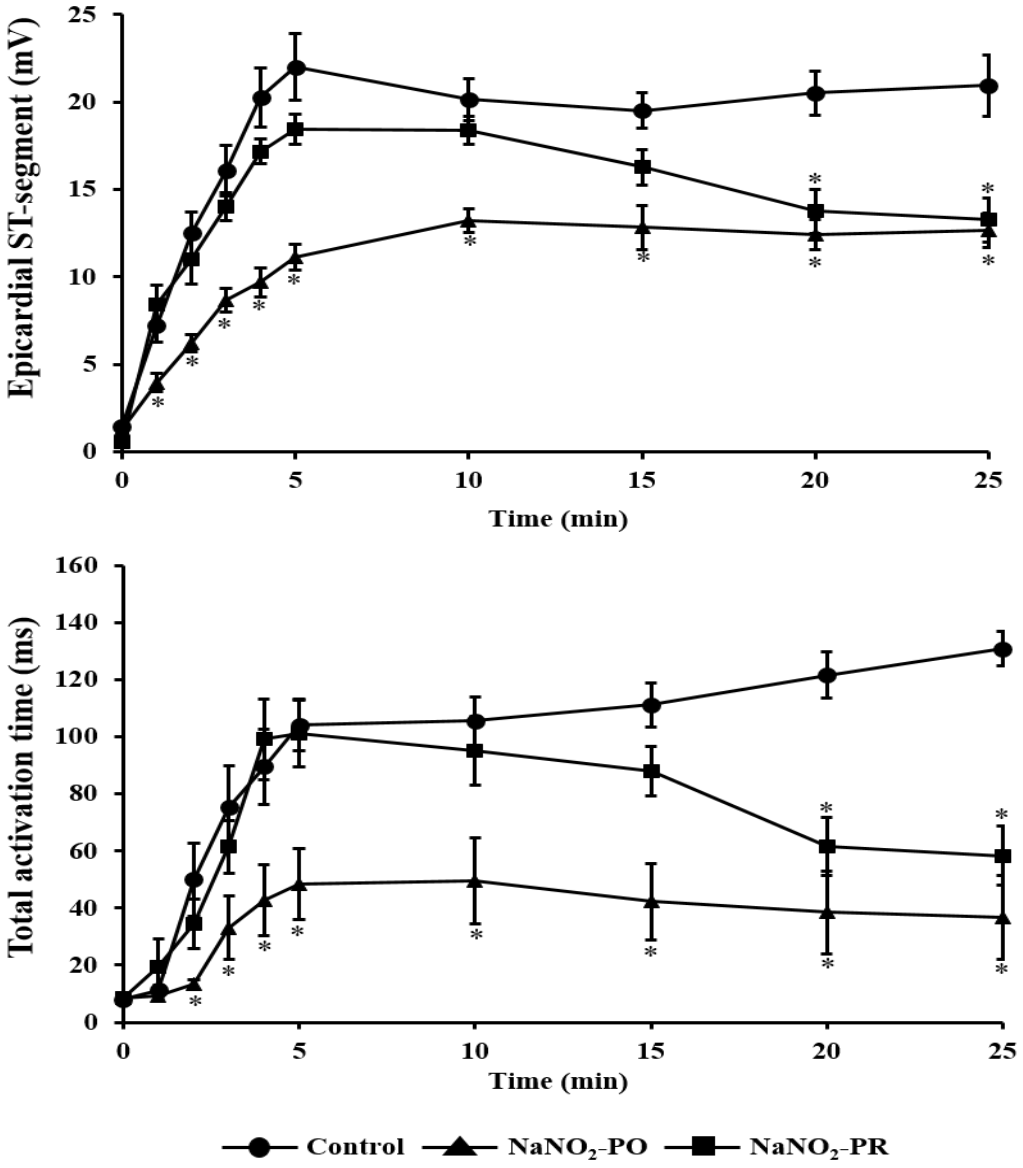

Figure 12.

*Changes in ischaemia severity during a 25 min coronary artery occlusion. Values are means ± s.e.m. * p<0,05 compared to the controls.*

4.2.5. Area at risk

There were no significant differences in the area at risk among the group. Thus in control dogs (n=7) the risk area was 36±2% in the $NaNO_2$-PO group (n=5) 42.3±1% and in the $NaNO_2$-PR group (n=7) 37±3%.

5. DISCUSSION

5.1. New findings

1. We have shown that gap junctions play a role in the delayed antiarrhythmic effect of preconditioning, induced by rapid cardiac pacing. The evidence for this resulted from studies which clearly showed that the inhibition of gap junction channels prior to ischaemia with carbenoxolone, a selective blocker of these channels, attenuates the protection against the ischaemia and reperfusion-induced arrhythmias, 24 hours later.

2. We have provided evidence for the first time that the inorganic sodium nitrite in a concentration of 0.2 μmol $kg^{-1}min^{-1}$ markedly reduces the severity of ventricular arrhythmias, resulting from a 25 min period of coronary artery occlusion and reperfusion in anaesthetised dogs. We have also suggested that the antiarrhythmic effect of nitrite is due to nitric oxide, which is formed from nitrite under reductive conditions, such as myocardial ischaemia. The present findings show that the antiarrhythmic effect of nitrite can be attributed, among the several other actions of nitric oxide, to its modulator effect on gap junction function.

5.2. The role of gap junctions in the delayed antiarrhythmic protection induced by rapid cardiac pacing

It has been shown previously that pacing the heart through the right ventricle with brief periods of rapid electrical impulses, markedly reduces the number and severity of ventricular arrhythmias that results from a coronary artery occlusion and reperfusion, 24 h later [65,93]. More recent studies have shown that in this delayed antiarrhythmic effect gap junctions may play a role [61]. This study clearly demonstrated that cardiac pacing (four times for 5 min at a rate of 240 beats min^{-1}) resulted in a time-dependent change in the mRNA and protein expressions of Cx43, which is the main structural protein of the ventricular gap junctions [24]. Further, more importantly, pacing preserved the structural integrity of the gap junctions during ischaemia, by keeping Cx43 in phosphorylated form [61] that indicates the open sate of gap junctions [94,95]. Although these findings strongly suggested the role of gap junctions in the delayed antiarrhythmic effect of cardiac pacing, we wanted to provide more direct evidence for the relationship between gap junctions and the pacing-induced suppression of arrhythmias. Therefore, studies were designed to examine whether closing of gap junctions by the gap junction uncoupler CBX would modify the antiarrhythmic effect of cardiac pacing. Carbenoxolone was administered in two concentrations (50 and 100 μmol L^{-1}) prior

to and during the occlusion, and changes in arrhythmia and ischaemia severity were assessed in parallel with the alterations in electrical impedance, an indirect measure of gap junction function [14].

We have found that CBX infused in paced dogs 24 h after the pacing stimulus, but prior to and during the entire occlusion period, attenuated the antiarrhythmic effect of cardiac pacing. In the presence of CBX there was a marked increase in the number and severity of arrhythmias resulted from a 25 min occlusion of the LAD. This effect was dose- dependent; CBX in a concentration of 100 μmol L^{-1} increased, interestingly, not only the phase 1b, but the phase 1a arrhythmias as well. This result suggests that CBX in higher concentrations may not selective for gap junctions. We have evidence that CBX in a concentration of 50 μmol L^{-1} does not influence the sodium, potassium and calcium channels [59], however, the higher concentrations (100 μmol L^{-1}) of CBX has been found to increase the calcium oscillations during diastole, resulting in delayed after-depolarisations and the subsequent generation of ventricular arrhythmias [96]. In order to clarify this question, in some *in vitro* experiments, using canine right ventricular papillary muscle preparations, we have determined the dose-dependent effects of CBX on the maximum upstroke velocity (dV_{max}), and on the duration of the action potential (APD). We have found that compared with the intact control samples, both concentrations of CBX (50 and 100 μmol L^{-1}) shortened APD_{90} from 236-±12 to 227±12 ms (ns.) and to 206±9ms ($P< 0.05$; using the Student t test) respectively, whereas the dV_{max} was not significantly changed by either concentration of CBX (88±16 V/s vs. 64±24 and 60±6 V/s, respectively). The results suggest that the higher concentration of CBX may modify the ion channels involved in impulse generation and conduction, and in the development of phase 1a arrhythmias.

Interestingly, the two concentrations of CBX had similar effects on the tissue resistivity changes; both concentrations resulted in marked and almost similar elevations in resistivity from the beginning of the occlusion. We suppose, therefore, that the increase in ectopic activity following the 100 μmol L^{-1} concentration of CBX is most probably not solely resulted from a higher rate of closure of gap junctions, but also from a rapid development in conduction disturbances due to the unspecific (not gap junction mediated effects) of this concentration of CBX. This assumption is also supported by the fact that there was a much faster increase in TAT in dogs that had been given 100 μmol L^{-1} concentration of CBX than that either in the controls or in dogs treated with the 50 μmol L^{-1} concentration of the drug.

Such concentration-dependent effect, however, was not seen in case of the epicardial ST-segment changes following the administration of CBX.

We have previous evidence that during ischaemia the phosphorylated form of Cx43 is going through marked dephosphorylation, shifting the phospho/dephospho ratio towards the dephosphorylated form [45]. Cardiac pacing, 24h earlier, markedly decreases the Cx43 dephosphorylation, and we suppose that the preservation of the phosphorylated form of Cx43, which can be associated with an open state of gap junctions [45,97], plays a role in the antiarrhythmic effect of cardiac pacing [61]. Therefore, in the present study we have examined, whether closing of gap junctions with CBX would influence Cx43 phosphorylation, and whether this can be associated with the attenuation of the antiarrhythmic effect. We have found that the administration of CBX increased the dephospho ratio of Cx43 compared with the paced dogs, but it did not completely abolish the effect of pacing on Cx43 phosphorylation. This result suggests that the gap junction uncoupling effect of CBX is, perhaps, not entirely due to a change in Cx43 phosphorylation. In this respect the data, available in the literature, are controversial. For example, it has been found that CBX had no effect on Cx43 phosphorylation in the rabbit heart [98], whereas it increased Cx43 dephosphorylation in the liver epithelial cells of rats [99].

Carbenoxolone, however, almost completely abolished the pacing-induced preservation of gap junction permeability, independently from the doses applied. Furthermore, CBX resulted in substantial structural alterations in the intercalated discs (ID). As we have reported earlier, a 25 min occlusion of the LAD causes significant structural rearrangements in the intercalated discs (e.g. dim contour and fragmentation of the end-to-end connections), and that pacing prevents these structural alterations [61]. When CBX was given in the paced dogs, similar structural changes appeared around the IDs to those observed in the control, unpaced dogs. Interestingly, the structural alterations were present in samples taken both from the ischaemic (supplied by the LAD) and the non-ischaemic (supplied by the intact LCX) myocardial regions. This observation indicates that although CBX was administered locally, into a side branch of the LAD, the drug distributed in the whole heart during the 20 min infusion period, and resulted in visible rearrangements of the intercalated discs within the non-ischaemic myocardium. A similar irregular arrangement of gap junction plaques has been found by others, following the administration of isoproterenol and CBX [100,101]. Yet, at this point, we may only speculate, how these morphological changes would account for the alteration in the function of gap junctions, and whether the observed structural alterations

resulting from ischaemia, cardiac pacing or CBX administration would entirely explain the functional changes of gap junctional coupling, and the subsequent modification in arrhythmia generation.

In summary, these of our studies demonstrate that the pharmacological inhibition of gap junction channels with the gap junction uncoupler CBX prior to and during a 25 min occlusion of the LAD attenuates the protection against arrhythmias that resulted from rapid cardiac pacing, 24h previously. In the presence of CBX this protective effect of pacing is reduced, indicating that closing of gap junctions in the already paced dogs eliminates, at least in part, the pacing-induced protection. Although the precise mechanisms, which underlie the gap junctional uncoupling effect of CBX is still not well understood, our results suggest that CBX by causing structural alterations in the intercalated disc, perhaps through the modification of Cx43 trafficking (and phosphorylation), interferes the protective (gap junction preserving) effect of cardiac pacing. Thus thee present data provide further evidence for the role of gap junctions in the delayed antiarrhythmic effect of preconditioning, induced by rapid cardiac pacing.

5.2. The effect of sodium nitrite on gap junction function during myocardial ischaemia

We have recently reported that sodium nitrite provides protection against the severe ventricular arrhythmias, resulting from coronary artery occlusion and reperfusion in anaesthetized dogs [83]. We have also evidence that gap junctions are involved in the generation of these acute arrhythmias, and also in the antiarrhythmic effect of preconditioning [59,61]. There is also substantial evidence that nitric oxide (NO) plays an important trigger and mediator role both in the early and the delayed antiarrhythmic effect of preconditioning [64-66,102]. It has been well documented that NO, among its several other effects, can modulate gap junction function [70,71,76,103], which effect would result protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias. [71]. On the basis of these previous observations it was obvious to examine, whether in the antiarrhythmic effect of inorganic sodium nitrite the modification of gap junction channels by NO would play a role. For this purpose, we administered sodium nitrite in intravenous infusion either 10 min prior to and throughout the occlusion ($NaNO_2$-PO) or 10 min prior to reperfusion ($NaNO_2$-PR).

We have found that sodium nitrite administered either before and during coronary artery occlusion the steep increase in resistivity and the decline in phase angle that usually occur

around the 14-15 min of ischaemia in the control animals were abrogated and the number of ectopic beats during phase 1b was markedly suppressed. There were also significant reductions in the ischaemia-induced elevation of epicardial ST-segment and increase in TAT. Similarly, in dogs in which the infusion of sodium nitrite was started at a time of the occlusion, when the phase 1b events are expected (i.e. 15 min of the ischaemia), there was an immediate reduction in the indices of ischaemia severity, as well as in the ischaemia-induced steep impedance changes, whereas the ectopic activity that occurred during phase 1b was simultaneously suppressed. We may draw, at least, three conclusions from these findings.

First, the effect of nitrite infusion is almost immediate, indicating a rapid conversion of nitrite to NO under ischaemic conditions [80,104,105]. We assume that by the time of the commencement of the infusion (i.e. 15 min of the occlusion) the ischaemic changes are sufficiently advanced to provide a milieu (low pH and oxygen tension) for the reduction of nitrite to NO [83].

Second, the short period of nitrite administration during ischaemia but prior to reperfusion can effectively increase NO bioavailability resulting in significant reduction in the ischaemic changes and its severe consequences, such as the occurrence of the life-threatening ventricular arrhythmias. The increased NO bioavailability resulting from a short period of sodium nitrite infusion prior to reperfusion, may favourably affect the reperfusion-induced severe ventricular arrhythmias, such as ventricular fibrillation, which is common in this canine model when the coronary artery is suddenly reopened [106]. We have found that only one nitrite-treated dog out of the remaining 12 dogs fibrillated during reperfusion in contrast with the controls, in which all the dogs that survived the occlusion died in VF on reperfusion. These of our findings are in accord with the results of Gonzales et al. [84]. They showed also in dogs that infusion of nitrite during the last 5 min of ischaemia significantly reduced infarct size and improved the recovery of contractile function after reperfusion [84]. The reduction in reperfusion-induced injury can be associated with the NO regulated suppression of the generation of oxidative stress products [66,83,107-109].

Third, the results of the present study confirm our hypothesis that NO, among its several other effects, may influence cardiac gap junctions and thereby modify the generation of the ischaemia and reperfusion-induced severe ventricular arrhythmias [70,71]. The effect of NO derived from sodium nitrite on gap junction channels is also immediate, since after the commencement of the infusion the second steep rise in resistivity and decline in phase angle were absent, and the number of VPBs during phase 1b was considerable suppressed. This

finding suggests that the infusion sodium nitrite just at a time of the occlusion period, when the serious, often life-threatening phase 1b arrhythmias start to appear, is able to inhibit the generation of these arrhythmias, and this effect can be associated with the preservation of gap junction by NO. Although it is still not known whether opening or closing of gap junctions by NO leads to the antiarrhythmic protection, our electrical impedance measurements suggests, by providing indirect evidence that sodium nitrite inhibits the further uncoupling of gap junctions, since it abolished those steep impedance changes that had preceded the occurrence of the phase 1b arrhythmias. Further information about the open/closed status of these channels would be provided by measuring Cx43 phosphorylation following nitrite administration. There is a consensus that the phosphorylated form of Cx43 can be associated with the open form of the channel, whereas a dephosphorylated form indicates the closure of gap junctions [45]. Although in this particular study the phospho/dephospho ratio of Cx43 was not determined, since the ischaemia-induced dephosphorylation of this protein is rapidly recovers following reperfusion, we have evidence from a previous study that NO, released, for example, from sodium nitroprusside [70] or from prolonged nitrite administration [71], maintains the phosphorylated form of Cx43 during ischaemia. Thus we may assume that NO, derived from any sources, such as nitrites, opens, or at least, keeps open these channels.

An interesting, and perhaps, clinically useful aspect of the rapid release of NO from nitrite during ischaemia the marked protection against the reperfusion-induced severe ventricular arrhythmias. The reduction in VF during reperfusion is particularly pronounced, resulting in a high rate of survival (85%) in the nitrite treated group. As to whether the attenuation of the reperfusion-induced VF in the nitrite infused dogs can be associated with a direct effect of NO on gap junctions during reperfusion we do not know, since the impedance changes is difficult to assess during reperfusion, particularly in control dogs, where VF commonly occurs soon after the reperfusion. Since, it is more than likely that the outcome of reperfusion, and thus the severity of reperfusion-induced arrhythmias, largely depends on the severity of the antecedent ischaemia, we may assume that the nitrite-induced less severe ischaemic changes and the suppression of arrhythmias during phase 1b, via the modulation of gap junctions, may contribute to the better survival of these animals from the combined ischaemia and reperfusion insult. These of our results confirm the findings of Gonzales et al. [84] who proposed that the administration of sodium nitrite during ischaemia, but prior to reperfusion might have a particular value for clinical point of view. They suggested that the administration of nitrite might be advantageous, as adjunctive pharmacological therapy to

coronary angioplasty interventions by improving myocardial salvage, and consequently, by providing a better cardiac function following reperfusion of an acute myocardial ischaemic insult [5]. Our results confirm these findings and provide evidence that, inorganic nitrites, administered prior to reperfusion, protect the myocardium against those severe, life-threatening ventricular arrhythmias that result from an acute ischaemia and reperfusion insult. This effect involves the modulation of gap junctions by NO, most probably by prolonging the opened status of these channels even during ischaemia.

In conclusion, this of our study provides further evidence that NO, derived from molecules which readily release NO, protects the heart against the acute ischaemia and reperfusion-induced early, often fatal rhythm disturbances. This antiarrhythmic protection can be, at least in part, due to the modulator effect of NO on gap junctions, since their function are immediately influenced and largely preserved after the administration of sodium nitrite. Although other effects, such as the preservation of endothelial function [81] or the favourable haemodynamic and anti-aggregator abilities of NO donors can not be excluded from the protective effect of sodium nitrite, the prompt inhibition of the impedance changes and, almost in parallel, the suppression of the phase 1b arrhythmias that occur immediately after the administration of sodium nitrite, suggest a direct effect on gap junctions. As to whether this involves the NO-induced increase in myocardial cGMP and a resultant decrease in the intracellular calcium level [110], which directly modulates gap junctions during ischaemia [111], or whether nitrite, through protein S-nitrosylation (e.g. S-nitrosylation of connexin43) result in modification in gap junction structure and/or function, warrants further investigations.

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9. ANNEX

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Regulation of gap junctions by nitric oxide influences the generation of arrhythmias resulting from acute ischemia and reperfusion *in vivo*

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Myocardial ischemia resulting from sudden occlusion of a coronary artery is one of the major causes in the appearance of severe, often life-threatening ventricular arrhythmias. Although the underlying mechanisms of these acute arrhythmias are many and varied, there is no doubt that uncoupling of gap junctions (GJs) play an important role especially in arrhythmias that are generated during phase Ib, and often terminate in sudden cardiac death. In the past decades considerable efforts have been made to explore mechanisms which regulate the function of GJs, and to find new approaches for protection against arrhythmias through the modulation of GJs. These investigations led to the development of GJ openers and inhibitors. The pharmacological modulation of GJs, however, resulted in conflicting results. It is still not clear whether opening or closing of GJs would be advantageous for the ischemic myocardium. Both maneuvers can result in protection, depending on the models, endpoints and the time of opening and closing of GJs. Furthermore, although there is substantial evidence that preconditioning decreases or delays the uncoupling of GJs, the precise mechanisms by which this attains have not yet been elucidated. In our own studies in anesthetized dogs preconditioning suppressed the ischemia and reperfusion-induced ventricular arrhythmias, and this protection was associated with the preservation of GJ function, manifested in less marked changes in electrical impedance, as well as in the maintenance of GJ permeability and phosphorylation of connexin43. Since we have substantial previous evidence that nitric oxide (NO) is an important trigger and mediator of the preconditioning-induced antiarrhythmic protection, we hypothesized that NO, among its several effects, may lead to this protection by influencing cardiac GJs. The hypotheses and theories relating to the pharmacological modulation of GJs will be discussed with particular attention to the role of NO.

Keywords: ischemia/reperfusion, arrhythmias, gap junction, nitric oxide

INTRODUCTION

Traveling on the London underground you may frequently hear "Mind the gap! Mind the gap!" This warning call is also valid for the heart when the genesis of arrhythmias is considered. Gaps not only separate but also connect cells by forming special channels, termed gap junctions (GJs), which allow fast electrical and metabolic cross-talk between the neighboring cells. In myocardial tissue, these GJ channels are accumulated in clusters located in the intercalated disks, and they represent low resistance pathways between the adjacent cells, allowing fast spread of impulse from the one cell to the other (electrical coupling). These channels can also transfer small molecules (less than 1,000 Da) resulting in tight metabolic intercellular communication (metabolic coupling). Since, the shape of the ventricular cardiomyocytes is elongated and the GJs are preferably located in the longitudinal end of the cell, under normal conditions, the action potential is propagated in longitudinal direction (Spach et al., 1981; Rudy and Quan, 1987; Peters and Wit, 1998; Rohr, 2004). This uniform anisotropy that mainly results from the structural arrangement (longitudinal vs. transversal) and electrical properties of GJs (low resistance), makes possible that the heart behaves as an electrical syncytium. However, under pathologic conditions, such as the acute myocardial ischemia, as the consequence of the rapid metabolic changes (Shaw and Rudy, 1997), these GJs are uncoupled, resulting in the closure of the low resistance pathways and changes in impulse propagation. In homogeneity (non-uniform anisotropy) develops within the cardiac tissue as regards the electrical conduction, which leads ultimately to arrhythmia generation (Spear et al., 1992; De Groot and Coronel, 2004).

The present paper will focus on the role of GJs in the generation of ventricular arrhythmias due to acute myocardial ischemia. We will discuss how the pharmacological modulation of GJs would influence these ischemia-induced early ventricular arrhythmias, and put forth a hypothesis, based mainly on our own studies in anesthetized dogs, that nitric oxide (NO), an important endogenous modulator of heart function, may also regulate cardiac GJs. We provide evidence that the effect of NO on GJs might have a

role in the cardioprotective (antiarrhythmic) effect of preconditioning and NO donors.

THE ROLE OF GAP JUNCTIONS IN THE ACUTE ISCHEMIA-INDUCED VENTRICULAR ARRHYTHMIAS

There seems to be consensus in respect that arrhythmias occurring soon (within 3 min) after the onset of the coronary artery occlusion result from those ionic and electrophysiological changes which are due to the rapid switch of myocardial metabolism from aerobic to anaerobic mode (Janse et al., 1986). These metabolic changes (loss of ATP, fall in intracellular pH, accumulation of lactate, etc.) are apparent within seconds or minutes after the onset of ischemia and directly affect the function of ion channels and exchangers, resulting in considerable alterations in impulse generation and conduction (Cascio, 2001). Without going into details, conditions develop during this early phase of ischemia favor reentry, which is thought to be the main mechanism underlying the phase Ia arrhythmias (Kléber, 1983; Janse et al., 1986).

Although processes underlying generation of phase Ib arrhythmias are less well understood, there is no doubt that uncoupling of GJs play an important role. As is mentioned above in the uniformly anisotropic heart the transfer of an impulse is largely dependent upon the resistance of GJs, which is lower in longitudinal than transversal direction (Hoyt et al., 1989; Saffitz et al., 1995). This provides longitudinal preference over transversal conduction (Spach et al., 1981; Peters and Wit, 1998) and a safety for normal cell-to-cell impulse propagation (Spach and Heidlage, 1995). However, under ischemic conditions, particularly with the progression of ischemia, the further loss of ATP and intracellular K^+, the accumulation of harmful metabolites and ions, the release of catecholamines, etc., would result in a milieu in which the uncoupling of GJs increases (White et al., 1990; Dhein, 1998). This leads to non-uniform changes in tissue resistance and inhomogeneous impulse conduction (Wojtczak, 1979; Kléber et al., 1987; Cascio et al., 2005) which initiate and maintain reentry during phase Ib (Spach et al., 1988). On the other hand, the increased resistance resulting from interruption of cell-to-cell coupling decreases the injury current, although at moderate levels of uncoupling this current would still be sufficient to induce delayed after-depolarization and trigger focal activity (Janse and van Capelle, 1982). Another consequence of the "metabolic overload" in the ischemic myocardium which largely accounts for the uncoupling of GJs is the reduced phosphorylation of connexin43 (Cx43), which is the primary structural protein of GJs in the ventricle (Söhl and Willecke, 2004). The ischemia-induced dephosphorylation of Cx43 results in conformational changes in connexin and leads to the closure of GJs and translocation of Cx43 from the membrane to the cytosol (Beardslee et al., 2000). This ischemia-induced Cx43 dephosphorylation and the subsequent closure of GJs occurs within 30 min (Beardslee et al., 2000; Schulz et al., 2003), making possible to use the measurement of Cx43 phosphorylation as a tool for the assessment of GJ function even during such a relatively short period of ischemia.

Functionally, GJ channels can be in open and closed state, although the conductance of a single channel may vary between several states – from closed, residual to the several levels of conducting (open) states – which are regulated by phosphorylation of the C-terminal of the connexin (Kwak and Jongsma, 1996). The assessment of GJ function particularly under *in vivo* conditions is rather difficult. Most of the currently used methods provide only indirect evidence on the coupling status of GJs. Measurement of GJ permeability using small molecular weight dyes (Ruiz-Meana et al., 2001) or the determination of connexin phosphorylation (Ando et al., 2005) allows evaluation of coupling only at a certain time point. Although measuring conduction velocity by activation mapping techniques (Rohr et al., 1998; Henriquez et al., 2001), or tissue impedance (resistivity and phase angle) changes by the use of a four-pin electrode method (Kléber et al., 1987; Cinca et al., 1997; Padilla et al., 2003) make possible continuous recording, these methods represent also only indirect assessment of GJ function. These methodological problems have been discussed in details previously (Garcia-Dorado et al., 2004; Végh and Papp, 2011). Nevertheless, despite these difficulties the combination of the available methods and techniques allow us to estimate the function of GJs and their role in arrhythmogenesis under various physiological and pathophysiological conditions.

THE ROLE OF GAP JUNCTIONS IN ARRHYTHMOGENESIS AND IN THE ANTIARRHYTHMIC EFFECT OF PRECONDITIONING

There were two studies (Smith et al., 1995; Cinca et al., 1997), both performed in anesthetized pigs, which provided the first *in vivo* evidence that GJs play an important role in the generation of the ischemia-induced ventricular arrhythmias. The first study pointed out a relationship between changes in tissue impedance and the occurrence of arrhythmias, showing that the appearance of phase Ib arrhythmias during a 60-min coronary artery occlusion was preceded by a steep increase in tissue resistivity around the 15 min of ischemia (Smith et al., 1995). The second study (Cinca et al., 1997) reported that ischemic preconditioning delays uncoupling of GJs and shifts the onset of the Ib phase arrhythmias to a later period of the ischemia. Our own studies in dogs (Papp et al., 2007) showed somewhat similar results, but the rise in tissue resistivity prior to the occurrence of the phase Ib arrhythmias was not as marked as either in pigs (Smith et al., 1995) or isolated heart preparation (Kléber et al., 1987). Furthermore, preconditioning in dogs not only delayed but significantly decreased the tissue impedance changes (Papp et al., 2007) and, as that we have pointed out previously (Végh et al., 1992a), preconditioning resulted in an absolute reduction in the number and severity of arrhythmias without shifting them to a later period of the occlusion. Preconditioning also preserved GJ permeability and phosphorylation of Cx43 determined both at 25 and 60 min of ischemia, suggesting that preconditioning in this species not only delays but indeed reduces the closure of GJs (Papp et al., 2007). There might be many explanations of these dissimilarities, among which the difference in the preexisting collateral system between dogs and pigs seems to play a major role. This has been thoroughly discussed previously (Végh and Papp, 2011).

Although the mechanisms by which preconditioning influences GJ coupling has not yet been elucidated, it seems reasonable to hypothesize that mediators and signaling pathways, which are thought to play role in this form of cardioprotection, may target

and modify GJs, perhaps at the level of connexins. This hypothesis is supported by the fact that GJ channels exist and can switch between various conductance states, which depend on the phosphorylation status of connexins (Kwak et al., 1995; Kwak and Jongsma, 1996). The phosphorylation of the C-terminal of connexins, which determines whether GJs are in open or closed state, involves kinases or kinase-mediated signaling pathways which are activated in response to a preconditioning stimulus. Thus, several kinases, such as protein kinase A (PKA), the various isoforms of PKC, PKG, as well as mitogen-activated protein (MAP) and tyrosine kinases (TK), etc., which have been identified as parts of the preconditioning-induced signaling cascade (Downey et al., 2008), were also shown to target connexins (Dhein, 2004; Salameh and Dhein, 2005). For example, the preconditioning-induced reduction in myocardial damage was associated with a PKC-activated enhanced Cx43 phosphorylation in the rabbit isolated hearts (Miura et al., 2004).

Since the different kinases and kinase isoforms may phosphorylate connexins differently, the resulting responses regarding the regulation of GJ coupling would also be different. Indeed, there are many, sometimes conflicting results reported in both normal and diseased hearts as concerns the activation of a certain kinase pathway and changes in GJ function (Salameh and Dhein, 2005; Dhein et al., 2011). These differences seem to largely depend on the preparations, models and species used, as well as on the experimental conditions applied. Since the regulatory role of the various kinase and signaling pathways on GJs have been excellently discussed previously (e.g., Dhein, 1998; Salameh and Dhein, 2005), it is not purposed to discuss these further. *Nota bene* the exploration of mechanisms which affect GJ function led to the idea that the generation of arrhythmias might be influenced through the modulation of GJs (Dhein et al., 2010).

PHARMACOLOGICAL MODIFICATION OF GAP JUNCTIONAL COUPLING AND ARRHYTHMIAS

During the past two decades, a number of drugs have been described and developed which facilitate or inhibit the coupling of GJs (reviewed by Dhein, 2004; Dhein et al., 2010). These were used, in part, as tools for obtaining information on the physiological and pathophysiological roles of GJs, in part, as drugs purposing to develop novel antiarrhythmic therapy (Dhein and Tudyka, 1995; Dhein, 2004; Salameh and Dhein, 2005). However, the pharmacological modification of GJ coupling raises also many questions, in particular, when the acute ischemia-induced ventricular arrhythmias are considered. It is still not clear whether opening or closing of GJs during ischemia would be advantageous for arrhythmia suppression. As we, and others (De Groot et al., 2001; De Groot and Coronel, 2004; Végh and Papp, 2011) have suggested both maneuvers can result in protection. There is no doubt that keeping GJs open during ischemia and thereby maintaining conduction velocity (De Groot and Coronel, 2004) would result in an antiarrhythmic effect. This has been proved by several *in vitro* and *in vivo* studies using synthetic antiarrhythmic peptides, such as AAP10 and rotigaptide (Dhein et al., 1994; Müller et al., 1997; Grover and Dhein, 2001; Xing et al., 2003, 2005; Végh and Papp, 2011). However, more controversial results were obtained with the use of uncouplers, indicating the complexity of the regulation of GJs in both normal and diseased hearts (Garcia-Dorado et al., 1997; Salameh and Dhein, 2005). These differences may be related to the uncoupler used, the model and endpoint examined, as well as the time of administration of the uncoupler to close GJs (Végh and Papp, 2011).

We have experimental evidence that in dogs both the GJ opener rotigaptide and the uncoupler carbenoxolone given prior to and during coronary artery occlusion protected against the ischemia-induced severe ventricular arrhythmias (Végh and Papp, 2011). The fact that the uncoupler carbenoxolone induced an antiarrhythmic effect was indeed surprising, since one would have expected that closing of GJs during ischemia result in enhanced gap junctional uncoupling and arrhythmias. The results of tissue resistivity measurements showed that immediately after the onset of the coronary artery occlusion the decline in phase angle (a measure of increased membrane capacitance due to closure of GJs; Padilla et al., 2003) was more marked in the carbenoxolone treated dogs than in the controls (Papp et al., 2008; Végh and Papp, 2011). Although these early impedance changes are thought not to be attributed to closure of GJs (Kléber et al., 1987), it cannot rule out the possibility that there might be cells within the ischemic area which are severely injured and uncoupled even soon after the onset of the coronary artery occlusion (Wolk et al., 1999; Daleau et al., 2001; Vetterlein et al., 2006). Furthermore, in dogs infused with carbenoxolone the steep increase in resistivity and decline in phase angle that occur usually around the 15 min of the occlusion were also absent. In these dogs the two characteristic arrhythmia phases disappeared, and although ectopic activity could be observed over the entire occlusion period, the total number of ectopic beats was significantly less than in the controls (Végh and Papp, 2011). We proposed that this finding could perhaps be associated with the phenomenon termed "paradoxical restoration of conduction" (Rohr et al., 1997). This suggests that in the border zone, the viable cells are electrically depressed through electrotonic interactions from their neighboring ischemic cells resulting in slowing of conduction (De Groot and Coronel, 2004). However, with the facilitation of uncoupling, such as may occur during ischemia in the presence of an uncoupler, this electrotonic interaction decreases, resulting in an improvement in conduction and, subsequently, a reduction in arrhythmia severity (De Groot and Coronel, 2004). Whatever the precise mechanism is, it seems that carbenoxolone given prior to and during ischemia attenuates impedance changes during the "critical" phase of ischemia and reduces phase Ib arrhythmias, and this effect is similar to that seen with the GJ opener rotigaptide and with preconditioning (Papp et al., 2008; Végh and Papp, 2011).

Interestingly, carbenoxolone almost completely abolished the antiarrhythmic effect of ischemic preconditioning. When it was given prior to and during the preconditioning procedure (two 5-min occlusion and reperfusion insults) both the impedance changes and the ectopic activity were markedly increased during the short ischemic periods compared to the preconditioned dogs without carbenoxolone administration (Papp et al., 2007). In these carbenoxolone treated preconditioned dogs the tissue impedance changes during the prolonged occlusion were as marked as in the non-preconditioned controls, and the severity of arrhythmias, particularly during phase Ib, was also substantially increased.

Furthermore, preservation of the phosphorylated form of Cx43 afforded by preconditioning was abolished with the administration of carbenoxolone. Our conclusion was that closing of GJs prior to preconditioning perhaps inhibits the transfer of endogenous substances that are released by the short preconditioning ischemia and reperfusion insults thus inhibiting the activation of signaling pathways leading to cardioprotection (Papp et al., 2007).

As has been mentioned above, many endogenous substances are thought to regulate GJs function by activating various protein kinases (Dhein, 1998; Salameh and Dhein, 2005). Our previous research focused on the exploration of mechanisms involved in the antiarrhythmic effect of ischemic preconditioning, provided substantial evidence that NO is one of the key mediators which plays essential trigger and mediator role in the preconditioning-induced cardioprotection (Végh et al., 1992c). Thus it seemed reasonable to hypothesize that the antiarrhythmic effect of preconditioning and of NO donors (György et al., 2000) may, in part, be accomplished through the modulation of GJ channels.

EVIDENCE FOR THE ROLE OF NITRIC OXIDE IN THE REGULATION OF CARDIAC GAP JUNCTIONS

The evidence that NO may modulate GJ function comes mainly from studies in non-cardiac tissues (Roh et al., 2002; Patel et al., 2006), especially from those which are dealing with vessel physiology where NO is one of the most important physiological mediators (Kameritsch et al., 2003; Rodenwaldt et al., 2007). These studies showed that NO is able to modify GJ permeability (Bolanos and Medina, 1996; Kameritsch et al., 2003) and the expression of connexin isoforms (Roh et al., 2002; Hoffmann et al., 2003; Yao et al., 2005). This latter would be especially important under chronic conditions where the regulatory role of NO on the expression of connexins has to be considered in terms of the development of chronic heart diseases (Poelzing and Rosembaum, 2004; Akar et al., 2007; Kontogeorgis et al., 2008; Kim et al., 2010; Radosinska et al., 2011). Changes in Cx43 expression play also an important role in the delayed phase of cardioprotection induced by rapid cardiac pacing 24 h prior to ischemia in dogs (Gönczi et al., 2012). In case of the acute and shorter periods of ischemic challenge (such as a 30- to 60-min ischemia) and its arrhythmia consequences, the alterations of GJ conductance, resulting from changes in connexin phosphorylation, seem to be the more likely mechanism through which NO may modify GJ function. However, the signaling pathways, which regulate the level and phosphorylation status of Cx43 and thus modulate the GJ channel properties, are even less well understood in the myocardium than in the other non-cardiac tissues. For example, it has been proposed that stimulation of both α_1 and β adrenoceptors, although through the activation of different pathways and protein kinases (PKC and PKA, respectively), leads to connexin phosphorylation and to the opening of GJs (Saez et al., 1997; Weng et al., 2002). 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 loss in total Cx43 protein content was restored by acetylcholine and also by the administration of the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP; Zhang et al., 2006). Since the protective effect of acetylcholine was inhibited by L-NAME, it was suggested that acetylcholine prevents the hypoxia-induced decrease of Cx43 and improves GJ coupling via a NO-mediated pathway.

In our own studies, using sodium nitroprusside (SNP) as an NO donor and administered in intracoronary infusion 20 min prior to and throughout a 60-min occlusion period of the left anterior descending (LAD) coronary artery in anesthetized dogs, we have found that SNP almost completely abolished the severe ventricular ectopic activity and attenuated the increase in tissue resistivity but it did not substantially influence the decrease in phase angle that resulted from occlusion (Gönczi et al., 2009). In the presence of SNP infusion, there was indeed a more marked reduction in phase angle during the first 10-min period of occlusion; and this effect was very similar to that seen with the administration of carbenoxolone (Papp et al., 2008; Végh and Papp, 2011). Furthermore, SNP, like carbenoxolone, abrogated the steep decline in phase angle that occurred in the controls just prior to the appearance of the phase Ib arrhythmias; i.e., the impedance changes remained virtually constant during this critical period of ischemia (i.e., between 15 and 20 min). Despite similarities of impedance changes of SNP and carbenoxolone, these *in vivo* impedance measurements do not provide an answer to the question, as to whether NO, derived from SNP, opens or closes GJs, and whether opening or closing of GJs leads to the antiarrhythmic effect of SNP. However, the fact, that in the presence of SNP the rapid impedance changes that precede the occurrence of phase Ib arrhythmias were markedly attenuated (and in parallel the ectopic activity was virtually disappeared), suggests a preserved GJ function during ischemia and confirms that of our previous supposition that the rate of uncoupling prior to phase Ib is of particular importance in the generation of arrhythmias (Papp et al., 2007; Végh and Papp, 2011). A further evidence that NO may preserve GJ function derived from the *in vitro* measurements. These showed that compared to the controls, SNP maintained GJ permeability and Cx43 phosphorylation even after 60 min of ischemia. In the presence of SNP, the membrane fraction of Cx43 remained largely in phosphorylated form and the metabolic coupling of the adjacent cells was significantly improved. Thus it seems from these results that NO, derived from NO donors, protects the heart against the ischemia-induced early ventricular arrhythmias, and that this effect, at least in part, can be attributed to the effect of NO, or of the NO-stimulated pathways on GJs, as their function is largely preserved in the presence of SNP (Gönczi et al., 2009).

More recent experimental data resulting from the administration of sodium nitrite support this hypothesis. Under experimental conditions sodium nitrite is used as an exogenous nitrite source to prove the importance and the potential therapeutic benefit of nitrite anion. Inorganic nitrites and nitrates, which are natural oxidative metabolites of NO, have been considered for a long time as inert molecules playing not a compelling role in NO physiology. However, over the last decade emerging evidence suggests that inorganic nitrites and nitrates may serve as important reservoirs for NO (reviewed, e.g., Lundberg and Govoni, 2004; Lefer, 2009), since these metabolites, particularly under hypoxic and anoxic conditions, can readily be reduced back to NO (Zweier et al., 1995; Bryan, 2006). This mechanism may provide an increased NO availability under ischemic conditions independently from NO

synthase (NOS) activity which is otherwise reduced in the absence of oxygen (Zweier et al., 1995). A number of studies in various experimental animal models have proved that nitrite anion has an important biological function and might represent an effective means to attenuate ischemia and reperfusion injury (e.g., Webb et al., 2004; Duranski et al., 2005; Shiva et al., 2007).

Thus in our anesthetized dog model, we infused sodium nitrite intravenously in a dose of 0.2 μg kg^{-1} min^{-1}, starting the infusion 10 min prior to and maintained throughout the entire 25 min occlusion of the LAD coronary artery, and changes in tissue impedance in parallel with arrhythmia distribution were assessed (Gönczi et al., 2010). We found that in the presence of sodium nitrite infusion the total number of ventricular premature beats during the occlusion was markedly reduced (472 $\pm$ 105 vs. 147 $\pm$ 77; $P < 0.05$) and the impedance changes were substantially less pronounced than in the controls (Gönczi et al., 2010). This is illustrated in **Figure 1** which clearly shows that in dogs infused with sodium nitrite, the steep increase in resistivity and the decline in phase angle that usually occur around the 14–15 min of ischemia in the control animals were abrogated and the number of ectopic beats during phase Ib was markedly suppressed. In these experiments we also used a mapping electrode, which collects signals from 31 unipolar electrode points of the epicardial surface of the ischemic area in order to evaluate changes in the epicardial ST-segment and in total activation time (TAT) by creating ST and activation maps. The results show that compared with control dogs, in dogs infused with sodium nitrite both the ischemia-induced increases in epicardial ST-segment and TAT were considerable reduced (**Figure 2**). In this study, at the end of the 25 min occlusion period, myocardial tissue samples were taken from the hearts for the assessment of metabolic coupling and Cx43 phosphorylation, as has been described previously (Gönczi et al., 2009). **Figure 3A** shows that the administration of sodium nitrite preserved the phosphorylated form of Cx43 within the ischemic LAD area compared with the control hearts in which the occlusion of the LAD resulted in marked dephosphorylation of Cx43. GJ permeability, determined by double dye loading (Ruiz-Meana et al., 2001; Papp et al., 2007), was also maintained even after the 25 min of ischemia in hearts infused with sodium nitrite (**Figure 3B**).

The results support our previous proposal (Végh and Papp, 2011) that in arrhythmia point of view the modification of GJ function, for example, by preventing the ischemia-induced dephosphorylation of Cx43, would particularly be important during that "critical" phase of ischemia when the rate of uncoupling of GJs rapidly increases, and when other factors, implicated in arrhythmogenesis, are also present. Furthermore, we suggest that NO might be one of the endogenous substances which would regulate GJs not only in vascular tissues (reviewed recently by Looft-Wilson et al., 2012) but also in cardiac myocytes. There is emerging evidence for a cross-talk between NO signaling and connexins in the vasculature which is essential for normal vascular function (Looft-Wilson et al., 2012). Although a strong proof is lacking for such an NO-mediated modulation of GJ proteins in cardiac myocytes, we assume that there might be similar interactions between NO and GJs also within the myocardium, since NO derives either from the "classical" NO donors or inorganic nitrites, or generated during a preconditioning stimulus influenced the electrical and metabolic properties of GJs and resulted in simultaneous alterations in arrhythmia generation. We have proposed previously the most likely scenario for the antiarrhythmic effect afforded by preconditioning is that the preconditioning stimulus triggers the generation and the release of NO from the vascular endothelial cells and also from cardiac myocytes (Parratt and Végh, 1996; Végh and Parratt, 1996). NO by diffusing to cardiac myocytes stimulates soluble guanylyl cyclase and increases cGMP within the myocardium since the inhibition of soluble guanylyl cyclase completely abolished the antiarrhythmic protection (Végh et al., 1992b). cGMP could modify arrhythmogenesis by a number of ways involving the inhibition of calcium entry through L-type calcium channels (Sun et al., 2007), modification of the cGMP/cAMP balance by influencing cGMP-dependent phosphodiesterase and/or the direct depression of cardiac myocytes, resulting in reduced oxygen demand during prolonged ischemia (Parratt and Végh, 1996; Végh and Parratt, 1996). There is evidence that in vascular endothelium both the endogenously produced (Straub et al., 2011) and the exogenously administered (Hoffmann et al., 2003; Rodenwaldt et al., 2007) NO can acutely increase GJ coupling by a cGMP-dependent mechanism. cGMP through the inhibition of the cGMP-dependent phosphodiesterase prevents the degradation of cAMP and stimulates the cAMP–PKA pathway (Francis et al., 2010). This has been shown to enhance the coupling of GJs (Hoffmann et al., 2003). The stimulation of the soluble guanylyl cyclase-cGMP pathway by NO and the subsequent activation of protein kinase G (Patel et al., 2006) might be another signaling mechanism which can lead to connexin phosphorylation and modification of GJ coupling (Lampe and Lau, 2004).

More recent studies suggests that NO can modify GJ function independent from the activation of the NO-induced cGMP–PKG pathway. Such a mechanism is *S*-nitrosylation during which NO reversible binds to the thiol groups of cysteine residue of proteins resulting in *S*-nitrosothiols (SNO). *S*-nitrosylation not only allows the storage and transport of NO (Dejam et al., 2004; Lima et al., 2010) but modulates the activity of several cardiac functions, including cardiac ion channels (Gonzalez et al., 2009), mitochondrial respiration (Sun et al., 2006, 2007), formation of reactive oxygen species (Sun et al., 2006), or gap junctional connexins (Straub et al., 2011). For example, in the myoendothelial junction, where the vascular endothelial and smooth muscle cells are connected NO has been found to enhance the opening of this special form of GJs through *S*-nitrosylation of Cx43 (Straub et al., 2011). It is reasonable to assume that *S*-nitrosylation of Cx43 would be a possible alternative mechanism by which NO regulates the function of GJs also in cardiac myocytes, especially under conditions of increased NO availability. This may occur, for example, after preconditioning (Kiss et al., 2010), the administration of NO donors (György et al., 2000; Gönczi et al., 2009), including sodium nitrite. There is evidence that *S*-nitrosylation plays an important role in cardioprotection afforded by preconditioning (Sun et al., 2007; Murphy and Steenbergen, 2008). As to whether *S*-nitrosylation of Cx43, indeed, plays a role in the modulation of GJ function by NO and, if so, how much this mechanism accounts for the antiarrhythmic effect is still not known and warrants further examinations.

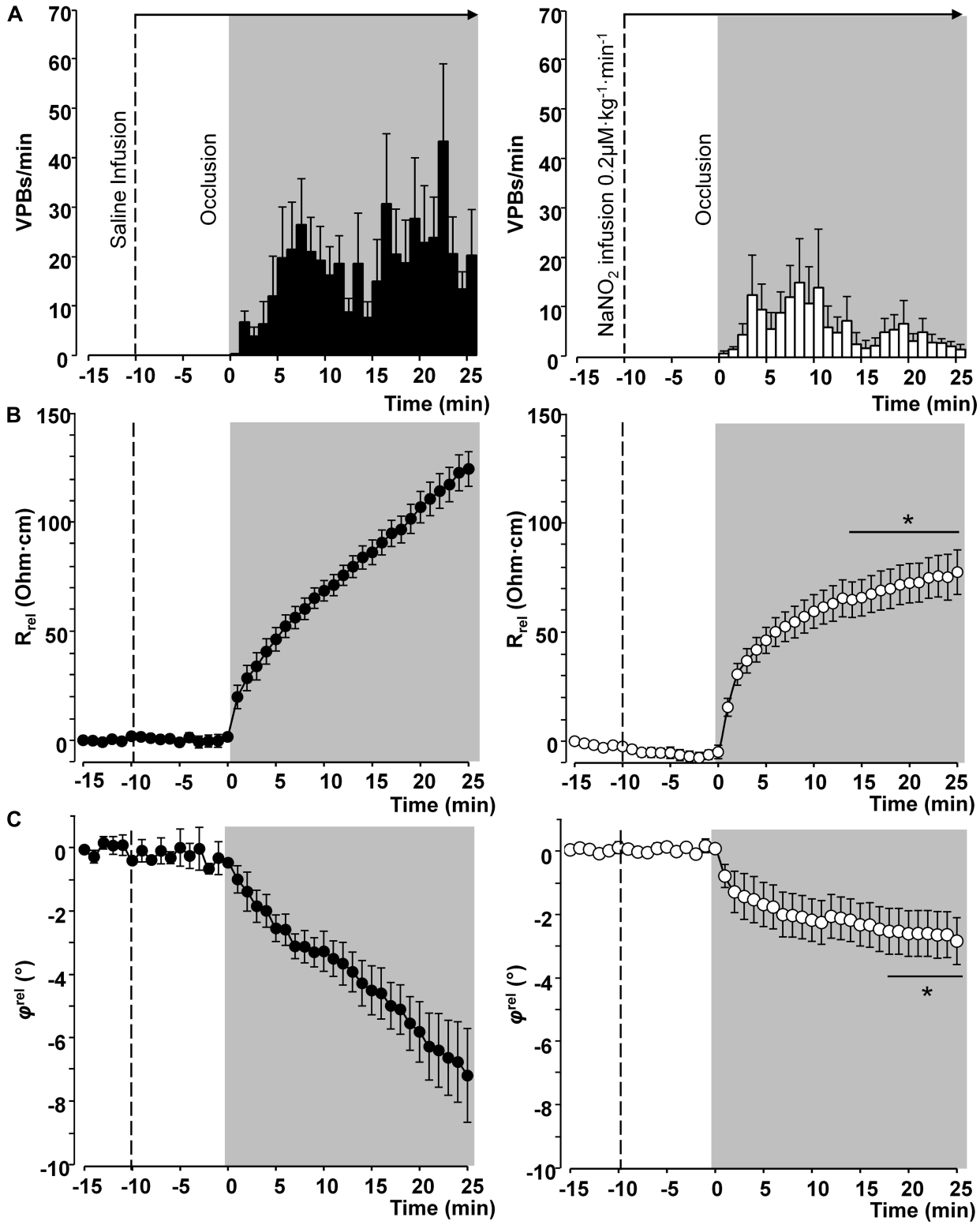

FIGURE 1 | Distribution of ventricular premature beats (VPBs) and relative changes in tissue impedance (resistivity and phase angle) at one minute intervals during a 25-min coronary artery occlusion in control dogs and in dogs infused with sodium nitrite. Compared with the controls, the infusion of sodium nitrite markedly reduced the number of VPBs **(A)** and attenuated the rise in tissue resistivity **(B)** and the decline in phase angle **(C)**, particularly during the critical period of ischemia (between 15 and 25 min) when the change of phase angle remained virtually constant. Values are means ± SEM obtained from nine dogs in each group. $*P < 0.05$ compared with the controls.

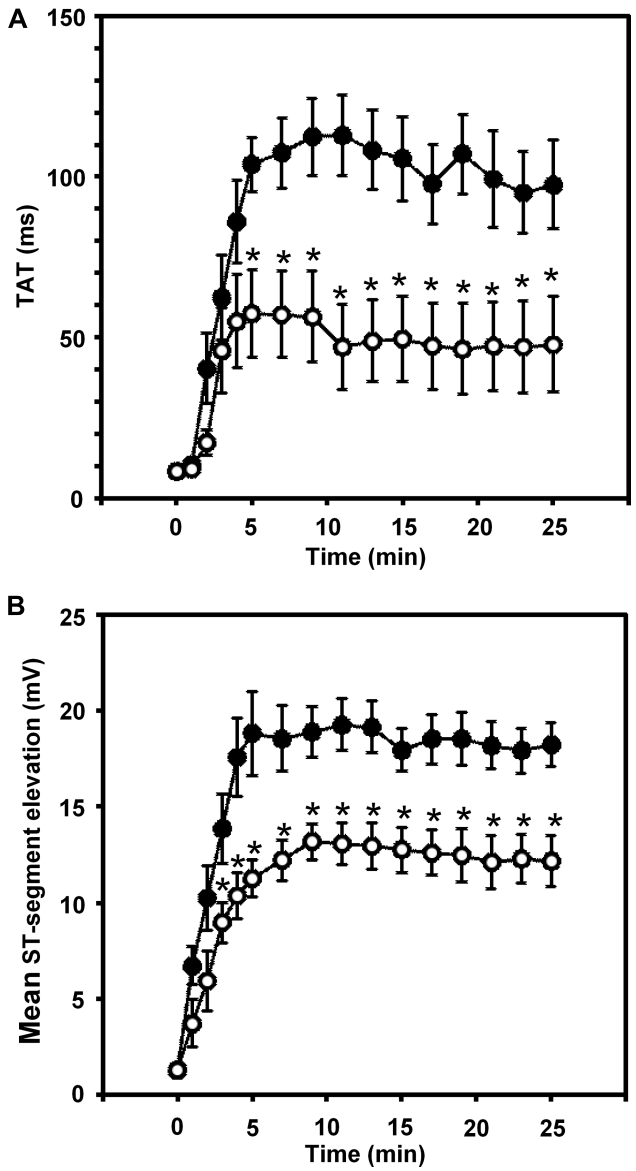

FIGURE 2 | Changes in the total activation time (TAT) (A) and in the epicardial ST-segment (B) during a 25-min occlusion of the anterior descending branch of the left coronary artery. In control dogs, both indices of ischemia severity were markedly increased, especially during the initial 5 min of the occlusion. These changes were significantly reduced in the presence of the intravenous infusion of sodium nitrite. Values are means ± SEM. $^{*}P < 0.05$ compared with the controls.

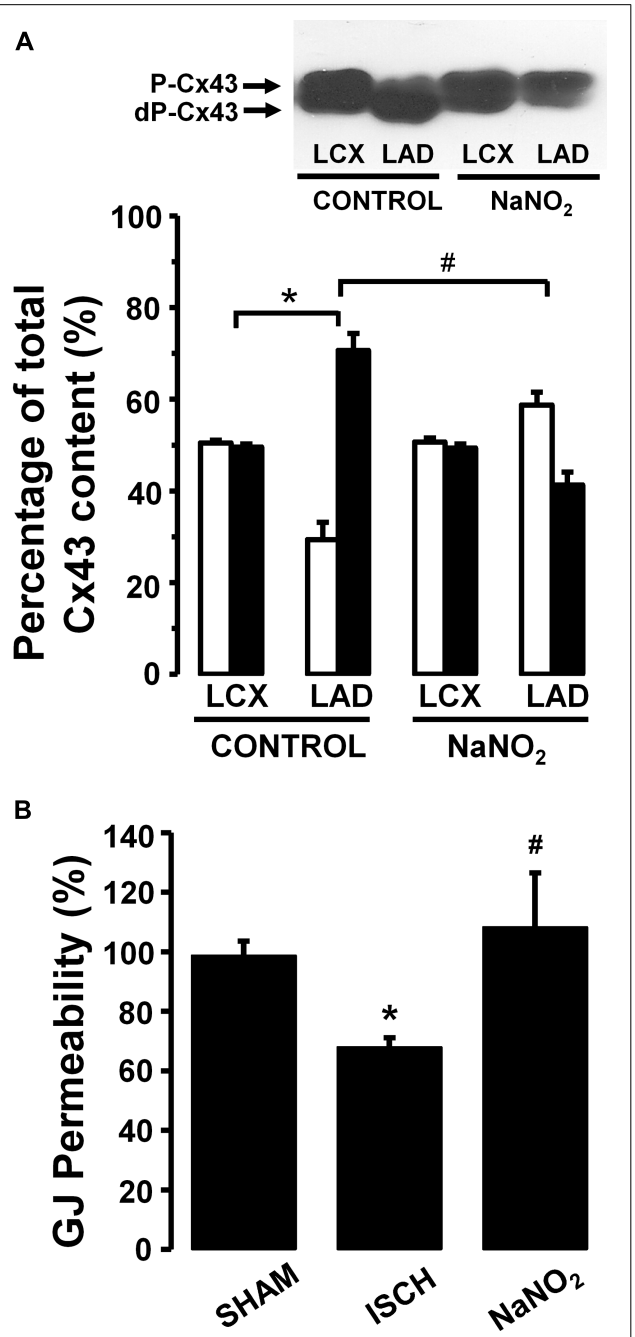

FIGURE 3 | (A) A representative Western blot and changes in the phosphorylated (P-Cx43; open columns) and dephosphorylated Cx43 (dP-Cx43; filled columns) isoforms as a percentage of the total sarcolemmal Cx43 content, following a 25-min LAD occlusion The phospho/dephospho ratio within the normal area is around 51/49 ± 1%. This shifted to 29/71 ± 4% in hearts of the control dogs when subjected to a 25-min occlusion. Infusion of sodium nitrite prevented this shift and preserved the phosphorylated form of this protein both within the normal non-ischemic (52/48 ± 1%) and the ischemic myocardial region (59/41 ± 3%). **(B)** Changes in gap junction permeability in sham-control (SHAM) and ischemic control (ISCH) hearts, as well as in hearts infused with sodium nitrite ($NaNO_2$). Sodium nitrite prevented the ischemia-induced reduction in gap junction permeability. Values are means ± SEM. $^{\#}P < 0.05$ compared with the ischemic control samples. $^{*}P < 0.05$ compared with non-ischemic samples.

SUMMARY

We hypothesized that NO derives from either endogenous (induced by preconditioning) or exogenous sources (administration of NO donors) is able to modulate GJ function, and that this effect of NO, in part, plays a role in the protection against the severe ventricular arrhythmias that results from an acute ischemia and reperfusion insult in anesthetized dogs. To support this hypothesis in the present article we summarized our results obtained from previous and more recent studies which aimed to examine

the regulatory role of NO on cardiac GJs in relation to arrhythmogenesis (Gönczi et al., 2009, Gönczi et al., 2010). The results give a strong support for this hypothesis, since in the presence of increased NO availability the function of GJs seems to be well preserved, as have been shown by both the *in vivo* and *in vitro* measurements. These measures, albeit provide only indirect evidence, clearly indicate that a maintained NO availability during a prolonged ischemic insult, resulting from either a preconditioning stimulus or the administration of drugs that liberate NO, inhibits the ischemia-induced tissue impedance changes and dephosphorylation of Cx43, and maintains the metabolic coupling between cells. These effects of NO are especially pronounced during that critical period of ischemia when factors and mechanisms, involved in the generation of the phase Ib arrhythmias are present and fully activated. As a result of the preserved GJ function, the Ib phase of arrhythmias are markedly suppressed. Although the precise mechanisms by which NO attains this GJ modulating effect is still not fully understood, we discussed hypotheses and theories which propose a role for NO in the regulation of GJs. These involve NO-mediated signaling cascades including protein kinases which might have a role in connexin phosphorylation, the classical NO-soluble guanylyl cyclase-cGMP pathway with the subsequent PKG activation and the cGMP-independent mechanism of NO through which NO is able to bind and modify proteins via *S*-nitrosylation. As to whether all these mechanisms are acting together or there is one particular mechanism which preferentially acts under certain circumstances is unknown and requires further investigations.

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RESEARCH ARTICLE

Effect of Sodium Nitrite on Ischaemia and Reperfusion-Induced Arrhythmias in Anaesthetized Dogs: Is Protein S-Nitrosylation Involved?

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Abstract

Background and Purpose

To provide evidence for the protective role of inorganic nitrite against acute ischaemia and reperfusion-induced ventricular arrhythmias in a large animal model.

Experimental Approach

Dogs, anaesthetized with chloralose and urethane, were administered intravenously with sodium nitrite (0.2 $\mu molkg^{-1}min^{-1}$) in two protocols. In protocol 1 nitrite was infused 10 min prior to and during a 25 min occlusion of the left anterior descending (LAD) coronary artery ($NaNO_2$-PO; n = 14), whereas in protocol 2 the infusion was started 10 min prior to reperfusion of the occluded vessel ($NaNO_2$-PR; n = 12). Control dogs (n = 15) were infused with saline and subjected to the same period of ischaemia and reperfusion. Severities of ischaemia and ventricular arrhythmias, as well as changes in plasma nitrate/nitrite (NOx) levels in the coronary sinus blood, were assessed throughout the experiment. Myocardial superoxide and nitrotyrosine (NT) levels were determined during reperfusion. Changes in protein S-nitrosylation (SNO) and S-glutathionylation were also examined.

Key Results

Compared with controls, sodium nitrite administered either pre-occlusion or pre-reperfusion markedly suppressed the number and severity of ventricular arrhythmias during occlusion and increased survival (0% vs. 50 and 92%) upon reperfusion. There were also significant decreases in superoxide and NT levels in the nitrite treated dogs. Compared with controls,

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increased SNO was found only in $NaNO_2$-PR dogs, whereas S-glutathionylation occurred primarily in $NaNO_2$-PO dogs.

Conclusions

Intravenous infusion of nitrite profoundly reduced the severity of ventricular arrhythmias resulting from acute ischaemia and reperfusion in anaesthetized dogs. This effect, among several others, may result from an NO-mediated reduction in oxidative stress, perhaps through protein SNO and/or S-glutathionylation.

Introduction

Inorganic nitrite and nitrate, the nautal metabolites of nitric oxide (NO) have been considered for a long time as inert molecules without further biological activity. More recently this concept has been challenged, due to the accumulating experimental and clinical evidence, which shows that these molecules may play an important physiological role in mediating the biological effects of nitric oxide [1, 2]. It has been recognized that these oxidative metabolites of NO are readily reduced back to NO, especially under hypoxic or anoxic conditions, when a drop in pH and oxygen tension promotes reductive processes [3]. This enzyme-independent NO formation might be of particular importance during ischaemia when in the absence of oxygen, the NO production by nitric oxide synthase (NOS) enzymes becomes limited. Thus nitrite can serve as a fundamental natural store of NO that serves to maintain cardiac function under ischaemic conditions [4, 5].

There is strong evidence that increasing NO bioavailability, for example, by the administration of organic nitrites and nitrates, which are thought to act as NO donors [6], suppresses the generation of arrhythmias that results from acute ischaemia and reperfusion in dogs [7–9] and also in humans [10, 11]. Although the precise mechanism of this antiarrhythmic effect is not fully elucidated, clinical studies have revealed that nitroglycerin causes electrophysiological alterations in the human heart [10], and reduces ectopic activity during acute myocardial infarction [11]. More recently we have reported that the antiarrhythmic effect of sodium nitroprusside is associated with the preservation of gap junctional function [9]. Another way to increase NO availability is via ischaemic preconditioning. We have evidence that preconditioning induced either by brief ischaemic episodes [12], cardiac pacing [13–15] or heavy physical exercise [16], stimulates the generation of NO in vascular endothelial cells and also in cardiac myocytes [17], and this NO plays an important trigger and mediator role in both the early and delayed cardioprotective (antiarrhythmic) effects of preconditioning [13–15, 18].

Nitric oxide exerts its cardioprotective effects via cGMP-dependent and cGMP-independent pathways [3, 19, 20]. For example, in dogs the antiarrhythmic effect of preconditioning was completely abolished by the inhibition of the NO-cGMP pathway [21]. On the other hand, we hypothesize that the preservation of NO availability following preconditioning and the subsequent reduction in superoxide production, which plays an essential role in the antiarrhythmic effect of preconditioning [22], could also contribute to the protection, at least in part, via cyclic-GMP-independent mechanisms, such as protein S-nitrosylation and S-transnitrosylation [23–25]. These are thought to provide protection by preventing the oxidation of proteins during the initial phase of reperfusion, when a burst of reactive oxygen species (ROS) usually occurs [26]. S-nitrosylation (SNO) is the covalent attachment of an NO moiety to a cysteine residue of proteins. Formation of SNO allows for storage and transport of NO [27], as well as

the regulation of several cardiac functions by NO, including the regulation of cardiac ion channels [28, 29], mitochondrial respiration [30] and ROS generation [23]; all of these may be connected to arrhythmogenesis [31].

Although a number of studies have shown that inorganic nitrites and nitrates, as natural reservoirs for NO, may regulate cardiovascular function and provide cardioprotection [32–34], there is a lack of information how these modify the severity of arrhythmias. Therefore, we designed studies in anaesthetized dogs to examine whether sodium nitrite provides protection against the acute ischaemia and reperfusion-induced severe ventricular arrhythmias, and if so, whether this effect involves protein SNO. Preliminary accounts of these studies were given at the ISHR World Congress in Kyoto [35] and at the meeting on 'Approaching the Clinic: Nitrite and Nitrate Pathophysiology and Therapy' in Atlanta, USA [36].

Methods

Ethics statement

The origin and upkeep of these dogs were in accord with 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 Institutes of Health (NIH Publication No. 85–23, revised 1996), and conformed to the Directive 2010/63/EU of the European Parliament. The protocols have been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/1211/2012).

Surgical procedures

Inbred mongrel dogs of either sex, and with a mean body weight in excess of 17 kg (mean 24 ± 3 kg) were anaesthetized with an intravenous bolus injection of pentobarbitone (0.5 mg kg-1 i.v., Euthanyl Bimeda-MTC Animal Health Inc.) and all efforts were made to minimize suffering. Prior to the studies the dogs were housed in an animal room (temperature:10–20°C, humidity: 40–70%, lightening: 12 h per day, 2 animals per pen), for at least two weeks and fed a standard diet with free access to water. Food was withdrawn 24 h before anaesthesia.

After catheterization of the right femoral vein, the anaesthesia was maintained with the intravenous administration of a mixture of chloralose and urethane (60 and 200 mg kg-1, respectively; Sigma, St. Louis, MO, USA). The depth of anaesthesia was monitored by the examination of the cornea and pain reflexes as well as by the measurement of blood pressure, and when it was necessary, a further bolus injection of the anaesthetic was given. The dogs were ventilated with room air using a Harvard respirator at a rate and volume sufficient to maintain arterial blood gases within normal limits [12, 22]. Body temperature was measured from the mid-esophagus and maintained at 37 ± 0.5°C.

Catheters (Cordis F4) were inserted into the right femoral artery for monitoring arterial blood pressure (systolic, diastolic, and mean), and into the left ventricle (LV) for the measurement of LV systolic and end-diastolic pressures and LVdP/dt. All pressure parameters, together with a chest lead electrocardiogram, were measured with a Plugsys Hemodynamic Apparatus (Hugo Sachs Electronics, Germany) and recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany).

A thoracotomy was performed at the left fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) prepared for occlusion just proximal to the first main diagonal branch. Through the right jugular vein, a catheter was positioned into the coronary sinus to obtain blood samples for the measurement of plasma nitrate/nitrite (NOx) levels. In

some control and treated dogs, the left circumflex (LCX) coronary artery was also prepared to measure coronary blood flow (CBF; ml min^{-1}) by means of a transit time Doppler flow probe (Hugo Sachs Electronics, Germany).

The severity of myocardial ischaemia was assessed by the measurement of changes in the degree of electrical activation and in epicardial ST-segment using a mapping electrode positioned within the ischaemic region [37]. Signals were collected from 31 unipolar electrodes (inter-electrode distance; 2 mm) at a frequency of 1 kHz, stored on a computer and analyzed offline by creating activation and ST maps. Changes in epicardial activation were assessed as a time delay between the first and a last point activated under the electrode and expressed as the total activation time (TAT) in milliseconds. Elevations of epicardial ST-segment, recorded in each minute from the unipolar electrodes were averaged and expressed in mV.

Ventricular arrhythmias were evaluated according to the Lambeth Conventions [38] with a modification as previously outlined [12]. Thus, the total number of ventricular premature beats (VPBs), the incidence and the 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), and the incidence of ventricular fibrillation (VF) were assessed during the occlusion period. During reperfusion, only the incidence of VF (which is a final event in this species) was determined. Dogs that were still alive 2 min after reperfusion (the end of the study) were considered to be survivors.

Measurement of plasma nitrate/nitrite (NOx) levels

These were performed as described previously [22]. Plasma nitrate/nitrite (NO_x) concentrations were determined by means of the Griess reaction in blood samples taken from the coronary sinus at various time intervals as described in the protocol. After sample preparation, the absorbance of the azo compound was measured spectrophotometrically at a wavelength of 540 nm and the total nitrate/nitrite (NO_x) concentration (μmol L^{-1}) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma St Louis, MO, USA).

Assessment of superoxide production

This has also been described previously [22]. In brief, tissue blocks (size: 0.5 x 0.5 x 2.0 cm), excised from the ischaemic area within 2 min of the reperfusion, were embedded in optimal cutting temperature compounds. Cryosections (20 μm) were produced and stained with dihydroethidium (Sigma, St Louis, MO, USA). A negative control was obtained by blocking the reaction with N-acetyl-L-cysteine (100 mmol L^{-1}, Sigma St Louis, MO, USA). Both from the stained and negative control samples 10–15 serial images were captured by a confocal laser scanning microscope (Olympus FV1000). The intensity of the fluorescent signals was analyzed by ImageQuant software (Molecular Dynamics) and expressed in arbitrary units.

Determination of peroxynitrite production

This was performed by the measurement of nitrotyrosine formation using Western immunoblot as described previously [22]. In brief, after tissue preparation (homogenization and centrifugation) the supernatant was collected and the protein concentration was determined by the Lowry method. Following electrophoresis the proteins were transferred onto PVDF membrane. After incubation with a monoclonal anti-nitrotyrosine antibody (MAB5404, Chemicon, USA), and horseradish peroxidase-conjugated rabbit anti-mouse IgG (P0161, Dakocytomation, Denmark) as the secondary antibody, the membrane was developed with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare, UK), exposed to X-ray film and scanned. ImageJ (NIH,

Bethesda, MD) was used to determine the density of nitrotyrosine bands. Equal loading was controlled using Coomassie Blue staining.

Assessment of protein S-nitrosylation and glutathionylation

Tissue homogenates from canine left ventricle were prepared as previously described [26], and protein concentrations were determined using the Bradford protein assay. The SNO-RAC protocol was used to examine protein SNO, as previously described [26]. Briefly, samples were incubated with 50 mmol L^{-1} n-ethylmaleimide for 20 minutes at 50°C in order to block non-modified (i.e., free) thiol groups. Samples were then incubated with thiopropyl sepharose resin (GE Healthcare, Piscataway, NJ) in the presence of 20 mmol L^{-1} ascorbate to reduce SNO, and rotated for four hours in the dark at room temperature. Resin-bound proteins were then subjected to trypsin digestion (sequencing grade modified; Promega, Madison, WI) overnight at 37°C. Resin-bound peptides were eluted in buffer containing (in mmol L^{-1}): DTT (20), NH_4HCO_3 (10), and 50% methanol (v/v), and identified using liquid chromatography-tandem mass spectrometry. Mass spectrometry results were analyzed using Proteome Discoverer v1.3 software (Thermo Fisher Scientific) with the NIH six-processor MASCOT cluster search engine (http://biospec.nih.gov, version 2.3). Proteome Discoverer v1.3 software was also used to provide relative quantification of SNO levels via label-free peptide quantification.

Glutathionylation was examined using Western immunoblot. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane, which was subsequently blocked with LI-COR blocking buffer (Lincoln, NE) containing 25 mmol L^{-1} n-ethylmaleimide. After this the membrane was incubated with an anti-glutathione antibody (Virogen, Watertown, MA), followed by incubation with a fluorescent secondary antibody (LI-COR). The membrane was imaged using a LI-COR Odyssey scanner. ImageJ (NIH, Bethesda, MD) was used to determine the density of glutathionylation bands.

Experimental protocol

The experimental protocol is illustrated in Fig 1. A total of 41 dogs were used and randomly divided into three groups. Each animal was subjected to a 25 min LAD occlusion followed by rapid reperfusion. Control dogs (C; n = 15) were infused with saline, commencing prior to and then throughout the 25 min occlusion period. The concentration of sodium nitrite has been selected on the basis of the study of Gonzalez et al., [39], and was administered by intravenous infusion at a concentration of 0.2 μmol kg^{-1} min^{-1} to two groups of dogs; in one group ($NaNO_2$-PO; n = 14) the infusion was started 10 min prior to and maintained over the entire occlusion period, whereas in the other group ($NaNO_2$-PR; n = 12), the infusion was given just 10 min prior to reperfusion. In dogs that survived the combined ischaemia and reperfusion insult, the hearts were stopped within 2 min with an overdose of the anaesthetic and myocardial tissue samples were collected from the ischaemic ventricular wall. In dogs that suddenly fibrillated on reperfusion, samples were quickly excised when the fibrillation had been observed. An additional six dogs served as sham-operated controls (not included in the protocol). These dogs were instrumented and infused locally with saline without subjection to ischaemia. At the end of the observation period the animals were euthanized with an excess of anaesthetic and myocardial samples were taken for further analyses.

In those dogs in which tissue samples were not taken (at least n = 5 dogs in each group), the heart was excised and the risk area was assessed by injecting Patent Blue V dye into the re-occluded artery using the same method that has been described in detail elsewhere [12].

Statistical analysis

All data were expressed as mean ± SEM and the differences between means were compared by ANOVA for repeated measures and by one-way ANOVA as appropriate, using the Fisher post hoc, Bonferroni and Newman-Keuls multiple comparison tests. Ventricular premature beats and episodes of ventricular tachycardia were compared using the Kruskal-Wallis test. The incidence of arrhythmia (such as VT and VF) and survival from the combined ischaemia and reperfusion insult was compared by the Fisher Exact test. Differences between groups were considered significant when $^{*}P < 0.05$.

Results

Haemodynamic effects of intravenous sodium nitrite and subsequent coronary artery occlusion

The administration of sodium nitrite resulted in a slight reduction in arterial blood pressure, LVEDP and in positive and negative $LVdP/dt_{max}$ without substantial changes in heart rate and

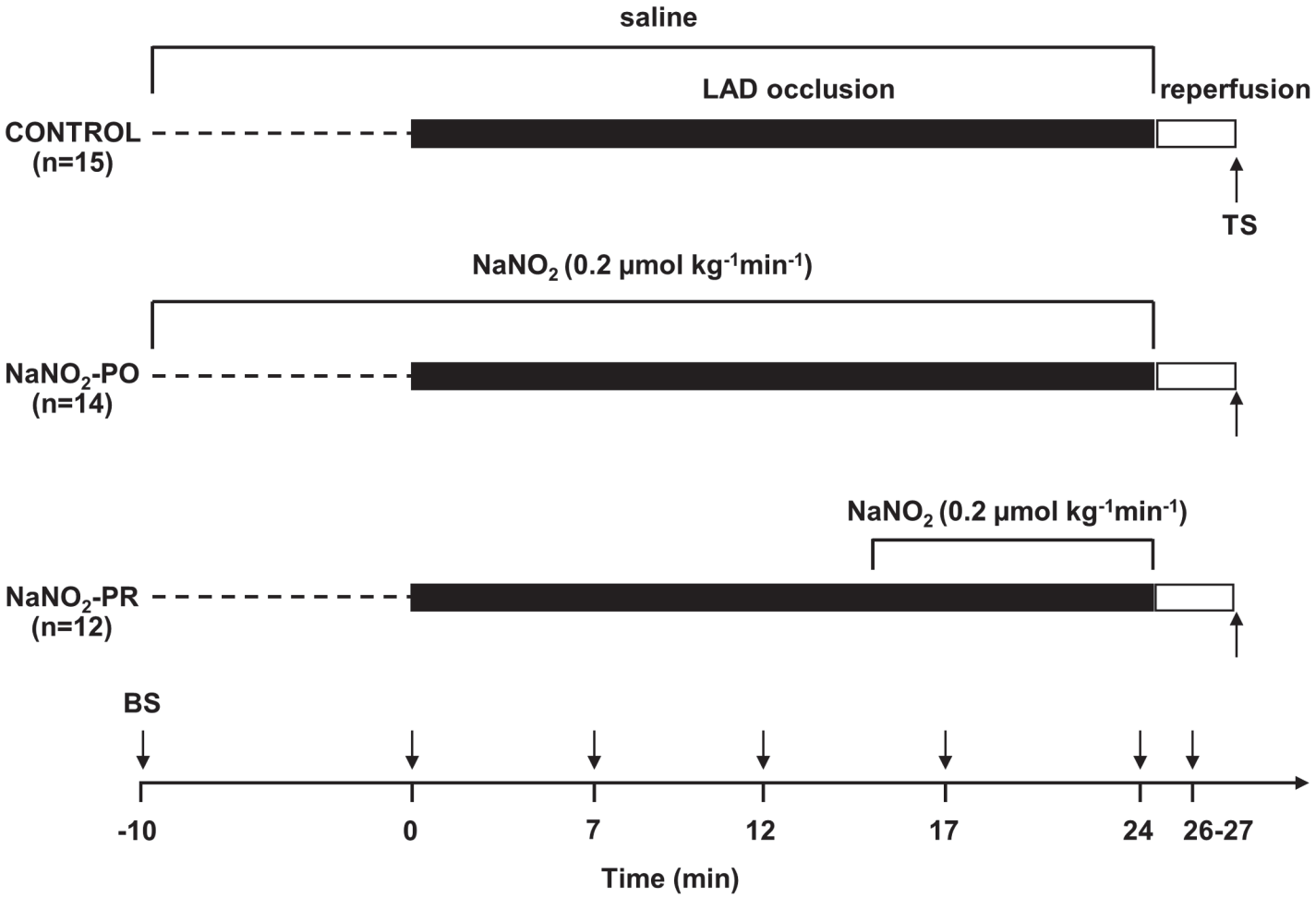

Fig 1. Experimental protocol. After surgery all animals were allowed to equilibrate for 20 min. Fifteen dogs (control group) were infused with pH 7.4 saline (rate: 0.5 ml min^{-1}) via intravenous route over the entire observation period. In another two groups of dogs, sodium nitrite was administered in intravenous infusion, starting the infusion either prior to and maintained throughout the occlusion ($NaNO_2$-PO; n = 14) or 10 min prior to reperfusion ($NaNO_2$-PR; n = 12). In all groups, ischaemia was induced by a 25 min occlusion of the LAD, followed by rapid reperfusion. During the experiment, blood samples (BS) were collected at various time intervals from the coronary sinus for the assessment of plasma nitrate/nitrite (NOx) levels. Within 2 min of reperfusion tissue samples (TS) were taken from the ischaemic myocardium for further analyses.

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Table 1. The haemodynamic effects of $NaNO_2$ infusion and coronary artery occlusion.

	SALINE				$NaNO_2$-PO				CONTROL				$NaNO_2$-PO				$NaNO_2$-PR			
	Baseline		Max. change		Baseline		Max.Change		Baseline		Max. changeon occlusion		Baseline		Max. changeon occlusion		Baseline		Max. changeon occlusion	
SABP (mm Hg)	143	±4	-2	±2	132	±7	-8	±3$^{\#}$	141	±5	-11	±3$^{\#}$	124	±6	-13	±1$^{\#}$	150	±3	-13	±1$^{\#}$
DABP (mm Hg)	97	±3	-3	±1	89	±6	-6	±2$^{\#}$	98	±4	-11	±1$^{\#}$	85	±5	-9	±1$^{\#}$	104	±5	-11	±2$^{\#}$
MABP (mm Hg)	112	±3	-2	±1	103	±6	-6	±2$^{\#}$	110	±3	-11	±1$^{\#}$	98	±5	-11	±1$^{\#}$	119	±4	-12	±1$^{\#}$
LVSP (mm Hg)	145	±4	-2	±1	135	±6	-6	±2	146	±4	-15	±1$^{\#}$	124	±6	-12	±12$^{\#}$	154	±6	-15	±2$^{\#}$
LVEDP (mm Hg)	3.7	±0.3	0.3	±0.2	4.6	±0.3	-1.1	±0.3$^{\#*}$	3.7	±0.3	12.2	±1.3$^{\#}$	4.8	±0.3	6.4	±0.3$^{\#*}$	4.0	±0.2	6.2	±0.4$^{\#*}$
+dP/dt (mm Hg s^{-1})	3081	±109	-22	±20	2924	±77	-98	±48$^{\#*}$	3081	±149	-629	±94$^{\#}$	2846	±106	-525	±44$^{\#}$	3083	±89	-430	±53$^{\#*}$
-dP/dt (mm Hg s^{-1})	2600	±92	-12	±9	2257	±59	-112	±32$^{\#*}$	2556	±107	-588	±92$^{\#}$	2157	±77	-456	±48$^{\#}$	2219	±59	-342	±49$^{\#*}$
HR (beats min^{-1})	167	±3	0	±1	162	±4	4	±3	167	±4	-3	±2	164	±4	1	±2	167	±1	2	±2
$mCBF_{LCX}$ (ml min^{-1})	30	±2	3	±1	32	±3	0	±1	28	±3	11	±1$^{\#}$	31	±3	12	±1$^{\#}$	29	±1	9	±2$^{\#}$

Values are mean ± SEM calculated from n = 12 to 15 experiments. Abbreviations: SABP: systolic blood pressure, DABP: diastolic blood pressure, MABP: mean arterial blood pressure, LVSP: left ventricular systolic pressure, LVEDP: left ventricular end diastolic pressure, HR: heart rate, $mCBF_{LCX}$: mean coronary blood flow determined on the LCX coronary artery.

$^{\#}P < 0.05$ vs. baseline value;

$^{*}P < 0.05$ vs. control group.

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in mean CBF measured on the LCX artery (Table 1). When the LAD was occluded for 25 minutes, the haemodynamic changes were similar in all groups, except that in dogs infused with sodium nitrite, the increases in LVEDP were less pronounced than in the controls (Table 1). Compared to controls, the compensatory blood flow changes occurring on the LCX when the LAD was occluded were not modified by sodium nitrite. There were also no significant alterations in blood methemoglobin concentrations following sodium nitrite administration. These values increased from around 0.15 ± 0.03% measured at baseline to 0.20 ± 0.01% in controls, to 0.38 ± 0.03% in $NaNO_2$-PO and to 0.26 ± 0.03% in $NaNO_2$-PR groups by the end of the occlusion.

Ventricular arrhythmias during coronary artery occlusion and reperfusion

The distribution of various arrhythmia types during a 25 min occlusion and reperfusion (only VF was assessed) is shown in Fig 2 and the summary of the arrhythmia events is given in Fig 3. Compared with controls, sodium nitrite infused either prior to and continued over the entire occlusion period or given just 10 min prior to reperfusion resulted in marked reductions in the total number of VPBs (442 ± 91 vs. 157 ± 78 and 85 ± 42; $P < 0.05$), in the incidence (87% vs. 29 and 50%; $P < 0.05$) and the number of episodes of VT (15.5 ± 5.1 vs. 3.4 ± 2.4 and 0.6 ± 0.2; $P < 0.05$) during occlusion. Furthermore, no dog infused with sodium nitrite fibrillated during occlusion compared with 40% incidence of VF in the control animals. When the ischaemic myocardium was rapidly reperfused, all of the control dogs fibrillated thus there was no survival in this group compared with the sodium nitrite treated groups in which 50% ($NaNO_2$-PO) and 92% ($NaNO_2$-PR) of the animals survived.

Ischaemia severity following coronary artery occlusion

This was assessed by changes in epicardial ST-segment and TAT during a 25 min occlusion of the LAD (Fig 4A and 4B). In control dogs both parameters were markedly elevated during the initial 5 min of the occlusion and this was maintained over the entire occlusion period. Administration of sodium nitrite prior to occlusion significantly reduced these indices of ischaemia

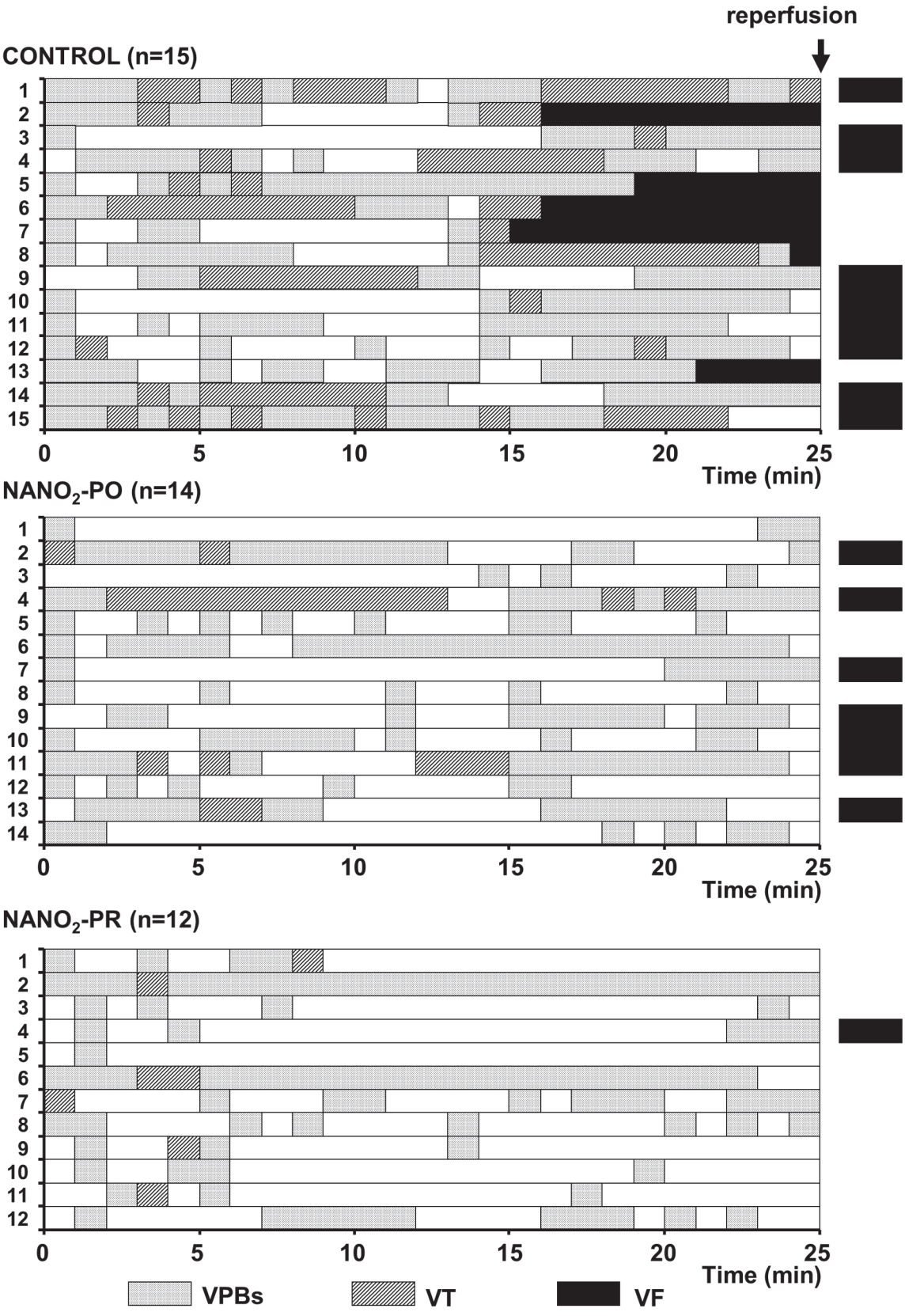
reperfusion
CONTROL (n=15)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0
5
10
15
20
25
Time (min)
NANO2-PO (n=14)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
5
10
15
20
25
Time (min)
NANO2-PR (n=12)
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
Time (min)
VPBs
VT
VF

Fig 2. The distribution of ventricular arrhythmias following coronary artery occlusion and reperfusion. This is shown in each individual control dog and in dogs that were infused with sodium nitrite prior to and during the occlusion ($NaNO_2$-PO group) and 10 min prior to reperfusion ($NaNO_2$-PR group). The filled coulumns are the times during which the dog was in ventricular fibrillation (VF), the shaded columns show periods of ventricular tachycardia (VT) and the lightly stippled coulumns are periods during which ventricular premature beats (VPBs) were evident. VF on reperfusion is also shown by the black horizontal columns and this was the only arrhythmia assessed during the reperfusion phase. The results show that in the controls VF occurred in 6 dogs and that all the other dogs that survived the occlusion period fibrillated on reperfusion. Thus there were no survival in this group from the combined ischaemia and reperfusion insult. In contrast infusion of sodium nitrite either prior to and during the occlusion or just prior to reperfusion significantly reduced the severity of the occlusion-induced arrhythmias and increased survival on reperfusion. This later was particularly marked in dogs in which sodium nitrite was infused 10 min prior to reperfusion.

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severity over the entire occlusion period. In contrast, compared with controls, in dogs infused with sodium nitrite 10 min prior to reperfusion, a significant reduction in epicardial ST (Fig 4A) segment and in TAT (Fig 4B) occurred only the last 7 min of the occlusion.

Changes in plasma nitrite and nitrate as well as in total NOx levels during coronary artery occlusion and reperfusion

These are shown in Fig 5. The plasma total NOx concentration in the coronary sinus blood was 21.36 ± 0.10 μmol L^{-1} as determined from 41 dogs at baseline. In control dogs, infusion of saline did not modify the plasma level of NO metabolites. However, when the LAD was occluded, the NOx levels, after a transient elevation occurring around the seventh min of ischaemia, were

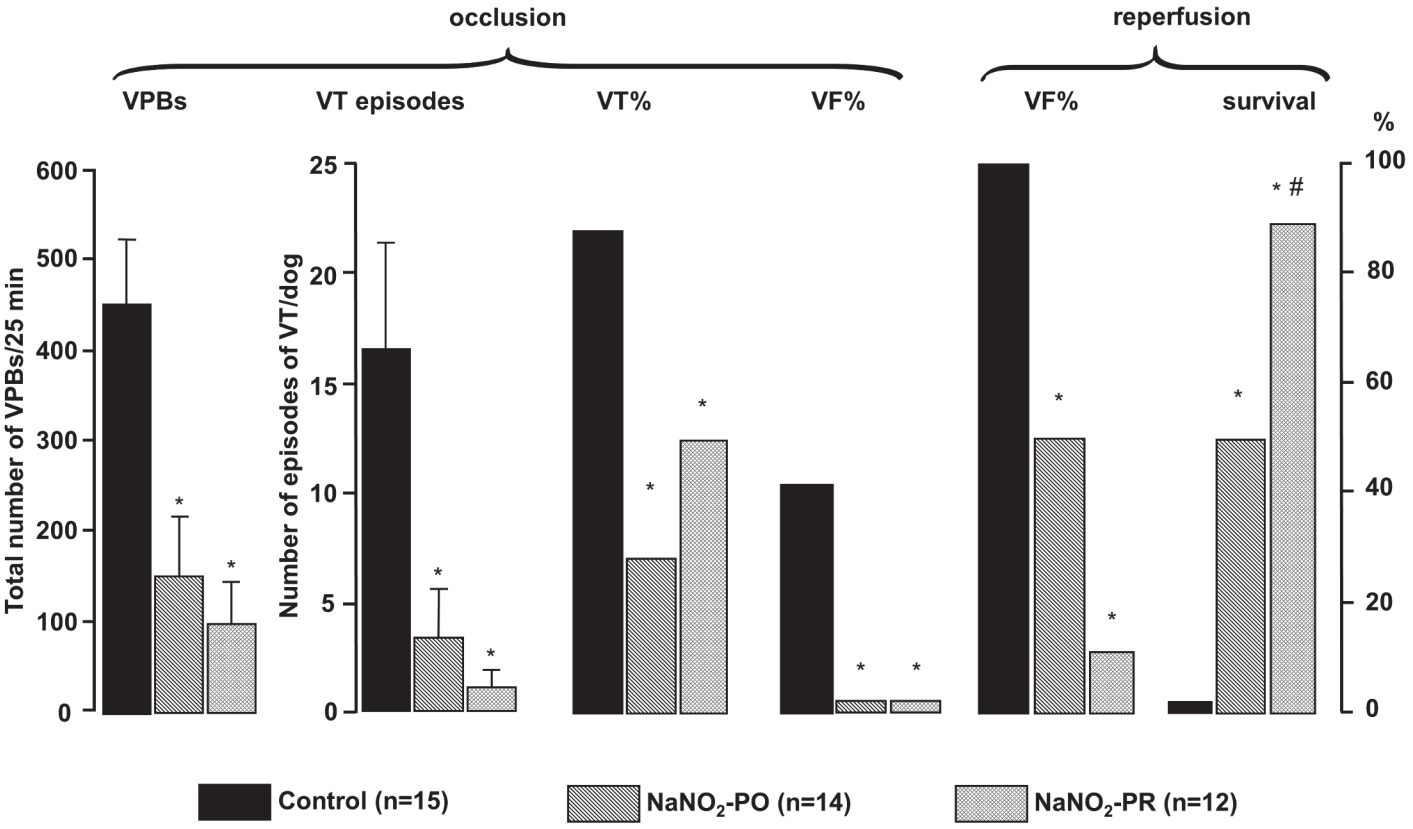

Fig 3. The severity of arrhythmias during a 25 min occlusion and reperfusion of the LAD in control dogs and in dogs infused with sodium nitrite. Both administration forms of sodium nitrite markedly reduced the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT) during occlusion, and increased survival from the combined ischaemia and reperfusion insult. Values represent mean ± SEM. $^{*}P < 0.05$ vs. controls, $^{\#}P < 0.05$ vs. $NaNO_2$-PO group.

doi:10.1371/journal.pone.0122243.g003

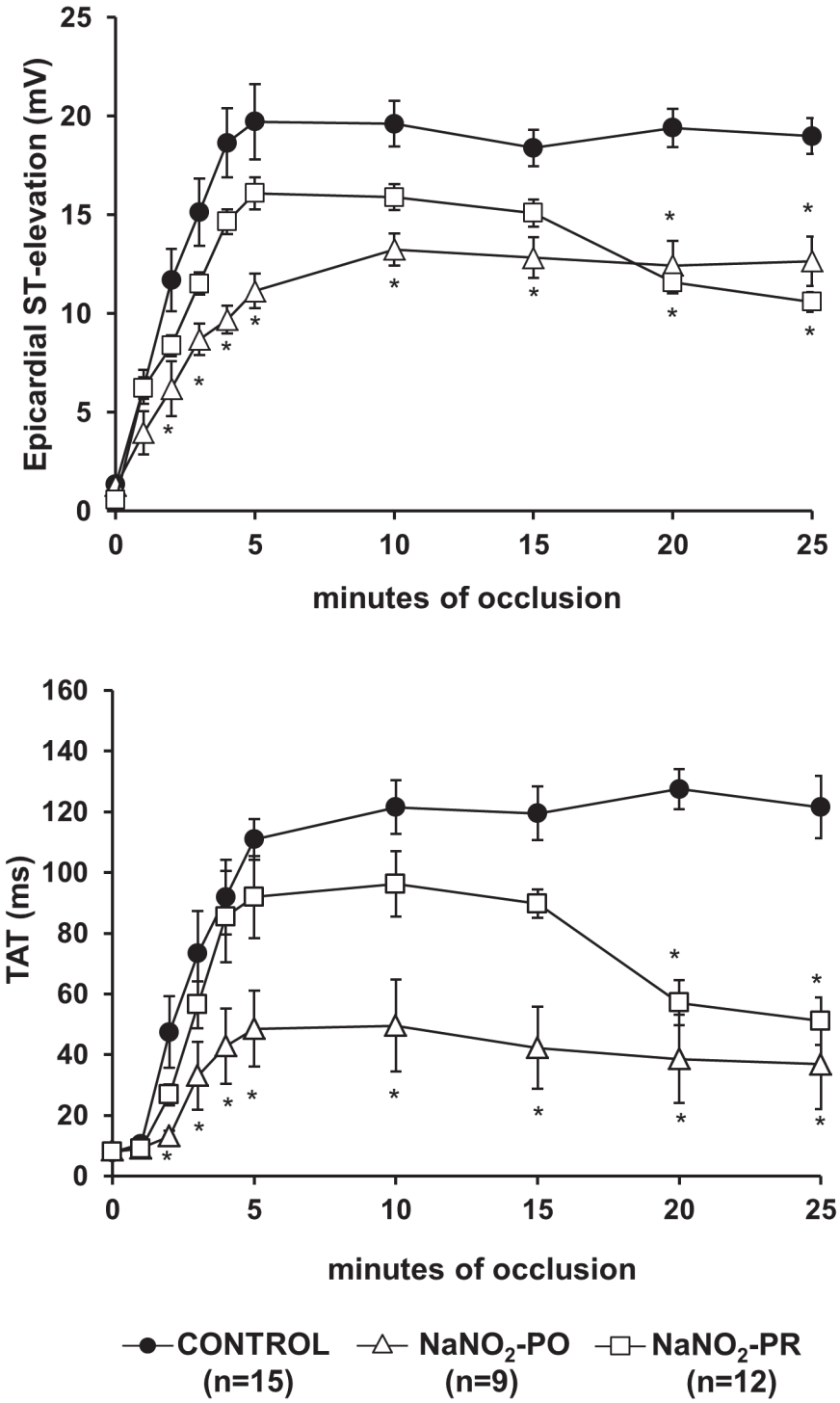

Fig 4. Changes in epicardial ST-segment (A) and in total activation time (TAT; B) during a 25 min occlusion of the anterior descending branch of the left coronary artery. In control dogs, both indices of ischaemia severity were markedly increased, especially during the initial 5 min of the occlusion. These changes were significantly less marked from the beginning of the occlusion in the $NaNO_2$-PO group and from the 15 min of the occlusion in the $NaNO_2$-PR group than in the controls. Values represent mean ± S.E.M. $^{*}P < 0.05$ compared with the controls.

doi:10.1371/journal.pone.0122243.g004

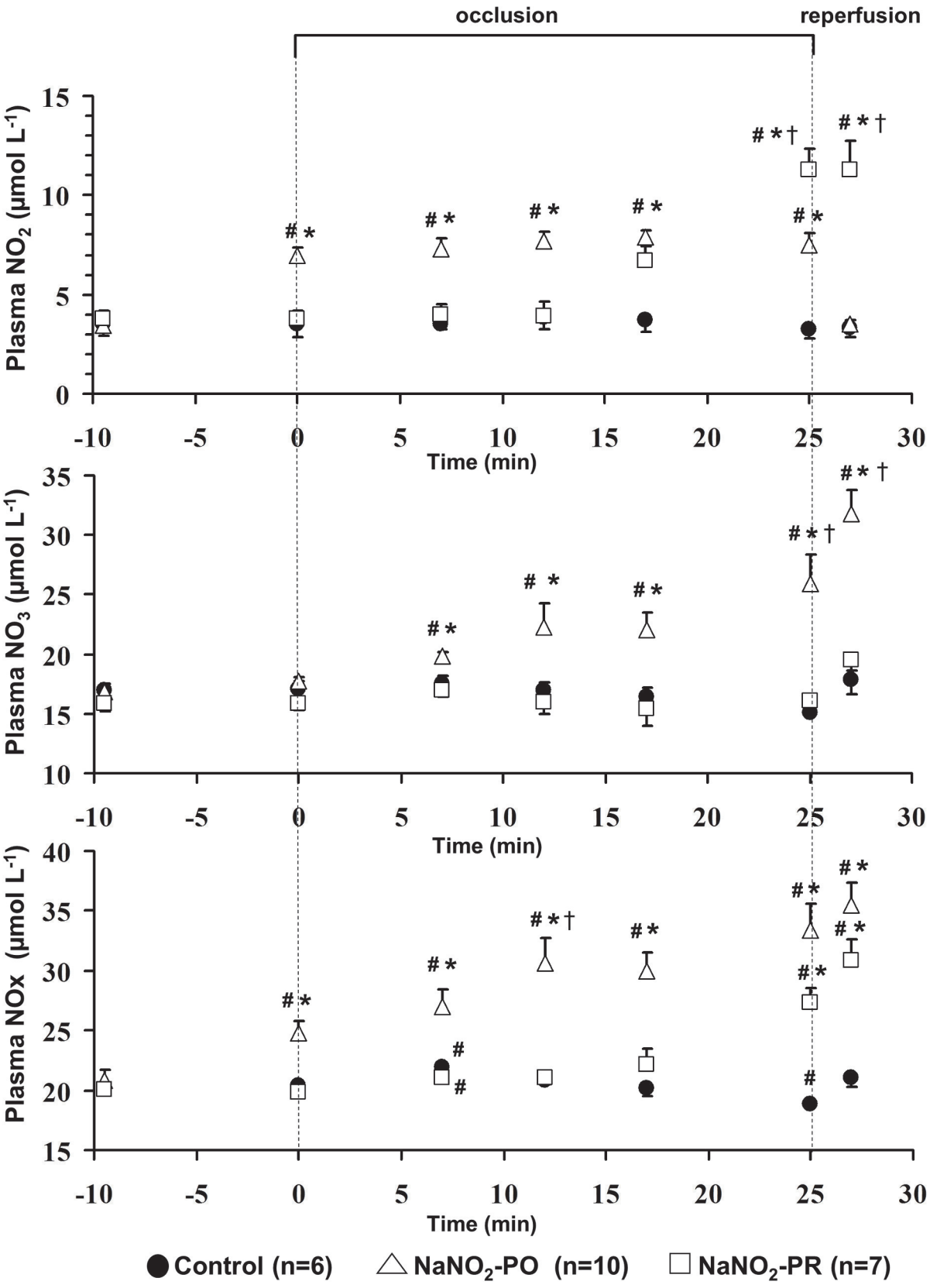
occlusion
reperfusion
Plasma NO_2 (μmol L^{-1})
Plasma NO_3 (μmol L^{-1})
Plasma NOx (μmol L^{-1})
Time (min)
Control (n=6)
$NaNO_2$-PO (n=10)
$NaNO_2$-PR (n=7)

Fig 5. Changes in plasma nitrite and nitrate as well as total nitrite/nitrate (NOx) levels in the blood of the coronary sinus. In control dogs the level of NO metabolites was significantly reduced by the end of the occlusion period. Infusion of sodium nitrite before and during occlusion increased nitrite, nitrate and NOx concentrations, whereas when nitrite was administered 10 min prior to reperfusion the increase in NOx resulted only from the elevation of nitrite concentration. Reperfusion caused almost similar increases of NOx in all groups. Values represent mean ± SEM. $^{*}P < 0.05$ vs. controls, $^{\#}P < 0.05$ vs. the initial baseline value and $^{\dagger}P < 0.05$ vs. $NaNO_2$-PO group.

doi:10.1371/journal.pone.0122243.g005

markedly reduced by the end of the occlusion period. Infusion of sodium nitrite prior to occlusion ($NaNO_2$-PO group), resulted in significant increases in both nitrite and nitrate levels, and these were maintained or even further increased during occlusion. In contrast, in dogs infused with sodium nitrite 10 min prior to reperfusion ($NaNO_2$-PR group), there was no substantial increase in nitrate levels up to the end of the occlusion. Reperfusion of the LAD resulted in almost similar step increases in plasma NOx levels in all groups, although the absolute concentration values were different among the groups.

The effect of sodium nitrite on the reperfusion-induced myocardial superoxide and nitrotyrosine formation

These were determined in all dogs during reperfusion, no matter whether dogs fibrillated or survived (Fig 6). Compared with sham controls (infused only with saline without subjection to ischaemia and reperfusion) 25 min of ischaemia and reperfusion resulted in marked increases in both superoxide (Fig 6A) and nitrotyrosine (Fig 6B) production. These were significantly less marked in those dogs that had been infused with sodium nitrite.

Effects of sodium nitrite on protein S-nitrosylation and glutathionylation

These were determined in tissue samples collected from control and sodium nitrite-treated dogs after 25 min of ischaemia and 2 min reperfusion. The results are summarized in Table 2 and Figs 7 and 8. Compared with controls, the number of proteins and peptides showing cysteine SNO was substantially increased only in dogs that had been infused with sodium nitrite 10 min prior to reperfusion ($NaNO_2$-PR group; Fig 7; Table 2). The lack of elevated protein SNO in the $NaNO_2$-PO group in comparison to the control and to the $NaNO_2$-PR groups led to the examination of protein S-glutathionylation in the same samples. Fig 8 shows that S-glutathionylation is increased in dogs infused with sodium nitrite over a longer period (i.e. 10 min prior to and throughout the 25 min occlusion) especially at the band noted at 52 kDa. Although these changes did not quite meet statistical significance ($p = 0.11$), the results are consistent with an increase in protein S-glutathionylation in the $NaNO_2$-PO group compared with the other two groups.

Area at risk

There were no significant differences in the area at risk among the groups. Thus in control dogs ($n = 6$) the risk area was 41.4 ± 1.0%, in the $NaNO_2$-PO group ($n = 5$) 42.3 ± 1.0% and in the $NaNO_2$-PR ($n = 5$) group 41.8 ± 1.1%.

Discussion and Conclusions

There is good evidence that nitrite, an oxidative metabolite of NO, represents a natural storage form of NO which under certain conditions, such as low pH and oxygen tension, can readily reduce back to NO [40–42]. Although a protective role for nitrite has been shown under conditions of ischaemia and reperfusion [39, 43, 44], the situation regarding arrhythmias is less clear and has not yet been examined in large animal models. We show here that intravenous administration of sodium nitrite in anaesthetized dogs markedly reduces the severity of arrhythmias

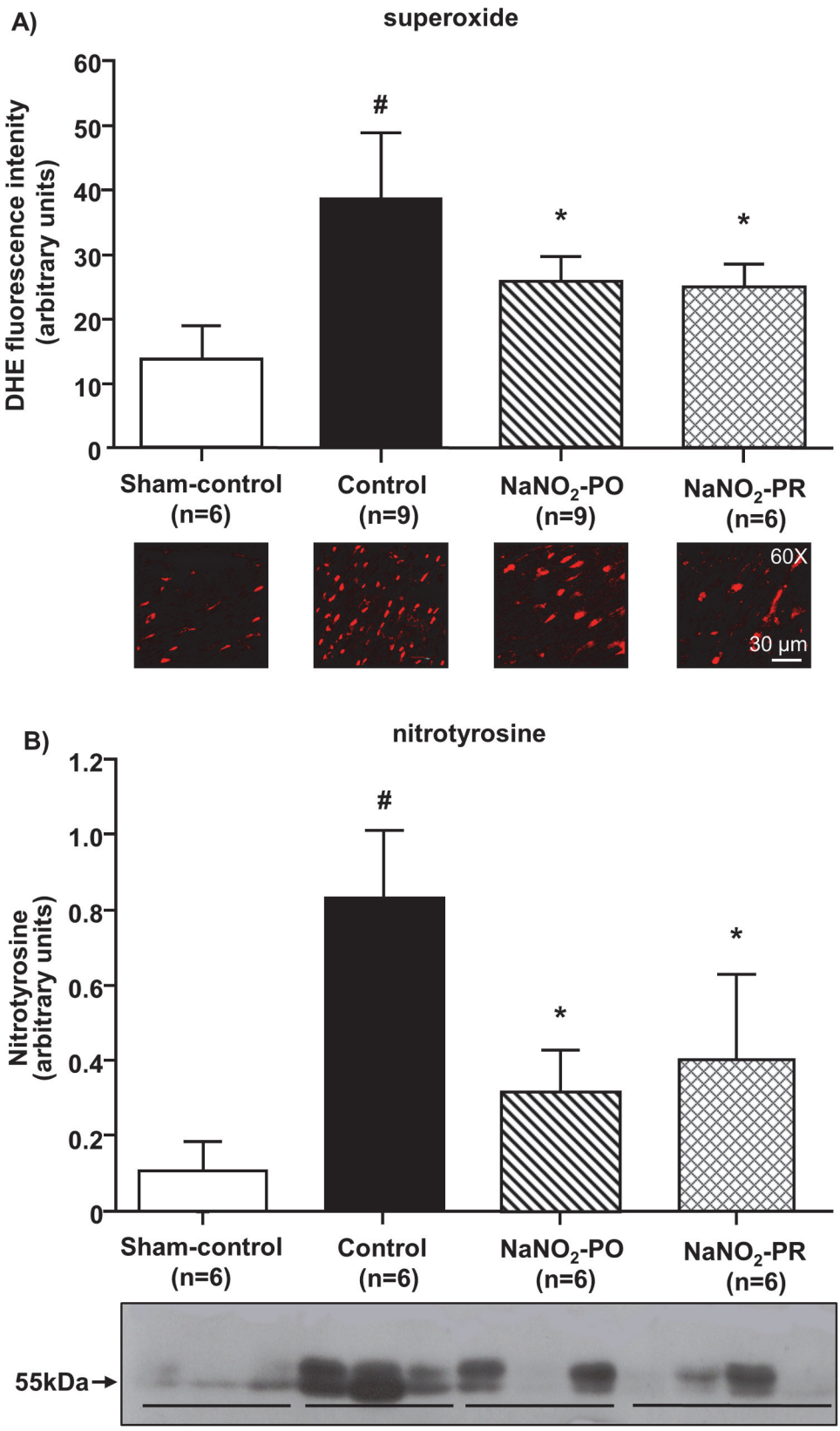
A)
superoxide
DHE fluorescence intenity (arbitrary units)
60
50
40
30
20
10
0
#
*
*
Sham-control (n=6)
Control (n=9)
NaNO2-PO (n=9)
NaNO2-PR (n=6)
60X
30 µm
B)
nitrotyrosine
Nitrotyrosine (arbitrary units)
1.2
1.0
0.8
0.6
0.4
0.2
0
#
*
*
Sham-control (n=6)
Control (n=6)
NaNO2-PO (n=6)
NaNO2-PR (n=6)
55kDa

Fig 6. Tissue superoxide (A) and nitrotyrosine (B) production following a 25 min occlusion and reperfusion of the LAD. Compared with the sham-control dogs (instrumented but not subjected to ischaemia and reperfusion; n = 6), there were significant increases in superoxide and nitrotyrosine (NT) formation in controls that had been subjected to ischaemia and reperfusion. Compared with controls, sodium nitrite significantly reduced superoxide and NT production, no matter when the infusion was initiated. Values represent mean ± SEM. $^{\#}P < 0.05$ vs. sham-control dogs, $^{*}P < 0.05$ vs. ischaemic controls.

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that results from a 25 min period of coronary artery occlusion and reperfusion Thus the number of VPBs and VT episodes, the incidence of VT and VF during occlusion were significantly decreased, and survival from the combined ischaemia and reperfusion insult was substantially increased, nonetheless there were great individual variability among dogs in their arrhythmia response to coronary artery occlusion. Interestingly, survival, assessed during the early period of reperfusion (within 2 min), was significantly higher in dogs that were infused with sodium nitrite 10 min prior to reperfusion (92%) than in dogs where nitrite was administered over a longer period of time, i.e. 10 min before and during the entire 25 min of LAD occlusion (50%). Moreover, sodium nitrite also significantly attenuated the ischaemic changes, such as the epicardial ST-segment and TAT. This effect in the $NaNO_2$-PR group became apparent shortly after the commencement of the nitrite infusion, when these indices of ischaemia severity started to markedly decline, whereas the phase Ib arrhythmias virtually disappeared. We may draw two conclusions from this finding. First, the effect of nitrite infusion is almost immediate, indicating a rapid conversion of nitrite to NO under ischaemic conditions [43, 44]. Indeed, around 15 min of the occlusion, the ischaemic changes are sufficiently advanced to provide a milieu for the reduction of nitrite to NO. This assumption is also supported by the measurements of NO metabolites (Fig 5). These show that in the $NaNO_2$-PR group the plasma nitrite, but not nitrate levels, were elevated over the 10 min period of infusion. It may well be that under these conditions nitrite almost fully reduces to NO, which by replacing the insufficient NO bioavailability due to coronary artery occlusion [22], would lessen the ischaemic changes and the arrhythmias. Second, the high incidence of survivors in the $NaNO_2$-PR group suggests that a short period of infusion of nitrite prior to reperfusion is sufficient to increase NO bioavailability and reduce the occurrence of the reperfusion-induced ventricular fibrillation which is common in this canine model when the coronary artery is suddenly reopened [12]. This finding is in accord with the results of Gonzales et al. (2008). They showed in dogs that infusion of nitrite during the last 5 min of ischaemia significantly reduced infarct size and improved recovery of contractile function after reperfusion [39].

There are a number of possible explanations through which NO may influence arrhythmias resulting from ischaemia and reperfusion. Some of these have already been discussed elsewhere [9, 22]. One of the mechanisms to which we paid particular attention in the present study is the regulatory role of NO in the generation of oxidative stress products. Our results showed that sodium nitrite, regardless of the duration of infusion, significantly suppressed the generation of superoxide and peroxynitrite during reperfusion (Fig 6). This is in accord with our [22] and others [45–47] previous findings that an increase in NO bioavailability during ischaemia may suppress the reperfusion-induced generation of ROS. Although there are a number of possible mechanisms through which NO may regulate superoxide production such as by directly acting on or indirectly influencing the components of the electron transport chain (ETC; reviewed e.g. by [46, 48, 49]), the most likely mechanism for the NO-mediated suppressed superoxide generation under conditions of ischaemia/reperfusion is thought to be the posttranslational SNO of mitochondrial complexI [50–53]. Another explanation which would support the marked antiarrhythmic effect of sodium nitrite is the modulatory effect of NO on calcium homeostasis. This also may involve both cGMP-dependent and cGMP-independent mechanisms

Table 2. Peptides showing cysteine S-Nitrosylation as identified by the SNO-RAC method.

				Control		$NaNO_2$-PO		$NaNO_2$-PR	
Function	**Protein Name**	**Protein ID**	**Peptide Sequence**	**Ratio**	**Ion Score**	**Ratio**	**Ion Score**	**Ratio**	**Ion Score**
glycolysis	triosephosphate isomerase	P00939	IAVAAQNCYK	1	45	0.15	38	7.19	50
			IIYGGSVTGATCK	1	89	0.94	72	20.74	90
			VPADTEVVCAPPTAYIDFAR	1	63	-	-	2.69	48
	glyceraldehyde-3-phosphate dehydrogenase	Q28554	VPTPNVSVVDLTCR	1	38	0.11	47	1.08	44
			IVSNASCTTNCLAPLAK	1	96	0.71	93	12.49	99
	fructose-bisphosphate aldolase A	P04075	ALANSLACQGK	1	75	0.16†	60	4.12	71
	6-phosphofructokinase	Q0IIG5	LPLMECVQVTK	1*	73	0.21	55	3.49 ‡	6 1
	beta-enolase	Q3ZC09	VNQIGSVTESIQACK	1*	67	-	-	11.40	86
	L-lactate dehydrogenase b chain	P13490	VIGSGCNLDSAR	1	74	0.76	75	41.63‡	80
tricarboxylic acid cycle	malate dehydrogenase, mitochondrial	Q32LG3	GCDVVVIPAGVPR	1	78	1.06	79	10.51‡	64
			EGVVECSFVK	1	45	1.68	45	16.56	49
			TIIPLISQCTPK	1	42	1.47	61	5.08	51
			GYLGPEQLPDCLK	1	72	1.02	69	9.06 ‡	68
	malate dehydrogenase, cytoplasmic	Q3T145	VIVVGNPANTNCLTASK	1	92	0.79	88	21.20‡	60
	pyruvate kinase isozyme M1	P11979	NTGIICTIGPASR	1	68	0.77	49	-	-
	isocitrate dehydrogenase	P54071	VCVQTVESGAMTK	1*	87	0.59	61	-	-
	aconitate hydratase, mitochondrial	P20004	VGLIGSCTNSSYEDMGR	1*	90	-	-	9.03	84
mitochondrial carrier proteins	ADP/ATP translocase 1	P02722	GADIMYTGTVDCWR	1*	67	0.85*	46	12.21‡	52
mitochondrial respiratory chain	ATP synthase subunit epsilon-like protein	Q5VTU8	YSQICAK	1*	45	0.09*	33	-	-
	cytochrome b-c1 complex subunit 1	P31800	LCTSATESEVLR	1*	74	-	-	20.18*	85
cytoskeleton	myosin light chain 3	P08590	ITYGQCGDVLR	1	67	-	-	21.25	75
			LMAGQEDSNGCINYEAFVK	1	63	-	-	0.80	61
	actin, gamma-enteric smooth muscle	Q5E9B5	LCYVALDFENEMATAASSSSLEK	1*	66	0.72	81	5.18*	72
fatty acid metabolism	fatty acid-binding protein, heart	O02772	LILTLTHGSAVCTR	1*	56	-	-	34.13	79
amino acid metabolism	aspartate aminotransferase, mitochondrial	P12344	VGAFTVVCKTCGFDFTGAIEDISK	11	5676	1.570.26	6456	9.633.77	5047
ATP metabolism	creatine kinase M-type	P05123	GYTLPPHCSR	1	41	1.00	34	-	-
	creatine kinase U-type	Q9TTK8	GLSLPPACTR	1*	35	1.91	41	-	-
	60 kDa heat shock protein	P86206	AAVEEGIVLGGGCALLR	1*	69	-	-	0.54*	57
Ca^{2+} storage	sarcoplasmic/endoplasmic reticulum calcium ATPase 2	O46674	VGEATETALTCLVEK	1	103	0.77*	70	3.93	115
others	serum albumin	P49822	YMCENQDSISTK	1	70	0.07†	66	0.77	59

(*Continued*)

Table 2. (*Continued*)

Function	Protein Name	Protein ID	Peptide Sequence	Control		$NaNO_2$-PO		$NaNO_2$-PR	
				Ratio	Ion Score	Ratio	Ion Score	Ratio	Ion Score
	pancreatic trypsin inhibitor	P00974	AGLCQTFVYGGCR	1*	80	-	-	4.14	67
	hydroxysteroid dehydrogenase-like protein 2	A4FUZ6	LAGCTVFITGASR	1*	86	0.14*	43	5.14*	89

S-nitrosylated proteins and peptides obtained from control and nitrite-treated dogs via SNO-RAC proteomic analysis. For comparison only proteins that were S-nitrosylated in control animals are shown in the table. Peptides were filtered at a false discovery rate of 5%. (C): SNO cysteine residue; (*) peptides were observed in only one SNO-RAC proteomic analysis for a given group. Peptides not detected under the specified condition contain line (-) in the ion score column. Proteome Discoverer v1.3 was used for determining the label free peptide ratio. Values represent mean ± SEM.

$^{\dagger}P < 0.05$ control vs. $NaNO_2$-PO,

$^{\ddagger}P < 0.05$ control vs. $NaNO_2$-PR group.

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[29]. The present study has now focused on the cGMP-independent NO-mediated mechanisms, such as the protein S-nitrosylation (SNO), using SNO-RAC proteomic analysis. We found that in dogs given sodium nitrite 10 min prior to reperfusion ($NaNO_2$-PR group), the number of SNO proteins and peptides was markedly increased compared with controls subjected to ischaemia and reperfusion without nitrite administration (Fig 7) or with dogs in which nitrite was administerd prior to and during ischaemia ($NaNO_2$-PO group). As shown in Table 2 twenty-five proteins exhibited an increase in SNO in the $NaNO_2$-PR group. Many of these proteins have been previously shown to undergo increase SNO with preconditioning [26, 30]. Of particular interest, there was an increase in SNO of SERCA2a. We have previously found that SNO of SERCA2a results in an increase in its activity [30], which might lead to better ion homeostasis and contribute to the reduction of arrhythmias. Although we did not observe SNO of other calcium handling proteins, there is evidence that SNO of the L-type Ca^{2+} channels reduces calcium entry [30], or the release of calcium from the sarcuplasmic reticulum is influenced by SNO of the ryanodine receptor [54–56]. These mechanisms may counteract the ischaemia and reperfusion-induced calcium overload and contribute to cardioprotection.

In the $NaNO_2$-PR group we also found an increase in SNO of many proteins that are involved in cardiac metabolism and energetics. For example, several proteins involved in glycolysis and in the Krebs cycle showed increased SNO (Table 2). These enzymes are rich in both thiols and metal centers, making them easily react with NO. Although the exact role of SNO in many of these metabolism-related proteins has not yet been well characterized, the results give a support for the regulatory function of this mechanism in glycolysis and energy metabolism by reversible shielding critical cysteins of these enzymes from irreversible oxidation, thereby allowing a more rapid metabolic recovery from an ischaemia/reperfusion insult [23]. It is also hypothesized that the reversible inhibition of the Krebs cycle enzymes would result in slower electron transfer into the ETC with a subsequent reduction in the superoxide production during reperfusion [46].

However, surprisingly, we did not find increased SNO in dogs where nitrite was administered prior to and during occlusion ($NaNO_2$-PO group), although the protection against the arrhythmias and ischaemic changes was present, and the generation of oxidative stress products was also significantly suppressed. This conflict requires further considerations. The lack of SNO in the $NaNO_2$-PO group might suggest that nitrite is oxidized to nitrate rather than reduced to NO in these hearts. This assumption seems to be supported by the fact that there was

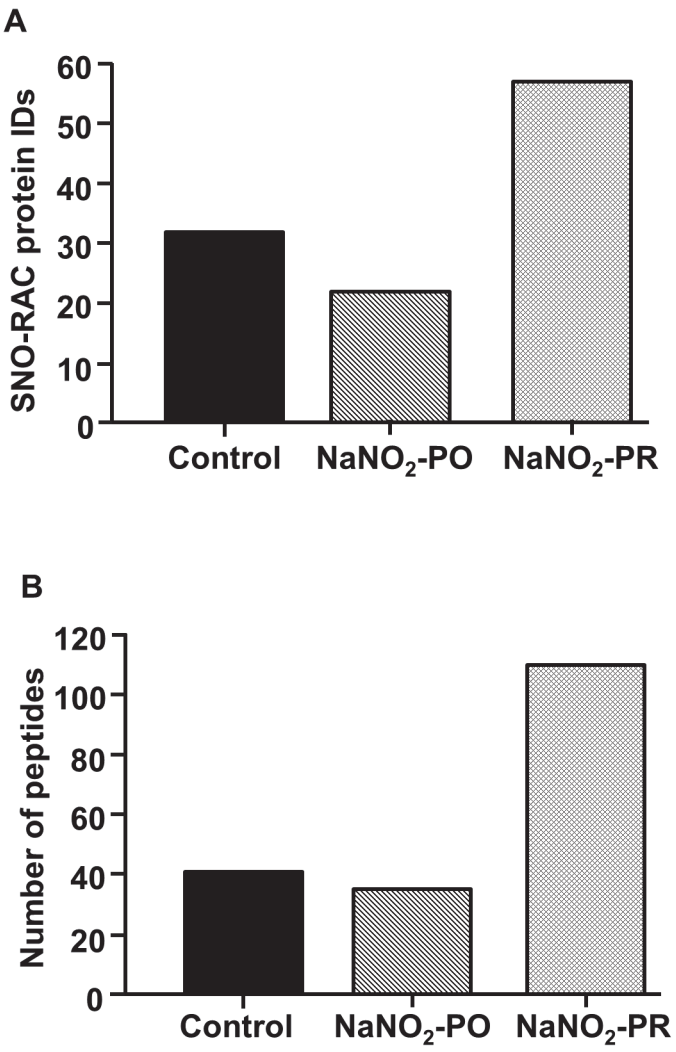

Fig 7. SNO-RAC protein identifications following 25 min ischaemia and reperfusion in control dogs and in dogs infused with sodium nitrite. Compared with controls there was an increase in the number of SNO proteins (A) and peptides (B) in dogs that had been infused with sodium nitrite prior to reperfusion ($NaNO_2$-PR group), as determined via liquid chromatography-tandem mass spectrometry for each treatment protocol at a false discovery rate of 5% (n = 4–5 hearts/group).

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a continuous increase in plasma nitrate levels in this group (Fig 5). However, considering that the samples were taken from the coronary sinus which collects blood from both the normal and ischaemic myocardium, it is unlikely that nitrate measured in the plasma is equivalent with nitrate concentrations in the ischaemic tissue and accurately reflects the oxidation/reduction of the infused nitrite. Another explanation for the reduced superoxide and nitrotyrosine production as well as for the obvious antiarrhythmic effect in these dogs might be that when nitrite is infused for a longer period of time the protective effects of NO are predominantly mediated through other mechanisms than SNO. Nitric oxide has many actions, which include both cGMP-dependent and cGMP-independent mechanisms. However, the reactions of NO are influenced by many different factors, such as the local concentration of NO and other molecules that react with NO (e.g. superoxide, heme proteins) or the surrounding milieu in which NO is produced etc. It might well be that the reduction in superoxide production in the

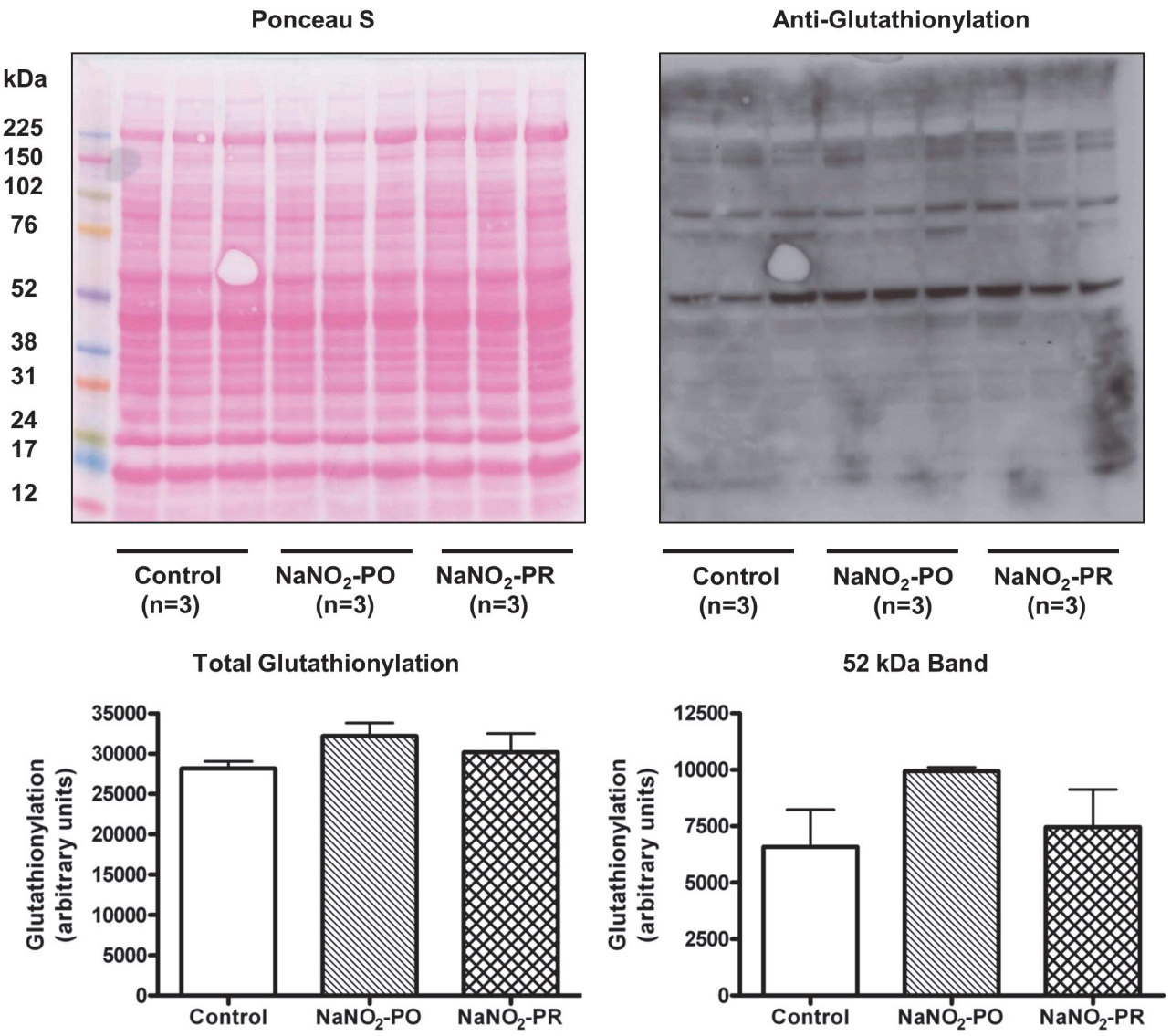

Fig 8. Protein S-glutathionylation levels in control dogs and in dogs infused with sodium nitrite. Compared with controls, there was an increase in protein S-glutathionylation in dogs that had been infused with sodium nitrite prior to occlusion ($NaNO_2$-PO group), as determined via Western immunoblot with an anti-glutathionylation antibody. Values represent mean ± SEM. n = 3 hearts/group.

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$NaNO_2$-PO group results primary from other actions of NO. These may include, among the others, the direct interaction of NO with heme proteins, such as soluble guanylyl cyclase, the terminal complex IV cytochrome oxidase [51], the NO mediated inhibition of neutropil superoxide production [57], as well as the NO-dependent activation of antioxidant enzymes [58] and signalling pathways, such as the cGMP/PKG pathway [59].

We also considered another possibility which might explain the lack of SNO in the $NaNO_2$-PO group. We supposed that when nitrite is administered over a longer period of time (i.e. prior to and during the entire occlusion interval), NO is generated and primarily promotes protein S- glutathionylation via GSNO. There is evidence that under conditions of continuous NO exposure resulting either from exogenous (administration of NO donors) or endogenous (activation of eNOS/iNOS) sources, NO induces protein S-glutathionylation [60]. This is thought

to regulate protein function and particularly under oxidative stress, protects protein thiols from irreversible protein oxidation by providing a "glutathione cap" [60]. If this concept is true, then the protection against arrhythmias in the $NaNO_2$-PO group can be explained by increased protein S-glutathionylation rather than protein SNO. To examine this possibility we measured, in the same tissue samples, protein S-glutathionylation. The results show that glutathionylation was somewhat more marked in the $NaNO_2$-PO group than in the control and the $NaNO_2$-PR groups (Fig 8). Although this change was not statistically significant ($p = 0.11$), the results indicate an increased number of glutathionylated proteins in this group and support the hypothesis that protein S-glutathionylation might also lead to protection [61, 62].

In summary, our results provide the first evidence that inorganic nitrite, administered in intravenous infusion in a concentration which results in a slight (approximate 7 mmHg) reduction in mean arterial blood pressure without substantially affecting coronary blood flow, markedly reduces the severity of ventricular arrhythmias that result from a 25 min occlusion and reperfusion of the LAD. This antiarrhythmic effect is particularly pronounced when the infusion of nitrite was started 10 min prior to reperfusion; in this case, survival from the combined ischaemia and reperfusion insult was 92%, compared with 0% in the controls and 50% in dogs in which nitrite was infused prior to and during the occlusion. Interestingly, the protective effect of nitrite was associated with increased protein SNO only when nitrite had been administered 10 min prior to reperfusion; the infusion of nitrite over a longer period seemed to prefer protein S-glutathionylation with subsequent protection against the serious consequences of the ischaemia and reperfusion. We propose that the marked antiarrhythmic effects of nitrite administration is associated with increased NO bioavailability during occlusion and the subsequent reduction in the effect of oxidative stress on reperfusion via those NO mediated mechanisms which reversibly protect proteins from oxidation during the early 'critical' phase of reperfusion.

Supporting Information

S1 Dataset. All the data presented in this paper are provided as an online supplement (S1_Dataset.xls).
(XLS)

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Author Contributions

Conceived and designed the experiments: ÁV EM. Performed the experiments: MK AK MG GM MJK. Analyzed the data: MK AK MG MJK. Contributed reagents/materials/analysis tools: GS JK. Wrote the paper: ÁV MK MG EM.

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ARTICLE

Further evidence for the role of gap junctions in the delayed antiarrhythmic effect of cardiac pacing[1]

Gottfried Miskolczi, Márton Gönczi, Mária Kovács, György Seprényi, and Ágnes Végh

Abstract: The objective of this study was to provide evidence that gap junctions are involved in the delayed antiarrhythmic effect of cardiac pacing. Twenty-four dogs were paced through the right ventricle (4 × 5 min, rate of 240 beats/min) 24 h prior to a 25 min occlusion of the left anterior descending coronary artery. Some of these paced dogs were infused with 50 (n = 7) or 100 μmol/L (n = 7) of the gap junction uncoupler carbenoxolone (CBX), prior to and during the occlusion. Ten sham-paced dogs, subjected only to occlusion, served as the controls. Cardiac pacing markedly reduced the number of ectopic beats and episodes of ventricular tachycardia (VT), as well the incidence of VT and ventricular fibrillation during occlusion. The changes in severity of ischaemia and tissue electrical resistance were also less marked compared with the unpaced controls. Pacing also preserved the permeability of gap junctions, the phosphorylation of connexin43, and the structural integrity of the intercalated discs. The closing of gap junctions with CBX prior to and during ischaemia markedly attenuated or even abolished these protective effects of pacing. Conclusion: Our results support the previous findings that gap junctions play a role in the delayed antiarrhythmic effect of cardiac pacing.

Key words: arrhythmias, carbenoxolone, gap junction, cardioprotection, ischaemia–reperfusion.

Résumé : Le but de la présente étude est de fournir des données probantes indiquant que les jonctions gap jouent un rôle dans l'effet antiarythmique retardé de la stimulation électrique cardiaque. Vingt-quatre chiens ont été soumis à une stimulation électrique du ventricule droit (4 × 5 min, fréquence de 240 battements/min) 24 h avant une occlusion de 25 min de l'artère interventriculaire antérieure. Chez certains des chiens soumis à la stimulation électrique, le carbénoxolone, un découpleur des jonctions gap, a été perfusé à des concentrations de 50 μmol/L (n = 7) et de 100 μmol/L (n = 7) avant et pendant l'occlusion coronarienne. Dix chiens « sham » soumis uniquement à l'occlusion coronarienne ont servi de témoins. La stimulation électrique du cœur a nettement diminué le nombre de battements ectopiques et d'épisodes de tachycardie ventriculaire, ainsi que la fréquence d'apparition de tachycardies et de fibrillations ventriculaires pendant l'occlusion coronarienne. En outre, nous avons observé moins de variations marquées de la gravité de l'ischémie et de la résistance électrique tissulaire que chez les témoins non stimulés. La stimulation électrique a aussi préservé la perméabilité des jonctions gap, la phosphorylation de la connexine43 et l'intégrité de la structure des disques intercalaires. La fermeture des jonctions gap induite par la carbénoxolone avant et pendant l'ischémie a nettement atténué ou même annulé les effets protecteurs de la stimulation électrique. Conclusion : Les résultats de la présente étude s'ajoutent aux résultats précédents qui indiquent que les jonctions gap jouent un rôle dans l'apparition des effets antiarythmiques retardés de la stimulation électrique du cœur. [Traduit par la Rédaction]

Mots-clés : arythmies, carbénoxolone, jonction gap, cardioprotection, ischémie–reperfusion.

Introduction

There is substantial evidence for the hypothesis that the electrical uncoupling of cells through gap junctions plays an essential role in the generation of severe ventricular arrhythmias that occur during the acute phase of myocardial ischaemia (e.g., Smith et al. 1995; Kléber and Rudy, 2004; Cascio et al. 2005). The first evidence for such a relationship between gap junctional uncoupling and the evolution of arrhythmias came from the observations showing that with the progression of ischaemia, the uncoupling of gap junctions is increased at the end-to-end connections, resulting in a steep elevation in tissue resistance (Kléber et al. 1987; Smith et al. 1995). The closure of these low-resistance pathways would lead to changes in impulse propagation and result in electrical inhomogeneity within the cardiac tissue. This electrical inhomogeneity initiates and maintains re-entry (deGroot and Coronel 2004; Cascio et al. 2005), and ultimately leads to the appearance of arrhythmias during phase Ib (Spach et al. 1988). There is strong experimental and clinical evidence that these acute Ib phase arrhythmias, which often end in ventricular fibrillation and thus are responsible for sudden cardiac death, result, in part, from the uncoupling of gap junctions (Smith et al. 1995; deGroot and Coronel 2004; Cascio et al. 2005).

A further support for the role of gap junctions in the early ischaemia-induced arrhythmias results from studies demonstrating that preconditioning, which has been proved to reduce these early life-threatening ventricular arrhythmias (Végh et al. 1992), modifies the function of gap junctions (Cinca et al. 1997; Papp et al. 2007). Preconditioning decreases the rate of uncoupling of gap junctions and delays their appearance (Cinca et al. 1997); moreover, preconditioning markedly reduces (Papp et al. 2007) the severity of the Ib phase of arrhythmias. This effect is also

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associated with the preservation of connexin43 (Cx43) phosphorylation and of the metabolic coupling of cells (Papp et al. 2007).

More recently, we have reported that gap junctions may also play a role in the delayed antiarrhythmic effects of preconditioning (Gönczi et al. 2012). Rapid right ventricular pacing, which has previously been shown to provide early and delayed protection against arrhythmias in the canine (Végh et al. 1991, 1994; Kaszala et al. 1996), preserved the structure and function of gap junctions during ischaemia, 24 h later. This preservation of gap junctional function was manifested in better electrical and metabolic coupling of cells during ischaemia, perhaps through maintaining Cx43 phosphorylation (Gönczi et al. 2012). This study also showed that pacing results not only in changes in Cx43 phosphorylation, but may also interfere with connexin turnover through the modification of the Cx43 gene, and protein expression during the 24 h period that elapses between pacing and the occlusion (Gönczi et al. 2012). We also have evidence that cardiac pacing induces time-course changes in gene expression encoding proteins involved in the delayed antiarrhythmic protection (Kovács et al. 2010, 2013).

A relationship between the preservation of gap junctional coupling and the antiarrhythmic protection following cardiac pacing would be largely strengthened if we could demonstrate that the closing of gap junctions by other means than ischaemia interfere with the protective effect of pacing. We already have evidence that carbenoxolone (CBX), a relatively selective uncoupler of gap junctions, significantly reduces the early antiarrhythmic effect of ischaemic preconditioning (Papp et al. 2007). Following this line of reasoning, in this study we have now examined whether the uncoupling of gap junctions with CBX would influence the antiarrhythmic protection afforded by cardiac pacing, 24 h previously.

Materials and methods

Ethics statement

Inbred mongrel dogs of both sexes were used in the experiments. The origin and upkeep of the dogs used in our experiments were in accord with Hungarian law (XXVIII, chapter IV, paragraph 31) regarding the use of large experimental animals, which conforms with the *Guide for the Care and Use of Laboratory Animals* (ILAR 1996), and also conformed to Directive 2010/63/EU of the European Parliament. The protocols were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/1211/2012).

Cardiac pacing protocol

This was performed as described previously (Kaszala et al. 1996). In brief, under light pentobarbitone anaesthesia (Euthanyl; Bimeda–MTC Animal Health; 0.5 mg/kg body mass, by intravenous (i.v.) injection), which allowed the dogs to breathe spontaneously, a Millar tip catheter (5F; Millar Instruments) was inserted into the left carotid artery for measuring arterial blood pressure. A 7F bipolar pacing electrode (Hugo Sachs Electronik–Harvard Apparatus) was introduced into the right ventricle through the jugular vein, and through this the heart was paced 4 times for 5 min at a rate of 240 beats/min. Twenty-four dogs were subjected to cardiac pacing, whereas in another 10 dogs the electrode was introduced into the right ventricle but the dogs were not paced. These "sham paced" dogs served as controls (Fig. 1). The dogs were then allowed to recover from the surgical interventions.

Coronary artery occlusion

Twenty-four hours after cardiac pacing, the dogs were reanaesthetized with a mixture of α-chloralose (60 mg/kg) and urethane (200 mg/kg) (Sigma, St. Louis, Missouri, USA), intubated, and ventilated with room air using a Harvard respirator (Harvard Apparatus, Natick, Massachusetts, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits (Végh et al. 1992). Body temperature was measured from the mid-oesophagus, and maintained by means of a heating pad at a temperature between 36.5 and 37.5 °C.

Polyethylene catheters were inserted into the right femoral vein and into the femoral artery for further administration of the anaesthetic and to measure arterial blood pressure. The Millar tip catheter, which had been inserted into the carotid artery, was now pushed into the left ventricle (LV) to detect changes in systolic (LVSP) and end-diastolic (LVEDP) pressures as well as in LV dP/dt. The depth of anaesthesia was monitored by the examination of the cornea and pain reflexes, as well as by measuring the blood pressure, and when it was necessary a further bolus injection of the anaesthetic was administered.

After thoracotomy, the anterior descending branch of the left coronary artery (LAD) was occluded for 25 min, as previously described (Végh et al. 1992). The severity of myocardial ischaemia was assessed by the measurement of changes in the degree of inhomogeneity in electrical activation and in epicardial ST-segment, using a mapping electrode positioned within the ischaemic myocardial region (Gönczi et al. 2012). Signals were collected from 31 unipolar electrodes (inter-electrode distance 2 mm) at a frequency of 1 kHz, stored on a computer, and analyzed offline by creating activation and ST maps. Changes in epicardial activation were assessed as the time delay between the first and a last point activated under the electrode, and is expressed as the total activation time (TAT) in milliseconds. Elevations of epicardial ST-segment, recorded each minute from the unipolar electrodes, were averaged and are expressed in millivolts.

Assessment of gap junctional electrical coupling

For the assessment of gap junctional electrical coupling, we used a method similar to one previously described in detail (Papp et al. 2007; Gönczi et al. 2012). In brief, 4 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 ventricular wall within the proposed ischaemic myocardial area. 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, California, USA). Changes in resistivity (Ω·cm) 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, 5 consecutive 4 s measurements were averaged at each minute.

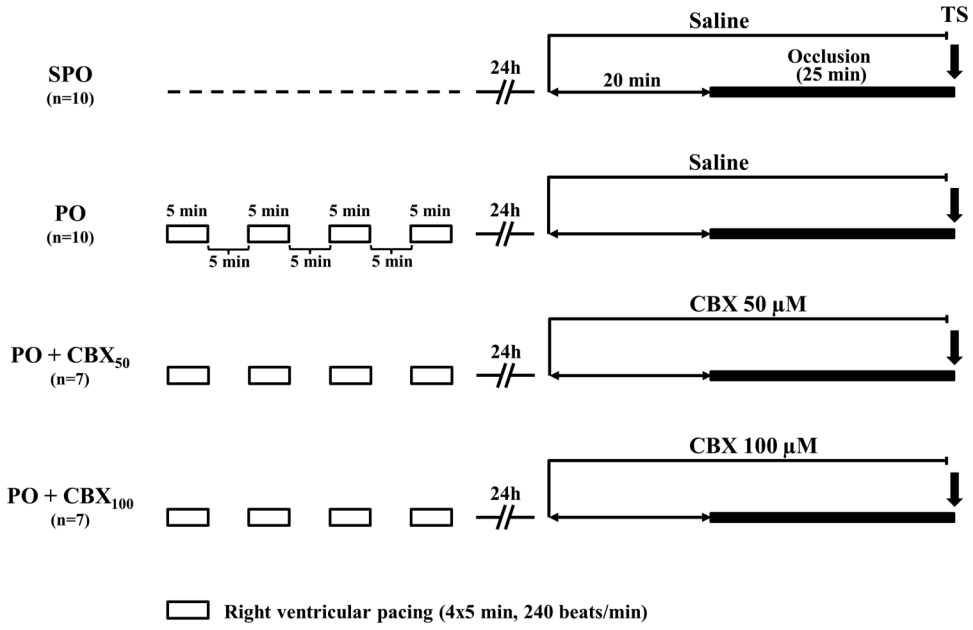
Assessment of ventricular arrhythmias

Ventricular arrhythmias during a 25 min coronary artery occlusion and following reperfusion were assessed according to the Lambeth Conventions (Walker et al. 1988), with modification as previously outlined by Végh et al. (1992). In brief, the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT; defined as a run of 4 or more consecutive VPBs at a rate faster than the resting heart rate), and the incidence of ventricular fibrillation (VF) were assessed during the 25 min of the LAD occlusion.

Immunofluorescence analysis

Immunofluorescence was used to determine changes in Cx43 signal intensity. Small transmural tissue blocks (0.5 cm × 0.5 cm × 2.0 cm) sectioned from myocardial regions supplied by the LAD and LCX branches of the left coronary artery were embedded in OCT compound, frozen in liquid nitrogen, and stored at −80 °C until processing. Longitudinal sections (8 μm thick) were cut from the mid-myocardium in a cryostat (Leica) at −20 °C, and the

Fig. 1. Experimental protocol. On day 1, under light pentobarbitone anaesthesia, the hearts were paced through the right ventricle 4 times for 5 min at a rate of 240 beats/min. On day 2 (24 h later), both the paced (PO; $n = 24$) and sham-paced dogs (SPO; $n = 10$) were subjected to a 25 min occlusion of the left anterior descending coronary artery. In 14 of the paced dogs, carbenoxolone (CBX) was infused into a small branch of the anterior descending branch of the left coronary artery (LAD) in concentrations of 50 μmol/L (PO+CBX_{50}; $n = 7$) and 100 μmol/L (PO+CBX_{100}; $n = 7$). The infusions were started 20 min prior to and maintained over the entire 25 min occlusion period. In the non-paced animals (SPO), on day 2, instead of CBX, saline was infused by the same route; these dogs served as the controls. In each group at the end of the occlusion period the animals were euthanized and tissue samples (TS) were taken from the areas supplied by the occluded LAD and the non-occluded left circumflex (LCX) coronary arteries, for further analyses.



slices were mounted on polylisine-coated slides. After blocking in 5% bovine serum albumin, the samples were labelled overnight with a 1:800 dilution of polyclonal rabbit anti-Cx43 antibody (Sigma–Aldrich) at 4 °C, then with FITC-conjugated secondary antibody (1:1500) for 1 h at room temperature, and finally with wheat germ agglutinin – Texas Red-X conjugate (1:500; Invitrogen) for 30 min at room temperature (25 °C). Images were captured with a laser scanning confocal microscope (FV1000; Olympus) using a 40× magnifying objective.

Immunoblot analysis

Changes in Cx43 protein phosphorylation was assessed by Western blot. The preparation and the determination procedures were as the same as described previously (Papp et al. 2007; Gönczi et al. 2012). 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 using the Bradford method (Bradford 1976). From each sample, 10 μg protein was separated on 10% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, Mass.). The blots were blocked with 5% non-fat milk, dissolved in TBST (Tris-buffered saline – Tween 20) for 1 h, and labelled overnight with a rabbit polyclonal anti-Cx43 antibody (Sigma–Aldrich) diluted to 1:500 000. This was followed by incubation for 1 h with HRP-conjugated anti-rabbit goat secondary antibody (Santa Cruz Biotech, Santa Cruz, Calif.) diluted to 1:10 000 at room temperature. Blots were visualized with the ECL Plus kit (GE Healthcare) using X-ray films (GE Healthcare). Band intensities were determined using the ImageJ software version 1.48 (NIH) and the relative amount of phosphorylated and dephosphorylated Cx43 isoforms are expressed as a percentage of the total sarcolemmal connexin content.

Determination of gap junctional metabolic coupling

This was evaluated by measuring tissue permeability using the "double-dye loading" method, as previously described in detail (Papp et al. 2007). In brief, freshly excised transmural tissue blocks from both the ischaemic and non-ischaemic ventricular walls were submerged for 15 min in a mixture of Lucifer yellow (LY, 1.5 mg/mL) and tetramethylrhodamine isothiocyanate – dextran (TD, 3.5 mg/mL; Sigma–Aldrich), fixed in paraformaldehyde, and then cryosections (20 μm) were prepared. Ten 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 ImageJ software. Gap junction permeability within the ischaemic area is expressed as a percentage of permeability measured within the non-ischaemic wall region.

Experimental protocol

This is illustrated in Fig. 1. A total of 34 dogs were used and randomly distributed among 4 groups. Both the paced (PO; $n = 24$) and sham-paced dogs (SPO; $n = 10$) were subjected to a 25 min occlusion of the LAD. In 14 dogs that were paced 24 h previously, CBX was infused into a small branch of the LAD at concentrations of either 50 μmol/L (PO+CBX_{50}; $n = 7$) or 100 μmol/L (PO+CBX_{100}; $n = 7$). The infusions were started 20 min prior to the occlusion period and maintained over the entire 25 min occlusion period. In the non-paced animals (SPO), on day 2, instead of CBX, saline was infused by the same route; these dogs served as controls. At the end of the occlusion period, the animals in each group were given an overdose of the anaesthetic, and the hearts were rapidly excised and placed in ice-cold saline. Tissue samples were taken from the areas supplied by the occluded LAD and the non-occluded left circumflex (LCX) coronary arteries, for further analyses.

Statistical analysis

All presented values are the mean ± SEM. Differences between groups, where normal distribution of data was verified, were compared using Student's t test, one-way ANOVA, or ANOVA for re-

Table 1. Haemodynamic effects of carbenoxolone and saline infusion.

	Saline		CBX 50 μmol/L		CBX 100 μmol/L	
	Baseline	Change	Baseline	Change	Baseline	Change
SABP (mm Hg)	159±5	−2±2	144±11	4±2	145±4	−2±2
DABP (mm Hg)	104±8	0±1	97±7	3±2	100±2	−2±2
MABP (mm Hg)	119±7	−1±2	113±8	3±2	115±2	−4±2
LVSP (mm Hg)	158±9	−3±1	131±11	3±2	124±3	−1±2
LVEDP (mm Hg)	5±1	0±1	8±1	1±1	9±2	2±0
$+dP/dt_{max}$ (mm Hg/s)	2980±50	−9±2	2834±202	145±86	2374±273	39±72
$-dP/dt_{max}$ (mm Hg/s)	2640±134	8±44	2477±127	203±113	1952±333	−120±100
HR (beats/min)	156±9	1±2	153±5	0±2	155±9	−2±2

Note: CBX, carbenoxolone; SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; 1 mm Hg = 133.322 Pa. Values are the mean ± SEM.

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

	SPO		PO		$PO+CBX_{50}$		$PO+CBX_{100}$	
	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change
SABP (mm Hg)	159±5	−11±4*	160±5	−9±4*	148±14	−9±1*	150±5	−10±4*
DABP (mm Hg)	108±4	−12±4*	107±3	−7±3*	100±9	−7±2*	104±5	−8±2*
MABP (mm Hg)	124±4	−14±2*	125±4	−8±3*	116±10	−8±1*	119±4	−9±3*
LVSP (mm Hg)	160±6	−13±4*	163±4	−4±3*	157±15	−7±1*	158±5	−9±2*
LVEDP (mm Hg)	8±1	8±1*	8±1	5±0*	9±1	7±1*	10±1	10±1*
$+dP/dt_{max}$ (mm Hg/s)	3039±199	−678±68*	2943±140	−362±163*	3120±299	−387±97*	2483±247	−487±110*
$-dP/dt_{max}$ (mm Hg/s)	2586±153	−551±164*	2708±153	−446±57*	2633±181	−346±104*	1394±247	−499±94*
HR (beats/min)	167±6	6±3	149±5	6±3	150±6	−1±3	154±9	3±6

Note: SPO, sham-paced dogs; PO, paced dogs; $PO+CBX_{50}$, paced dogs infused with 50 μmol/L CBX; $PO+CBX_{100}$, paced dogs infused with 100 μmol/L CBX; CBX, carbenoxolone; SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; 1 mm Hg = 133.322 Pa; *, $P < 0.05$ compared with the baseline values. Values are the mean ± SEM.

peated measures with Fisher's post-hoc test, as appropriate. Data with non-normal distribution, e.g., the number of VPBs and of VT episodes and incidences of VT, VF, were compared with a Mann–Whitney *U* test and Fisher's exact test, respectively. Differences between groups were considered statistically significant at $P < 0.05$.

Results

Haemodynamic effects of carbenoxolone and of coronary artery occlusion

These are summarized in Tables 1 and 2. Infusion of saline prior to occlusion in the control and also in paced dogs resulted in no significant alterations in the haemodynamic parameters (data are summed from the SPO and PO groups). There were also no significant haemodynamic changes following the local administration of CBX in either concentration (Table 1). Occlusion of the LAD resulted in significant reductions in arterial blood pressure, left ventricular systolic pressure, and in positive and negative dP/dt_{max}, and an increase in LVEDP in all groups. These changes were somewhat less pronounced in the paced dogs, irrespective of whether they were treated with CBX or not. In anaesthetized dogs, occlusion of the LAD resulted in no significant changes in the heart rate (Table 2).

The severity of ventricular arrhythmias during a 25 min occlusion of the LAD

This is illustrated in Fig. 2. Compared with the sham-paced controls, in dogs subjected to rapid right ventricular pacing 24 h prior to the occlusion, the number of VPBs (267 ± 31 vs. 36 ± 8) and the episodes of VT (8.7 ± 2.8 vs. 0.3 ± 0.3), as well as the incidences of VT (90% vs. 10%) and VF (50% vs. 10%) were significantly reduced. This protective effect of cardiac pacing was significantly attenuated by the administration of CBX, especially when it was given at a concentration of 100 μmol/L. Thus, in the presence of 50 or 100 μmol/L CBX, there were more ectopic beats (76 ± 11 and 236 ± 101) and episodes of tachycardia (1.3 ± 0.8 and 5.3 ± 4.2) in the paced dogs. Further, in these groups, more dogs exhibited VT (43% and 57%) and VF (57% and 29%) during the occlusion than in the PO group.

Changes in tissue resistivity in relation to the distribution of VPBs during a 25 min of occlusion

The distribution of VPBs in relation to changes in tissue electrical resistance over a 25 min coronary artery occlusion are illustrated in Fig. 3. The sudden occlusion of the LAD resulted in an immediate increase in tissue resistivity and the occurrence of ectopic beats, which peaked around 5 min of ischaemia. After this, the phase Ia arrhythmias were decreased. At around 12 min of occlusion, the tissue resistivity started to steeply increase again, and that was accompanied by the appearance of the phase Ib arrhythmias. This phase of arrhythmias peaked around 20 min of ischaemia, after which the ectopic activity declined again, whereas the resistivity continued to increase. In contrast, in dogs subjected to pacing 24 h previously, the changes in resistivity caused by occlusion of the LAD were significantly less marked, particularly during phase Ib. Thus, in this case, the steep increase in resistance virtually disappeared, and, in parallel, the ectopic activity was almost completely abolished. The administration of CBX to the paced dogs, irrespective of the concentration used, resulted in a continuous and steep elevation in tissue resistance. However, it is interesting to note that CBX influenced the number of ectopic beats in a concentration-dependent manner, i.e., whereas the lower concentration (50 μmol/L) preferentially increased the number of VPBs during phase Ib, the higher concentration (100 μmol/L) of the drug enhanced ectopic activity during both phase Ia and Ib.

Changes in ischaemia severity during coronary artery occlusion

The severity of ischaemia was assessed by 2 parameters: changes in epicardial ST-segment (Fig. 4A), and in the total activation time

Fig. 2. Arrhythmia events during a 25 min period of coronary artery occlusion in the control (SPO) and in paced dogs (PO), as well as in those paced animals that were treated with carbenoxolone (CBX) at either 50 μmol/L (PO+CBX_{50}) or 100 μmol/L (PO+CBX_{100}). Compared with the controls, for dogs paced 24 h previously, the total number of ventricular premature beats (VPBs), the incidence (%) and the number of episodes of ventricular tachycardia (VT) and the incidence (%) of ventricular fibrillation (VF) during occlusion were significantly reduced. This marked antiarrhythmic effect of pacing was attenuated or even abolished when CBX was administered before the occlusion in concentrations of 50 or 100 μmol/L, respectively. Values are the mean ± SEM. *, $P < 0.05$ compared with the SPO group; #, $P < 0.05$ compared with the PO group.

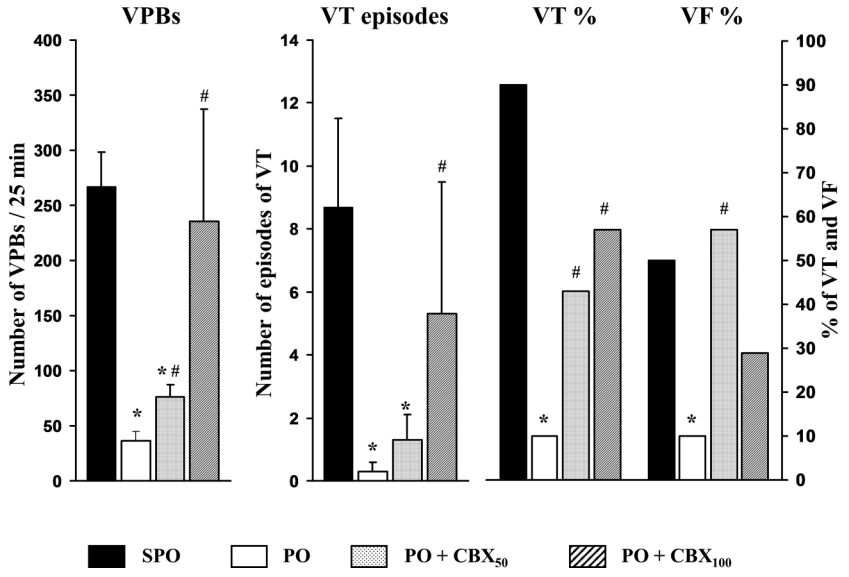


Fig. 3. Distribution of ventricular premature beats (VPBs) and relative changes in tissue resistivity during a 25 min occlusion of the anterior descending branch of the left coronary artery (LAD) in control (SPO) and in paced dogs (PO), as well as in those paced animals that were treated with carbenoxolone (CBX). Compared with the controls, cardiac pacing 24 h prior to occlusion markedly reduced the number of VPBs and attenuated the changes in tissue resistivity. In contrast, for dogs paced 24 h before occlusion that were infused with CBX, the number of VPBs and changes in tissue impedance were again markedly increased. Following treatment with 50 μmol/L of CBX (PO+CBX_{50}), the number of VPBs was only increased during the Ib phase (between 13 and 25 min of the occlusion), whereas 100 μmol/L (PO+CBX_{100}) also enhanced the ectopic activity during phase Ia. Values are the mean ± SEM. *, $P < 0.05$ compared with the SPO group.

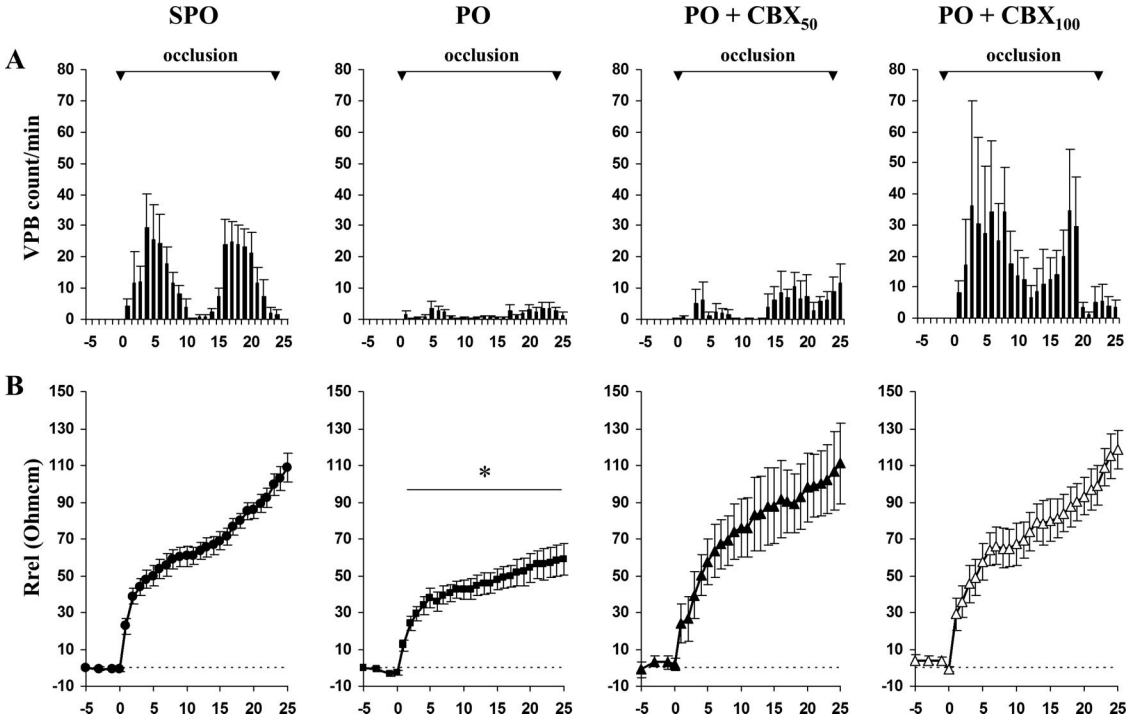
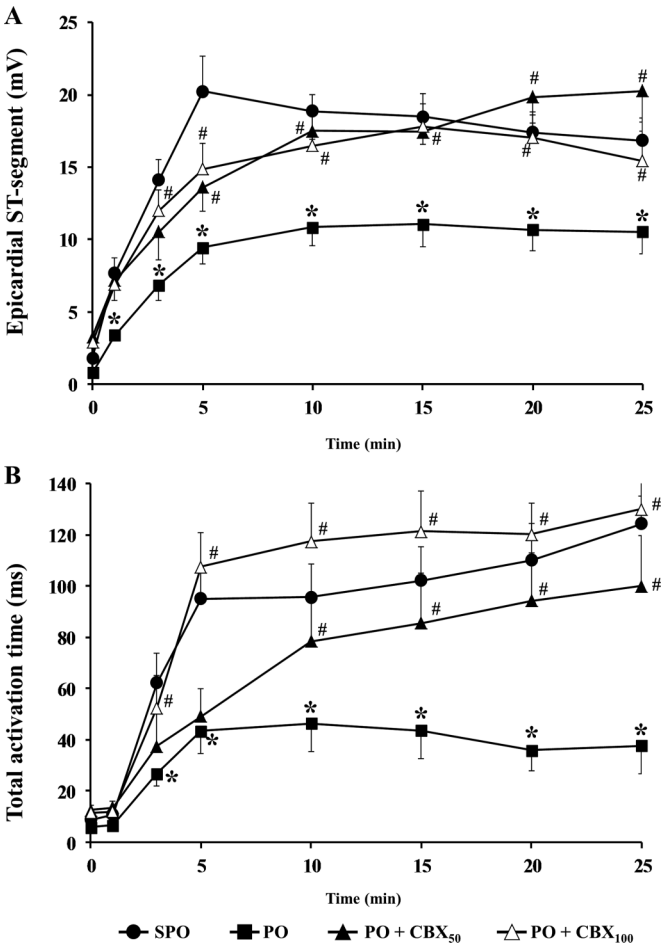


Fig. 4. (A) Changes in total activation time (TAT) and (B) epicardial ST-segment during a 25 min occlusion of the LAD. Compared with the controls, cardiac pacing 24 h previously significantly reduced the elevations of TAT and epicardial ST-segment over the entire occlusion period. In the presence of carbenoxolone (CBX), these indices of ischaemia severity were again increased; the elevation of TAT was particularly marked following treatment with 100 μmol/L CBX. Values are the mean ± SEM. *, $P < 0.05$ compared with the SPO group; #, $P < 0.05$ compared with the PO group.



(TAT; Fig. 4B). In the non-paced control dogs, occlusion of the LAD resulted in immediate and marked increases in both indices of ischaemia severity, reaching the maximum values around 5 min of the ischaemia. In contrast, in dogs paced 24 h previously, these ischaemia-induced changes were significantly reduced (Fig. 4). However, when CBX was infused in these paced dogs prior to and during occlusion, both the epicardial ST-segment and TAT were again significantly increased.

Changes in connexin43 phosphorylation in paced dogs following CBX administration

These changes are illustrated in Fig. 5. In samples taken from the myocardial wall supplied by the LCX coronary artery, the phosphorylated form of Cx43 usually predominates over the dephosphorylated form, indicating that the majority of gap junctions are in an open state in this normal, non-ischaemic region (SC group). In contrast, in samples collected from the area supplied by the occluded LAD, the phospho/dephospho ratio shifts towards dephosphorylation, indicating that a part of the gap junctions has already closed within the ischaemic myocardial region (SPO group). Cardiac pacing performed 24 h before the coronary artery occlusion, dephosphorylation induced by 18 prevented the ischaemia-induced dephosphorylation of Cx43, resulting in the distribution of the phospho/dephospho forms in a manner similar to that seen in the normal, non-ischaemic samples. Although the administration of either 50 or 100 μmol/L CBX in paced dogs significantly increased the dephosphorylation of Cx43, this dephosphorylation was still less pronounced than in the non-paced (SPO) controls. Indeed, in the CBX-treated paced dogs, the levels of the phosphorylated and dephosphorylated forms of Cx43 were almost equal.

The effect of CBX on gap junctional permeability following cardiac pacing

These results are shown in Fig. 6. In dogs subjected to a 25 min period of occlusion, gap junctional permeability, as assessed within the ischaemic area, was significantly decreased to 68% of the normal non-ischaemic (100%) value. This reduction in permeability was completely abolished in dogs paced 24 h previously. However, when CBX was infused in the paced dogs, the permeability was again significantly reduced.

Assessment of the structural alterations of the intercalated discs following cardiac pacing and CBX administration

The structural alterations occurring after a 25 min period of coronary artery occlusion in control dogs and in paced dogs with and without CBX administration are illustrated by a series of representative confocal immunofluorescence images (Fig. 7), prepared from tissue samples collected from both the ischaemic (supplied by the LAD) and the non-ischaemic (supplied by the LCX) myocardial wall. For the dogs subjected to a 25 min occlusion without cardiac pacing (SPO group), the images captured from the ischaemic LAD area showed signs of structural impairment of the intercalated discs (indicated with arrows); i.e., the contour of these end-to-end connections became "blurred" compared with that from the images obtained from samples taken from the non-ischaemic (LCX) myocardial region (Fig. 7A). In contrast, for the dogs subjected to cardiac pacing 24 h previously, even after a 25 min coronary occlusion, the integrity of the intercalated discs was preserved within the LAD region, and there were no visible differences between the 2 areas (Fig. 7B). Carbenoxolone (CBX), administered in concentrations of 50 or 100 μmol/L to the paced dogs prior to and during the occlusion, resulted in again perceivable alterations (dim membrane density, blurred contour) around the disc areas; however, it is interesting to note that these changes occurred not only in the area supplied by the occluded LAD, but also in the region supplied by the patent LCX artery (Fig. 7C and D). These results suggest that CBX may alter the structure of gap junctions.

Discussion

We have prior evidence that gap junctions play a role in both the early and the delayed antiarrhythmic effects of preconditioning induced either by short coronary artery occlusion or rapid cardiac pacing (Papp et al. 2007; Gönczi et al. 2012). One piece of evidence for this was that the closing of gap junctions during the preconditioning procedure, using CBX, which is a relatively selective uncoupler of gap junction channels, attenuates the early protection against arrhythmias resulting from brief periods of ischaemia–reperfusion (Papp et al. 2007, 2008; Végh and Papp 2011). In those studies in which the participation of gap junctions in the delayed phase of the protection was examined, we have pointed out that pacing induces time-dependent changes in connexin43 mRNA and protein expression, further it preserves the phosphorylation of Cx43 and structural integrity of the gap junction during occlusion, 24 h later (Gönczi et al. 2012). We proposed that these effects may have a role in the delayed antiarrhythmic effect associated with cardiac pacing (Kaszala et al. 1996; Gönczi et al. 2012). These experiments, however, did not provide adequate evidence for a direct relationship between the pacing-

Fig. 5. Changes in the ratio of phosphorylated/dephosphorylated connexin43 (Cx43) in the ischaemic myocardial samples (supplied by the occluded LAD) taken from control (SPO), paced (PO), and carbenoxolone (CBX) treated dogs (50 μmol/L, PO+CBX50; 100 μmol/L, PO+CBX100), in comparison with samples obtained from the non-ischaemic (supplied by the LCX) area of the untreated sham controls (SC). Ischaemia induced marked dephosphorylation of Cx43, which was prevented by cardiac pacing, 24 h previously. The administration of either 50 or 100 μmol/L CBX significantly reduced the phospho/dephospho ratio of Cx43, compared with the untreated paced dogs. Values are the mean ± SEM. †, $P < 0.05$ compared with the SC group; *, $P < 0.05$ compared with the SPO group; #, $P < 0.05$ compared with the PO group.

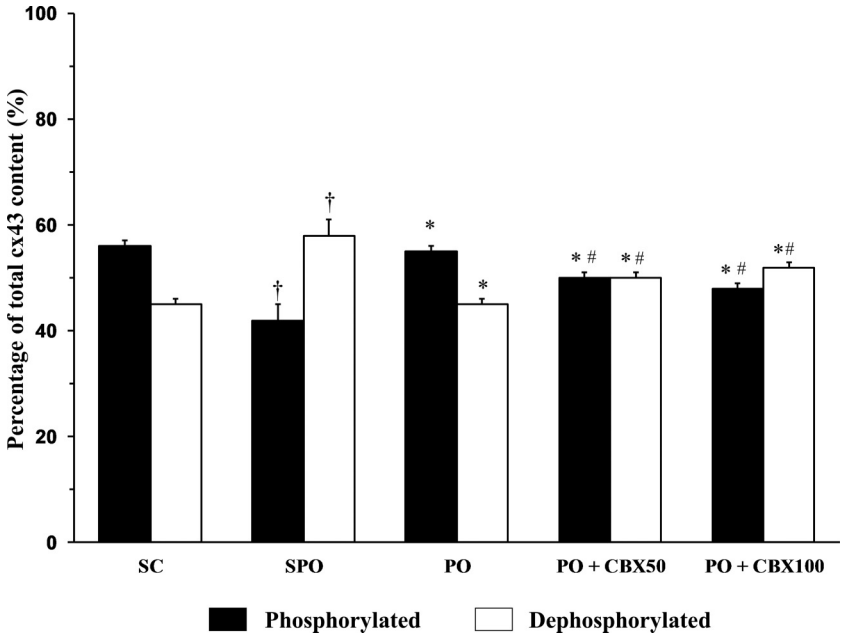
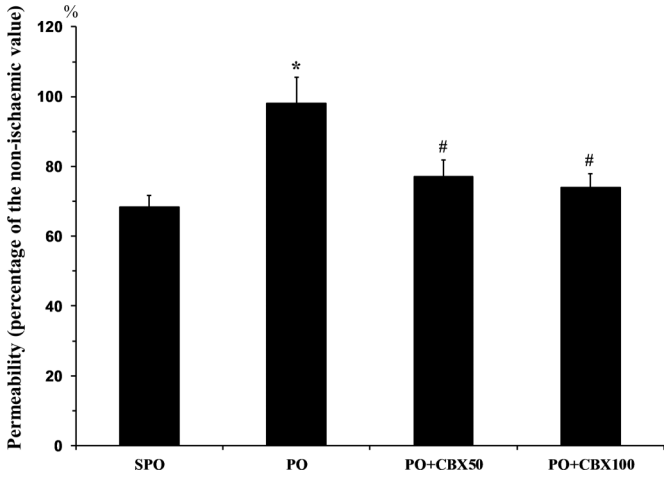


Fig. 6. The effect of carbenoxolone (CBX) on gap junctional permeability in dogs subjected to cardiac pacing, 24 h previously. In the paced dogs (PO group), gap junctional permeability within the ischaemic area (expressed as percentage of permeability measured in the non-ischaemic area) is well-preserved compared with the unpaced controls (SPO). CBX at either 50 μmol/L (PO+CBX50) or 100 μmol/L (PO+CBX100), reduced this preserving effect of pacing on metabolic coupling. Values are the mean ± SEM. *, $P < 0.05$ compared with the SPO group; #, $P < 0.05$ compared with the PO group.

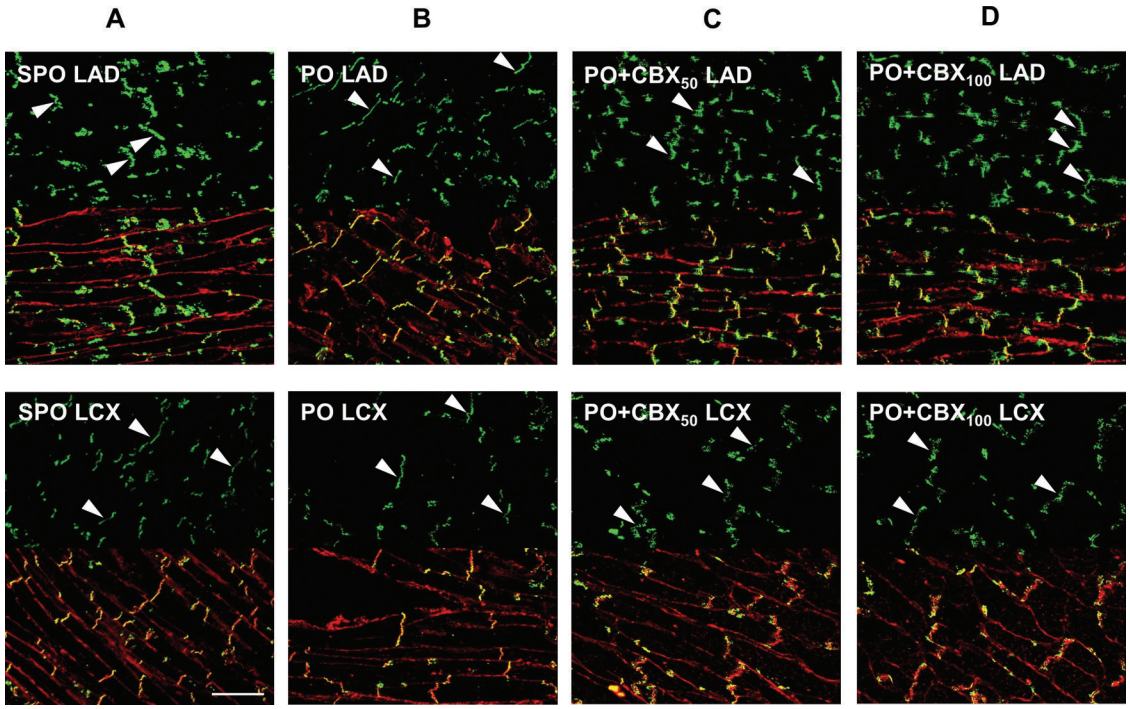


induced suppression of arrhythmias and gap junctions. Therefore, we have now raised the question and designed studies to examine how the antiarrhythmic protection is modified in the paced dogs if the gap junctions are closed by the gap junction uncoupler CBX. In this study we used CBX in 2 concentrations: 50 and 100 μmol/L.

We have found that CBX infused in paced dogs 24 h after the pacing stimulus, but prior to and during the entire occlusion period, attenuated the antiarrhythmic effect of cardiac pacing. Thus, in the paced dogs, following the administration of CBX there was a significant increase in the number of VPBs and VT episodes, as well as in the incidence of VT and VF during occlusion (Fig. 2). Some of these arrhythmia types, such as the number of ectopic beats and the episodes of VT, showed a concentration-dependent increase in the CBX-treated animals compared with the untreated paced dogs. However, it needs to be noted that the marked increase in ectopic activity in those paced dogs that were infused with 100 μmol/L of CBX, most probably resulted from the increase in VPBs occurring during phase Ia, as can clearly be seen from the distribution of these arrhythmias (Fig. 3A). This result indicates that the higher concentration of CBX affects not only the Ib but also the Ia phase of arrhythmias, suggesting that CBX in the concentration of 100 μmol/L acts not only on gap junctions, but may modify those ion channels that are involved in the development of phase Ia arrhythmias. Although in a previous study we showed that 50 μmol/L CBX does not affect sodium, potassium, and calcium ion channels (Papp et al. 2007), it could be that CBX at this concentration is highly selective for gap junctions (deGroot et al. 2003), as we have not found any evidence that 100 μmol/L of CBX has the same effect.

Therefore, we performed in-vitro experiments using canine right ventricular papillary muscle preparations, in which we measured the effects of CBX on the maximum upstroke velocity (dV_{max}) and on the duration (APD) of the action potential. We found that compared with the control samples, 50 and 100 μmol/L CBX concentration-dependently shortened APD90 (from 236 ± 12 ms to 227 ± 12 and 206 ± 9 ms, respectively; this latter change was significant at $P < 0.05$ (using the Student t test)), but there was no significant change in dV_{max} (88 ± 16 V/s vs. 64 ± 24 and 60 ± 6 V/s, ns). In Langendorff-perfused guinea pig hearts, it was found that 100 μmol/L of CBX increased the calcium oscillations during diastole, and caused delayed after-depolarisations, as well as an enhancement in arrhythmia generation (Plummer et al. 2011). These results suggest that CBX in a higher concentration range may

Fig. 7. Representative immunofluorescence images obtained from control (A), paced (B), and carbenoxolone (CBX) treated (C and D) dogs, in samples collected from myocardial regions supplied by the anterior descending branch of the left coronary artery (LAD; ischaemic) and LCX (non-ischaemic) coronary arteries. In dogs subjected simple to a 25 min ischaemia, the immunofluorescence images captured from the ischaemic LAD area indicated signs of structural impairment of the intercalated discs compared with the non-ischaemic LCX zone. Such a structural impairment was not seen in the paced dogs, indicating that pacing preserves the integrity of the end-to-end connections. The administration of CBX to paced dogs markedly attenuated this beneficial effect of pacing; in the presence of CBX infusion, signs of structural impairment occurred again around the intercalated discs, not only within the ischaemic area, but also in the non-ischaemic LCX region. This suggests that CBX is distributed in the whole heart during that 20 min infusion period, which precedes the occlusion, and may associated with structural rearrangements of the end-to-end connections caused by CBX.



influence ion channels other than gap junctions, and this observation would also explain why we found a marked increase in the number of VPBs during phase Ia following the administration of 100 μmol/L of CBX (Fig. 3). Since there were no substantial differences between the effects of the 2 concentrations of CBX on the tissue resistivity changes (both concentrations resulted in a marked and almost similar elevations in resistivity from the beginning of the occlusion), the increase in ectopic activity following administration of 100 μmol/L CBX is most probably not only resulting from a higher rate of closure of gap junctions, but also from a rapid development in conduction disturbances due to the unspecific (not gap junction mediated) effects of this concentration of CBX. This assumption is also supported by the fact that there was a much faster increase in TAT in dogs that had been given 100 μmol/L of CBX than was found in the controls or in the dogs treated with 50 μmol/L (Fig. 4B). Such a concentration-dependent effect was not seen in case of the epicardial ST-segment changes following the administration of CBX (Fig. 4A).

Our previous study showed that cardiac pacing not only results in time-dependent alterations in mRNA and protein expression of Cx43, but it also modifies the changes in Cx43 phosphorylation that results from a 25 min period of coronary artery occlusion, 24 h later (Gönczi et al. 2012). We hypothesized that the preservation of the phosphorylated form of Cx43 that resulted from pacing, and which can be associated with an opened state of gap junctions (Beardslee et al. 2000; Matsushita et al. 2009) would play a role in the protection against arrhythmias. Thereafter, we subsequently wondered whether closing of the gap junction with CBX in these paced dogs influenced Cx43 phosphorylation, and whether this could be related to the attenuation of the anti-arrhythmic protection. We have found that compared with the sham-control (SC) samples that had been taken from the non-ischaemic areas (supplied by the LCX) of the untreated dogs, the phospho/dephospho ratio of Cx43 was shifted towards dephosphorylation in the ischaemic samples (SPO) collected from the occluded LAD myocardial wall region. Pacing inhibited this ischaemia-induced dephosphorylation of Cx43, and preserved the normal phospho/dephospho ratio within the ischaemic area (Fig. 5). The administration of CBX decreased the phosphorylated form and increased the dephosphorylated form of Cx43, resulting in an almost equal distribution between the phospho and dephospho forms of Cx43, but it did not completely abolish the effect of pacing on Cx43 phosphorylation. This result may suggest that the gap junction uncoupling effect of CBX is perhaps not entirely due to the dephosphorylation of Cx43. In this respect, data in the literature are also controversial. For example, it has been found that in the liver epithelial cells, CBX increased the dephosphorylation of Cx43 (Guan et al. 1996), whereas in rabbit hearts CBX had no effect on Cx43 phosphorylation (deGroot et al. 2003).

However, CBX almost completely reversed the pacing-induced preservation of the metabolic coupling of cells, as assessed by measuring gap junction permeability (Fig. 6). Furthermore, in the presence of CBX the intercalated discs (IDs) underwent substantial structural changes. As we have reported previously (Gönczi et al. 2012), and as also shown in the representative immunofluorescence images of the present study (Fig. 7), pacing prevented those structural rearrangements (dim contour and fragmentation of the end-to-end connections) that occurred around the IDs within the ischaemic area, following a 25 min occlusion of the LAD. When CBX was given in these paced dogs prior to and during the occlu-

sion, similar structural changes appeared around the IDs to those observed in the control, unpaced dogs. Interestingly, however, these structural changes occurred not only within the ischaemic LAD region, but also in those samples that had been taken from the non-ischaemic LCX area. This finding indicates that although CBX was administered locally (into a side branch of the LAD), the drug was distributed throughout the heart during the 20 min infusion period, and resulted in visible structural alterations, also within the normal non-ischaemic myocardium. This observation agrees with other reports which found that gap junction uncouplers such as CBX and isoproterenol cause an irregular arrangement of the gap junction plaques (Inoue et al. 1987; Goldberg et al. 1996). Nevertheless, it is difficult to assess how these morphological changes would account for the alteration in the function of gap junctions, and whether the observed structural alterations resulting from ischaemia, cardiac pacing, or CBX administration could entirely explain the functional changes of gap junctional coupling, and the subsequent modification in arrhythmia generation.

In summary, this study demonstrated that the pharmacological inhibition of gap junction channels with the gap junction uncoupler CBX prior to and during a 25 min occlusion of the LAD attenuates the protection against arrhythmias that resulted from rapid cardiac pacing, 24 h previously. In the presence of CBX this protective effect of pacing is reduced, indicating that closing of gap junctions in the already paced dogs eliminates, at least in part, the pacing-induced protection. Although the precise mechanisms that underlie the gap junctional uncoupling effect of CBX are still not well-understood, our results suggest that CBX, by causing structural alterations in the intercalated disc, perhaps through the modification of Cx43 trafficking (and phosphorylation), interferes with the protective (gap junction preserving) effect of cardiac pacing. We believe that the data from this study provide further evidence for the role of gap junctions in the delayed antiarrhythmic effect of preconditioning, induced by rapid cardiac pacing.

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EXAMINATION OF THE EFFECT OF SODIUM NITRITE ON GAP JUNCTION FUNCTION DURING ISCHAEMIA AND REPERFUSION IN ANAESTHETIZED DOGS

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It has previously been proved that sodium nitrite, infused prior to coronary artery occlusion or before reperfusion, results in marked antiarrhythmic effect in anaesthetized dogs. We have now examined whether this protection involves the modulation of gap junction (GJ) function by nitric oxide (NO), derived from nitrite administration under ischaemic conditions. Two groups of chloralose and urethane anaesthetized dogs, each containing 13 animals, were subjected to a 25 min period occlusion of the left anterior descending (LAD) coronary artery, followed by reperfusion. One group was infused with sodium nitrite (0.2 μmol/kg/min, i.v.), the other group with saline 10 min prior to reperfusion. The severities of arrhythmias and of ischaemia (epicardial ST-segment, total activation time), parallel with changes in myocardial tissue impedance, a measure of electrical coupling of gap junctions, were assessed during the experiments. Compared to the controls, nitrite infusion administered prior to reperfusion significantly attenuated the severity of ischaemia, the ischaemia-induced impedance changes and, consequently, the severity of arrhythmias, occurring during the 1B phase of the occlusion, and increase survival following reperfusion (0% vs. 85%). It is concluded that the marked antiarrhythmic effect of sodium nitrite is partly due, to the preservation of the electrical coupling of GJs by NO.

Keywords: Arrhythmia – sodium nitrite – gap junction – ischaemia/reperfusion – myocardial tissue impedance

INTRODUCTION

We have just recently shown that inorganic sodium nitrite, administered in intravenous infusion either before or during a 25 min coronary artery occlusion in anaesthetized dogs, resulted in a marked protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias [14]. We have also pointed out that sodium nitrite was particularly effective, when it was given during the occlusion, but 10 min prior to reperfusion [14]. In this case, compared with the control group, in which no dog survived the combined ischaemia and reperfusion insult, 92% of the nitrite received dogs survived [14]. This marked protection was attributed to the generation of nitric oxide (NO) from nitrite. There is now substantial experimental and clinical

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evidence that inorganic nitrate and nitrite, the natural oxidative metabolites of NO, may serve as stores of NO [9, 10, 15, 17], since they are able to rapidly reduce back to NO, especially under hypoxic or anoxic conditions, when a drop in pH and oxygen tension promotes reductive processes [30]. This enzyme-independent NO formation may particularly be important during ischaemia, when in the absence of oxygen, the generation of NO by nitric oxide synthase (NOS) becomes limited [10, 30]. Indeed, the infusion of nitrite, even for a short period (10 min) before reperfusion, markedly increased the plasma nitrate/nitrite (NOx) levels in the coronary sinus blood of the dogs [14]. We concluded that the antiarrhythmic effect of nitrite is associated with better NO availability during ischaemia, and with a subsequent NO-mediated reduction in the harmful consequences of oxidative stress [13], most probably through protein S-nitrosylation and/or S-glutathionylation [14].

There are, however, several other mechanisms, through which nitrite may influence arrhythmia generation. For example, we have shown previously that the antiarrhythmic effect of sodium nitroprusside can be associated with the modulator effect of NO on gap junctional function [6]. There is no doubt that the intercellular connections between cells, called gap junctions (GJs), play an important role in the maintenance of normal impulse propagation, and that the uncoupling of GJs under ischaemic conditions largely contributes to arrhythmia generation, particularly during phase 1B [20, 21]. In contrast to the phase 1A arrhythmias, which appear soon after the occlusion (between 3 and 8 min), these later occurring phase 1B arrhythmias (around 15 min of the occlusion) are often terminated in sudden cardiac death [21]. The association between the acute ischaemia-induced ventricular arrhythmias and GJs, as well as the influence of arrhythmia generation through the modification of GJs, has already been discussed earlier [22, 24].

Following this line, in the present study we have examined, whether GJs play a role in the marked antiarrhythmic effect of sodium nitrite, infused in dogs during occlusion, but just prior to reperfusion. In this experimental arrangement we have observed a marked protection against the phase 1B arrhythmias, and a high rate of survival in our previous study [14].[1]

MATERIALS AND METHODS

The experiments were performed on dogs of both sexes, weighing between 17 and 25 kg (mean body weight 21 ± 2 kg). The origin and upkeep of the dogs were in accordance with the Hungarian law (XXVIII, chapter IV, paragraph 31) regarding the use of large experimental animals, which conforms to the Guide for the Care and Use of Laboratory Animals (ILAR, 1996), and also conformed to Directive 2010/63/EU of the European Parliament. The protocols were approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged,

[1]The arrhythmia data, obtained from 6 control and 6 sodium nitrite treated dogs, were used in this previous paper (KOVÁCS et al., 2015).

Hungary (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/1211/2012*)*.

Before the experiments, the dogs were housed in an animal room (temperature 20 °C; humidity from 40 to 70%; lighting 12 h per day; two animals per pen) for at least two weeks and fed a standard diet with *ad libitum* access to water. Food was withdrawn 24 h before anaesthesia. The surgical procedures were similar to those described previously [23]. In brief, the dogs were lightly anaesthetized with sodium pentobarbitone (30 mg/kg i.v.; Sigma, St. Louis, MO, USA) and the right femoral vein was prepared and catheterized for the administration of further anaesthetic (mixture of chloralose and urethane, 60 and 200 mg kg^{-1}, respectively; Sigma, St. Louis, MO, USA) to maintain anaesthesia. The left femoral vein was cannulated for the intravenous administration of sodium nitrite and saline. The depth of anaesthesia was monitored by the examination of the cornea and pain reflexes, as well as by measuring the blood pressure. The animals were then intubated, and ventilated with room air using a Harvard respirator (Harvard Apparatus, Natick, MA, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits [23]. Body temperature was measured from the mid-oesophagus, and maintained by a heating pad at 37 ± 0.5 °C.

A polyethylene catheter was inserted into the right femoral artery to measure arterial blood pressure (systolic and diastolic). A Millar tip catheter (5F, Millar Instruments, USA) was introduced, via the left carotid artery, into the left ventricle (LV) to detect changes in systolic (LVSP) and end-diastolic (LVEDP) pressures as well as in LVdP/dt.

Thoracotomy was performed 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. Myocardial ischaemia was induced by a 25 min period occlusion of the LAD, followed by rapid reperfusion. The severity of ischaemia was assessed by measuring changes in total activation time (TAT) and in epicardial ST-segment, using a mapping electrode positioned within the ischaemic myocardial region [6]. Signals were collected from 31 unipolar electrodes (inter-electrode distance; 2 mm) at a frequency of 1 kHz, stored on a computer and analyzed offline by creating activation and ST maps. Changes in epicardial activation were assessed as a time delay between the first and a last point activated under the electrode and expressed as the total activation time (TAT) in milliseconds. Elevations of epicardial ST-segment, recorded in each minute from the unipolar electrodes, were averaged and expressed in mV.

The electrical coupling of gap junctions was assessed by measuring tissue impedance[2] as described in detail previously [6, 19, 20]. In brief, four stainless steel electrodes, mounted on a non-conductive panel, were inserted into the left ventricular

[2]Since the myocardial tissue is not purely resistive (the cell membrane contains capacitance elements), there will be a time delay between the voltage (V measured across the tissue) and the sinusoidal current (I) applied through it. Therefore, myocardial impedance ($Z = V/I$) can be precisely defined by two com-

wall within the proposed ischaemic myocardial area. A sub-threshold 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). 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.

Ventricular arrhythmias during a 25 min coronary artery occlusion and following reperfusion were assessed according to the Lambeth Conventions [27] with that modification as has been previously outlined [23]. Thus we determined the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT), and the incidence of ventricular fibrillation (VF) during the occlusion period. During reperfusion, only the incidence of VF, which is a fatal event in this species, was determined. Dogs that were alive 5 min after reperfusion were considered to be survivors.

All parameters, together with a chest lead electrocardiogram, were measured with a Plugsys Haemodynamic Apparatus (Hugo Sachs Electronics, Germany) and recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany).

Experimental protocol

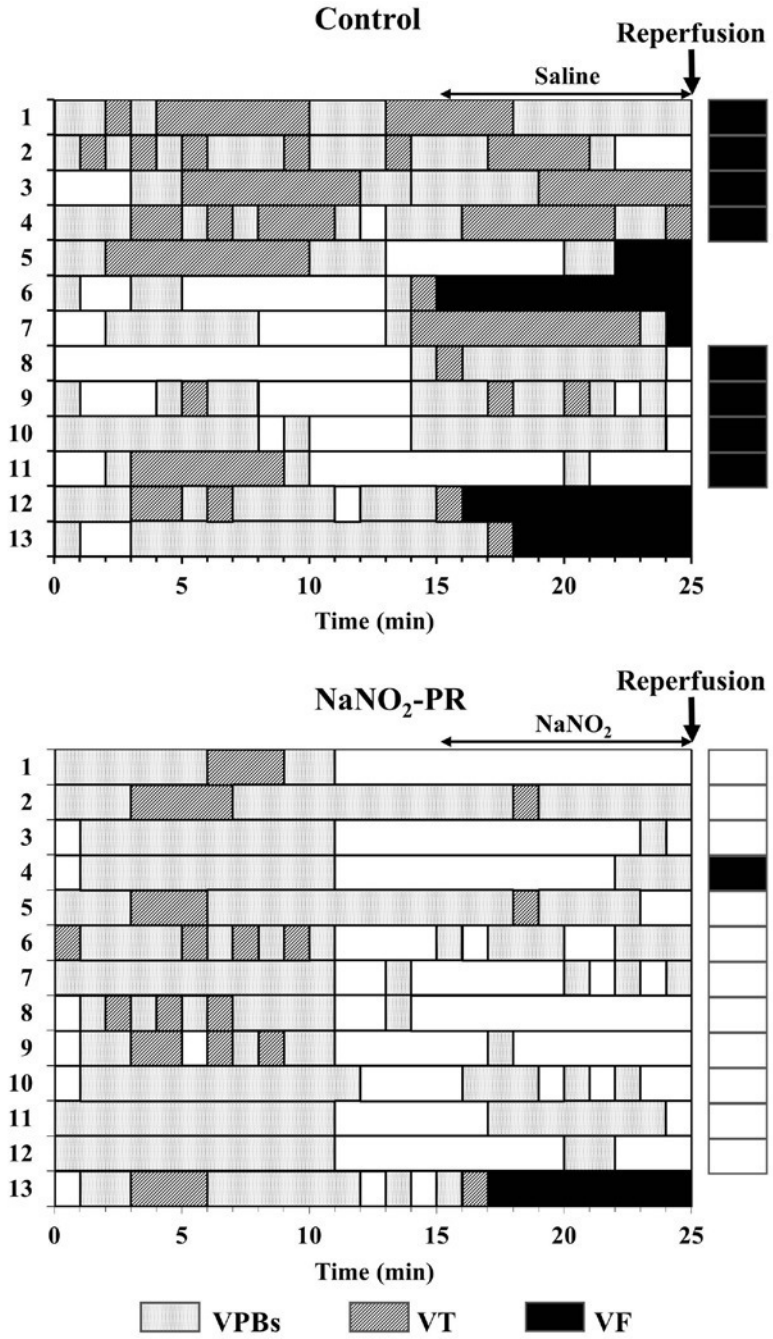
A total of 26 dogs were used and randomly divided into two groups. Each animal was subjected to a 25 min LAD occlusion followed by rapid reperfusion. Control dogs (C; $n = 13$) were infused with saline, commencing 10 min prior to the end of the coronary artery occlusion. In the other group ($NaNO_2$-PR; $n = 13$) sodium nitrite was administered by intravenous infusion at a concentration of 0.2 μmol kg^{-1} min^{-1} also 10 min prior to reperfusion as has been described previously [14]. In dogs that survived the combined ischaemia and reperfusion insult, the hearts were stopped with an overdose of the anaesthetic either within 2 min (where myocardial tissue samples were taken) or 5 min (where the area at risk was determined). In dogs that suddenly fibrillated on reperfusion, the heart was quickly excised when the fibrillation had been observed. In 7 control and 7 nitrite treated dogs, after excising the heart, the risk area was assessed by injecting Patent Blue V dye into the re-occluded artery using the same method that has been described in detail elsewhere [23].

ponents: tissue resistance (R) and phase angle (θ). In tissues, the impedance (Z) is a complex number ($Z = R + jX$), where R is the resistance (in phase component of V with respect to I), j is the imaginary unit ($j = \sqrt{-1}$), and X is the reactance (in quadrate component of V with respect to I). The time delay between the voltage and current waves can be determined from the phase angle [$\theta = \arctan(X/R)$] of tissue impedance.

Table 1
Haemodynamic changes during a 25 min occlusion of the LAD

	Control			$NaNO_2$-PR		
	Baseline	0–15 min	15–25 min	Baseline	0–15 min	15–25 min
		Max. change	Max. change		Max. change	Max. change
SABP (mm Hg)	143±4	–12±3*	–14±3*	152±3	–13±1*	–15±2*
DABP (mm Hg)	101±4	–9±1*	–11±2*	104±4	–13±2*	–14±3*
MABP (mm Hg)	115±3	–10±1*	–12±2*	121±9	–13±2*	–14±2*
LVSP (mm Hg)	144±4	–14±1*	–15±1*	150±5	–14±2*	–16±2*
LVEDP (mm Hg)	3.7±0.3	11.4±1.4*	12.0±1.3*	4.0±0.4	8.8±0.4*	6.2±0.4*#
+dP/dt (mm Hg s^{-1})	3161±118	–635±92*	–654±87*	2972±134	–411±62*	–423±60*
–dP/dt (mm Hg s^{-1})	2635±83	–596±95*	–609±91*	2333±79	–423±63*	–444±56*
HR (beats min^{-1})	164±4	–3±2	–2±1	170±3	1±2	2±2

Values are means ± S.E.M. *P<0.05 compared with baseline value, #P<0.05 compared with the control group. SABP = systolic arterial blood pressure; DABP = diastolic arterial blood pressure; MABP = mean arterial blood pressure; LVSP = left ventricular systolic pressure; LVEDP = left ventricular end-diastolic pressure; HR = hearth rate.


Control
Reperfusion
Saline
1
2
3
4
5
6
7
8
9
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11
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0
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15
20
25
Time (min)
NaNO2-PR
Reperfusion
NaNO2
1
2
3
4
5
6
7
8
9
10
11
12
13
0
5
10
15
20
25
Time (min)
VPBs
VT
VF

←

Fig. 1. The distribution of arrhythmia events following a 25 min occlusion and reperfusion of the LAD in 13 control and 13 sodium nitrite-treated dogs. The filled columns represent ventricular fibrillation (VF), the shaded columns show periods of ventricular tachycardia (VT) and the lightly stippled columns are periods during which ventricular premature beats (VPBs) were evident. VF on reperfusion is shown by the black horizontal columns, and this was the only arrhythmia event assessed during reperfusion. In control dogs there were many VPBs and episodes of VT over the entire occlusion period, and in 5/13 dogs VF occurred during the 1B phase of the occlusion. Since, all the dogs that survived the occlusion, fibrillated on reperfusion, there was no survivor in this group from the combined occlusion and reperfusion insult. Sodium nitrite infused 10 min prior to reperfusion reduced the number of VPBs and VT episodes during phase 1B. In this group VF occurred in one dog during the occlusion, and in one dog following reperfusion, and 11 dogs out of the 13 animals survived reperfusion

Statistical analysis

All data are expressed as means ±S.E.M. and the differences between means were compared by ANOVA for repeated measures and by the one-way ANOVA as appropriate, using the Fisher post-hoc test. VPBs and episodes of VT 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's exact test. Differences between groups were considered significant at $P<0.05$.

RESULTS

Haemodynamic changes following a 25 min occlusion of the LAD

These are summarized in Table 1. Occlusion of the LAD resulted in marked and similar decreases in arterial blood pressure, left ventricular systolic pressure (LVSP), positive and negative dP/dt_{max}, and increases in LVEDP in both groups during the first 15 min of ischaemia. In dogs infused with sodium nitrite 10 min prior to reperfusion, there were no further significant alterations in the haemodynamic parameters compared to the saline treated controls, except that the LVEDP significantly reduced by the end of the occlusion period. The heart rate in anaesthetized dogs was practically unchanged both during occlusion and following sodium nitrite administration.

The severity of ventricular arrhythmias during coronary artery occlusion and reperfusion

The distribution and the incidence of various types of arrhythmias are illustrated in Figure 1. In control dogs subjected to a 25 min occlusion of the LAD, there were many ectopic beats (525 ± 90) and episodes of VT (19.8 ± 5.6) that occurred 92%

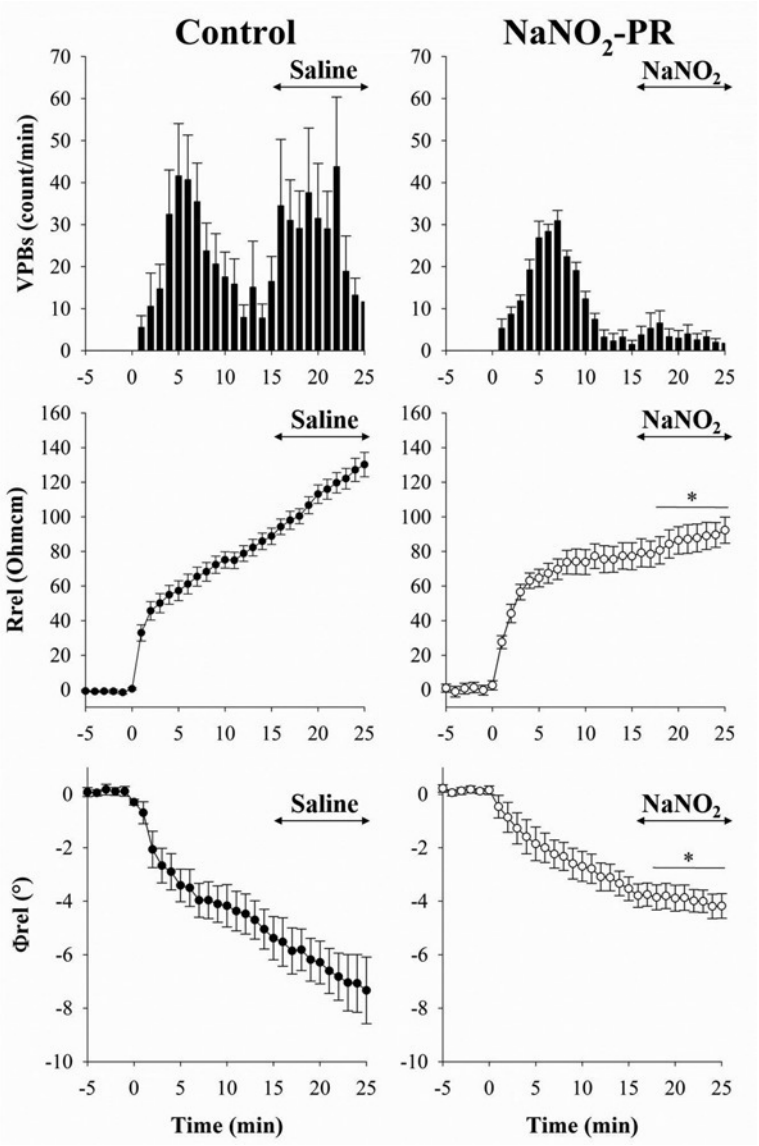

Fig. 2. Distribution of VPBs and relative changes in tissue impedance (resistivity and phase angle) at one minute intervals over a 25 min occlusion of the LAD occlusion in control dogs and in dogs infused with sodium nitrite 10 min prior to reperfusion. The infusion of nitrite significantly reduced the number of VPBs occurring during phase 1B, and inhibited the second steep rise in tissue resistivity, as well as the decline in phase angle that was obvious in the controls between 15 and 25 min of the ischaemia. Values are means ± S.E.M. *$P<0.05$ compared to the controls

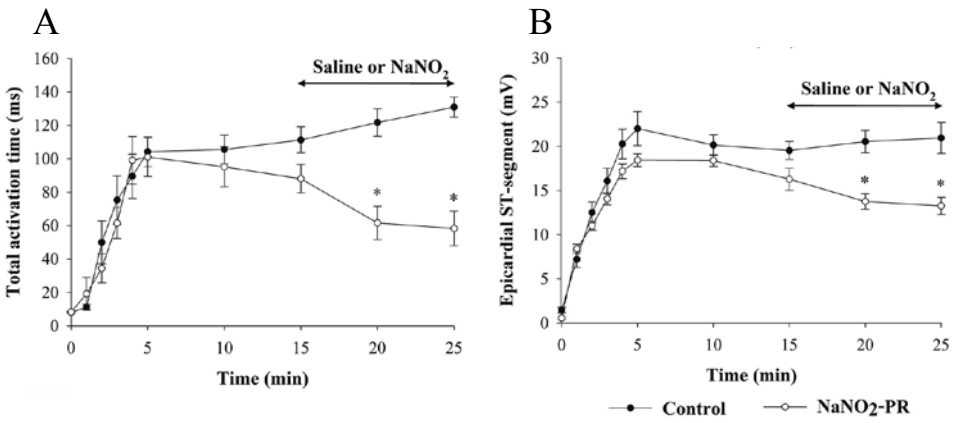

Fig. 3. Changes in the epicardial ST-segment (A) and in the total activation time (B) during a 25 min occlusion of the LAD in control and nitrite infused dogs. In control animals both indices of ischaemia severity were markedly increased especially during the initial 5 min of the occlusion. These ischaemic changes were significantly reduced after the infusion of sodium nitrite. Values are means ± S.E.M. *$P<0.05$ compared to the controls

of the dogs. Furthermore, in this group 5 out of the 13 animals (38%) exhibited VF during occlusion and all the remaining dogs (62%) died in VF following reperfusion, thus no control dog survived the combined ischaemia and reperfusion insult. In contrast, dogs infused with sodium nitrite 10 min prior to reperfusion showed significantly less VPBs and episodes, particularly during the later phase of the occlusion. Although in this group one dog (8%) died during the occlusion period and another one (8%) when the myocardium was reperfused, 85% of the animals infused with sodium nitrite survived the combined ischaemia and reperfusion challenge (Fig. 1).

Distribution of VPBs and changes in tissue impedance during a 25 min LAD occlusion

The data are shown in Figure 2. In control dogs occlusion of the LAD resulted in an immediate increase in tissue resistivity and a decrease in phase angle accompanied by the occurrence of many ectopic beats. These changes were apparent during the first 10 min of ischaemia (phase 1A), after which the impedance changes became slower and the number of VPBs significantly decreased. A second steeper rise in resistivity and decline in phase angle started around 13 min of the occlusion, followed immediately by the re-appearance of the phase 1B arrhythmias. In dogs that received sodium nitrite, the resistivity and the phase angle changes during phase 1A were similar to the controls, although incidentally the phase 1A arrhythmias (Figs 1, 2) were somewhat less pronounced in the nitrite treated than in the control animals. Nevertheless, in this

group, the infusion of sodium nitrite almost completely inhibited the ischaemia-induced marked impedance changes, and significantly suppressed the ectopic activity, which was apparent in the controls during phase 1B.

Changes in epicardial ST-segment and total activation time during LAD occlusion

We used two indices to assess the severity of ischaemia during coronary artery occlusion; i.e. the changes in total activation time (TAT; Fig. 3A) and in epicardial ST-segment (Fig. 3B). In control dogs occlusion of the LAD resulted in immediate and marked increase in both parameters, reaching the maximum values within the first 5 min of the occlusion. The infusion of sodium nitrite 10 min prior to reperfusion resulted in an immediate decrease in the epicardial ST-segment and in the TAT, and these were significantly less pronounced by the end of the occlusion period than in the controls.

Area at risk

There were no significant differences in the area at risk between the control ($n = 7$; 36 ± 2%) and sodium nitrite ($n = 7$; 37 ± 3%) groups.

DISCUSSION

We have previously provided evidence that in dogs gap junctions play an important role in the generation of arrhythmias that accompany the sudden occlusion of one of the main branches of the left coronary artery, and that preconditioning by modifying the function of gap junctions may exert the protection against these severe ventricular arrhythmias [19, 20, 24]. We have also reported that nitric oxide (NO), which plays a mandatory role, both as trigger and mediator of the preconditioning-induced early and delayed antiarrhythmic protection [1, 11, 25, 26], attains its protective effect, among the others, through the modification of gap junctions [6, 22]. Since we have just recently published that the inorganic sodium nitrite, infused in dogs prior to and during the entire occlusion period, or just prior to reperfusion, results in marked anti-arrhythmic effect [14], we have now examined whether this considerable protection is due, at least in part, to the regulation of gap junction function by NO, derived from sodium nitrite. It should be noted that the present study involves 6 control and 6 treated dogs, of which the arrhythmia data, but not the results of the impedance measurements, had already been included in a previous publication [14]. In the present study 7 new control and 7 sodium nitrite-treated animals were added, in order to precisely assess the relationship between gap junctions and the sodium nitrite-induced antiarrhythmic effect.

It was found that sodium nitrite, administered just that period of the occlusion when the phase 1B arrhythmia events are expected (i.e. around 15 min), resulted in immediate haemodynamic effects (e.g. a decrease in LVEDP), a reduction in ischaemia severity (epicardial ST-segment and total activation time), abolished the ischaemia-induced steep impedance changes, and simultaneously, suppressed the severe ventricular arrhythmias, occurring during phase 1B.

At least three conclusions can be drawn from these findings. First, the effect of nitrite is almost immediate, indicating a rapid conversion of nitrite to NO under ischaemic conditions [4, 28, 30]. We assume that by the time of the commencement of the infusion (i.e. 15 min of the occlusion) the ischaemic changes are sufficiently advanced to provide a milieu (low pH and oxygen tension) for the reduction of nitrite to NO. This assumption is supported by the finding that the plasma NOx levels (indicator of NO formation) in the blood of the coronary sinus were markedly increased following nitrite infusion [14]. Since the plasma nitrate level was unchanged during nitrite infusion, we assume that nitrite, under these reductive conditions, most probably converted to NO [14].

Second, the short period of nitrite administration during ischaemia but prior to reperfusion can effectively increase NO bioavailability. This usually decreases following coronary artery occlusion, since in the absence of oxygen, the generation of NO from NOS activation, is reduced or even abolished [12]. Thus under such conditions, replacement of NO, for example, by an enzyme-independent source, may reduce the ischaemic changes and the occurrence of arrhythmias during the rest of the ischaemia. The increased NO bioavailability, resulted from a short period of sodium nitrite infusion prior to reperfusion, may favourably affect the reperfusion-induced severe ventricular arrhythmias, such as ventricular fibrillation, which is common in this canine model when the coronary artery is suddenly reopened [23]. We have found that only one nitrite-treated dog out of the remaining 12 dogs fibrillated during reperfusion in contrast with the controls, in which all the dogs that survived the occlusion died in VF on reperfusion. These findings are in accordance with the results of Gonzalez et al. [5], who showed also in dogs that infusion of nitrite during the last 5 min of ischaemia, significantly reduce the infarct size and improve the recovery of contractile function after reperfusion. One of the mechanisms, which may play a role in the protection against the reperfusion-induced injury, is the suppression of the generation of oxidative stress products. There is substantial evidence that NO can regulate the production of reactive oxygen species (ROS) during ischaemia and reperfusion [3, 7, 12]. For example, we have shown previously that the increase of NO bioavailability during ischaemia by either preconditioning or the administration of an NO donors, suppresses the reperfusion-induced ROS generation and the arrhythmias [12]. Similarly, we have found that sodium nitrite, regardless of the duration of infusion, significantly suppressed the superoxide and peroxynitrite production during reperfusion [14].

Third, the results of the present study confirm our hypothesis that NO, among its several other effects as discussed in detail elsewhere [e.g. 12, 14, 24], may influence

cardiac gap junctions and thereby modify the generation of the ischaemia and reperfusion-induced severe ventricular arrhythmias [6, 22]. The effect of NO derived from sodium nitrite on gap junction channels is also immediate, since after the commencement of the infusion the second steep rise in resistivity and decline in phase angle were absent (the changes of these parameters after 15 min of ischaemia remained virtually constant), and the ectopic activity during phase 1B was considerable suppressed. This finding suggests that the infusion of sodium nitrite, just at a time of the occlusion period, when the serious, often life-threatening phase 1B arrhythmias start to appear, is able to inhibit the generation of these arrhythmias, and this effect can be associated with the preservation of gap junctions by NO. Although it is still not known whether opening or closing of gap junctions by NO leads to the antiarrhythmic protection, our electrical impedance measurements suggest that sodium nitrite inhibits the further uncoupling of gap junctions, since it abolished those steep impedance changes that had preceded the occurrence of the phase 1B arrhythmias. Although we are aware that the measurement of impedance changes only provide indirect evidence for the opened/closed status of gap junctions, since a substantial portion of gap junctions needs to be closed to detect changes in tissue resistivity [8], we assume that NO, derived from sodium nitrite, keeps open, rather than close gap junction channels. The assessment of the phosphorylation of connexin 43 (Cx43), the main structural protein of gap junctions, would provide further information about the open/closed status of these channels. There is a consensus that the phosphorylated form of Cx43 can be associated with the open form of the channel, whereas a dephosphorylated form indicates the closure of gap junctions [2]. In the present study we did not determine connexin phosphorylation, since we know that the ischaemia-induced dephosphorylation of this protein rapidly recovers following reperfusion, but we have previous evidence that NO, released, for example, from sodium nitroprusside, keeps Cx43 in phosphorylated form and the gap junctions in opened state during ischaemia [6].

The other consequence of the rapid release of NO from sodium nitrite during ischaemia is the marked protection against the reperfusion-induced severe ventricular arrhythmias. The reduction in VF during reperfusion is particularly pronounced, resulting in a high rate of survival (85%) in the nitrite treated group. As to whether this marked protection against the reperfusion-induced VF is associated with a direct effect of NO on gap junctions occurring during reperfusion we do not know, since it is difficult to measure the impedance changes soon after reperfusion, when VF commonly occurred particularly in control dogs. However, it is more than likely that the outcome of reperfusion, and thus the severity of reperfusion-induced arrhythmias, largely depends on the severity of the antecedent ischaemia, we assume that the nitrite-induced less severe ischaemic changes and the suppression of arrhythmias during phase 1B, via the modulation of gap junctions, may contribute to the better survival of these animals from the combined ischaemia and reperfusion insult. Gonzalez et al. [5] proposed that administration of nitrite just prior to reperfusion might have a particular clinical value, as adjunctive pharmacological therapy to coronary angioplasty interventions, by improving myocardial salvage, and consequently, by providing a better cardiac function following reperfusion of an acute myocardial ischaemic

insult [5]. We have now confirmed these findings and provided further evidence that nitrite by modulating gap junctions may protect the heart against the ischaemia and reperfusion-induced severe, life-threatening ventricular arrhythmias.

In conclusion, the present study provides evidence that NO, deriving from molecules which readily release NO, protects the heart against the acute ischaemia and reperfusion-induced severe, often fatal rhythm disturbances. Although other effects, such as the preservation of endothelial function [16] or the favourable haemodynamic (e.g. the reduction in preload [LVEDP], and consequently in myocardial stretch) and anti-aggregator abilities of NO donors, cannot be excluded, as indirect factors, from the antiarrhythmic effect of sodium nitrite, the prompt inhibition of the impedance changes and, almost in parallel, the suppression of the phase 1B arrhythmias that occur immediately after the administration of sodium nitrite, suggest a direct modulator effect of NO on gap junctions. As to whether this involves the NO-induced increase in myocardial cGMP and a resultant decrease in the intracellular calcium level [18], which directly modulates gap junctions during ischaemia [29], or whether nitrite, through protein S-nitrosylation (e.g. S-nitrosylation of connexin43) result in modification in gap junction structure and/or function, warrants further investigations.

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