Functional role of gap junctions in early and delayed cardioprotection induced by nitric oxide donors and rapid cardiac pacing

PhD Thesis

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LIST OF PUBLICATIONS

Full Papers

1. Gönczi M, Papp R, Kovács M, Seprényi Gy, Végh Á. Modulation of gap junctions by nitric oxide contributes to the antiarrhythmic effect of sodium nitroprusside? *Br J Pharmacol* **2009**; 156:786-93. IF:5.204

2. Gönczi M, Kovács M, Seprényi Gy, Végh Á. The involvement of gap junctions in the delayed phase of protection induced by cardiac pacing in dogs. *Clin Sci* **2012**; 123:39-51. IF: 4.613

Full Papers not related to the thesis

1. Papp R., Gönczi M., Kovács M., Seprényi Gy., Végh Á. Gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischaemic preconditioning. *Cardiovasc Res*, **2007;** 74: 396-405. IF:5.820

2. Papp R., Gönczi M., Végh Á A réskapcsolatok szerepe akut miokardiális iszkémia okozta aritmiákban *Cardiologia Hungarica* **2008;** 38: 109-115.

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1. Gönczi M, Papp R, Seprényi Gy, Végh Á. A réskapcsolatok szerepe a nitrogén-monoxid antiaritmiás hatásában. Magyar Kardiológus Társaság Kongresszusa, Balatonfüred, 2008. *Cardiologia Hungarica* **2008**; 38: B15.

2. Gönczi M, Papp R, Seprényi Gy, Végh Á. Role of nitric oxide in the modulation of gap junction channels. XXVIII. European Section Meeting of the International Society for Heart Research (ISHR), Athens *J Mol Cell Cardiol* **2008**; 44: 752.

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5. Gönczi M, Kiss A, Végh Á. A nátrium-nitrit ($NaNO_2$) védelmet nyújt az iszkémia/reperfúzió során megjelenő kamrai aritmiákkal szemben a réskapcsolatok befolyásolása által Magyar Élettani Társaság LXXIV. Vándorgyűlése 2010 *Abstract book:* 97:E5.

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6. Gönczi M. Kovács M, Seprényi Gy, Végh Á. Role of gap junctions in cardiac pacing-induced delayed antiarrthythmic protection 75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, 2012. *Abstract book:* 118: P-C9.

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LIST OF ABBREVIATIONS

Cx43	Connexin 43
DABP	Diastolic arterial blood pressure
FITC	Fluorescein isothiocyanate
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
NO	Nitric oxide
NOS	Nitric oxide synthase
OTC	Optimal cutting temperature
PC	Preconditioning
PVDF	Polyvynildene-fluoride
SABP	Systolic arterial blood pressure
SDS	Sodium dodecyl-sulphate
SNP	Sodium nitroprusside
TAT	Total activation time
TD	TRITC conjugated dextran
TRITC	Tetramethyl-rhodamine isothiocyanate
VF	Ventricular fibrillation
VPB	Ventricular premature beats
VT	Ventricular tachycardia
WGA	Wheat germ agglutinin

SUMMARY

It is well-established that gap junction channels contribute to arrhythmia generation during myocardial ischaemia and they play an important role in the early antiarrthymic effect of ischaemic preconditioning (PC). There is also evidence that nitric oxide (NO) is a key mediator of the PC-induced protection and that the administration of NO donors mimics this antiarrhythmic protection. Since NO may modulate gap junction function in non-cardiac tissues first we aimed to investigate, whether in an anaesthetised canine model the antiarrhythmic effect is due to the modification of cardiac gap junctions by NO.

It is also well-described that the protection of PC occurs immediately after the PC stimulus and several hours later, in a delayed phase as well. It is also known that the protective state could be induced by stimuli other that brief periods of ischaemia, e.g. rapid cardiac pacing or heavy physical exercise. Since cardiac pacing has been found to influence gap junction function and the expression of Cx43, secondly we aimed to examine the role of gap junctions in the delayed phase of the antiarrhythmic protection induced by rapid right ventricular pacing

In order to examine the regulatory role of NO on cardiac gap junctions in anaesthetised dogs we administered the NO donor sodium nitroprusside (SNP) in an intracoronary infusion in a dose of $0.2\mu g \cdot kg^{-1} \cdot min^{-1}$prior to and during the occlusion of the left anterior descending coronary artery (LAD). We evaluated the severity of ventricular arrhythmias, changes in myocardial tissue impedance, gap junction permeability as well as the phosphorylation status of Cx43. We showed that compared to the controls, the administration of SNP markedly reduced the severity of ischaemia and of ventricular arrhythmias and prevented the ischaemia-induced gap junctional electrical and metabolic uncoupling and dephosphorylation of Cx43.

Thus we concluded that the antiarrhythmic protection of SNP infusion may, at least in part, be due to the modification of gap junctions by NO.

In the studies aimed to examine the role of gap junctions in the delayed antiarrhythmic effect we paced dogs at a frequency of 240beat/min for 4x5min and then subjected them to coronary artery occlusion 24h later. Compared to the non-paced controls, pacing significantly reduced ventricular arrhythmias and attenuated tissue impedance changes during occlusion,. Pacing also prevented the ischaemia and reperfusion-induced structural impairment of the intercalated discs and preserved gap junction permeability and the phosphorylation of Cx43. We also observed time-dependent changes in Cx43 mRNA and protein expression in response

to cardiac pacing, with a significant reduction in Cx43 protein expression 12h after cardiac pacing. This reduced Cx43 protein content was accompanied by increased susceptibility to arrhythmias and more marked impedance changes if the LAD was occluded at this time point.

We conclude that cardiac pacing results in time-dependent changes in Cx43 expression, which may be related to the altered gap junction function and may influence arrhythmia generation during ischaemia.

1. INTRODUCTION

Myocardial ischaemia - caused by severe stenosis or complete occlusion of a coronary artery – often leads to the generation of severe ventricular tachyarrhythmias and ultimately to sudden cardiac death. During the past few decades, despite the development of the facilities in interventional cardiology and pharmacological treatments, sudden cardiac death still remains one of the leading factors contributing to cardiovascular mortality in the modern world. Therefore, better understanding of the mechanisms underlying these acute ischaemia and reperfusion-induced life threatening arrhythmias would promote experimental and clinical cardiologists to find and develop novel therapeutic strategies.

1.1 Temporal distribution and mechanisms of acute ischaemia-induced ventricular arrhythmias

The sudden occlusion of a major coronary artery acutely induces the generation of severe ventricular arrhythmias, occurring in a characteristic biphasic distribution [1]. The first phase of these arrhythmias - termed as phase Ia – generally lasts from the 2^{nd} to the 10^{th} minute of ischaemia. The proposed mechanisms of these phase Ia arrhythmias implicate immediate reduction of oxygen and energy nutrients within the affected myocardial area, prompting the myocytes to switch from aerobic to anaerobic metabolism [2]. As a result of this, ATP stores are quickly depleted and the accumulation of lactate in the cytosol causes further glycolytic enzyme inhibition and intracellular pH drop [3]. These fast metabolic events directly affect ion pump functions and create ionic imbalance across the cell membrane due to Na^+/K^+ pump dysfunction [4], opening of the sarcolemmal K_{ATP} channels [5], extracellular pH reduction [3] and Na^+ channel inactivation [6]. Furthermore, as a consequence of altered function of Na^+/Ca^{2+} exchanger, intracellular Ca^{2+} overload occurs, which is a source of triggered activity [7]. These changes are the most prominent in the central zone of the ischaemic area and lead to dramatic changes in action potential morphology and duration [7] as well as impulse generation and conduction [8]. Within those areas most affected by ischaemia, even conduction block may develop. In addition, a high level of spatial inhomogeneity in excitability, conduction velocity and tissue refractoriness occurs within the border, the area between the normal and ischaemic regions [9] creating the substrate for re-entry arrhythmias. This is considered to be a major mechanism underlying phase Ia arrhythmias [7, 10].

The first phase is generally followed by a relatively arrhythmia free interval [1Kaplinsky 1979] or a so-called “compensatory phase”, during which ionic changes become balanced and

impulse conduction returns near to normal state for a short period of time [11]. Although the mechanism of this arrhythmia free interval is not fully understood, one of the explanations likely be that the release of catecholamines [11] possibly counteracts the acidification and K^+ efflux [12], and enhances ion pump functions e.g. Na^+/K^+ pump [13] and normalise impulse conduction. It is also likely that endogenous protective substances [14, 15] are released soon after the occlusion such as bradykinin [16] and nitric oxide [17], by their favourable haemodynamic effects may transiently improve myocardial function during this period.

However, if ischaemia is further maintained, these compensatory processes are attenuated or even exhausted, resulting in further deterioration of myocardial function and the appearance of the phase Ib arrhythmias [1]. This phase generally occurs between the 15^{th} and the 30^{th} min of ischaemia and often terminates in ventricular fibrillation [18, 19]. Although processes underlying the generation of phase Ib arrhythmias are less well understood than that of the phase Ia, it is proposed that uncoupling of gap junctions play a pivotal role. This is supported by the finding that by this time of ischaemia, the accumulation of catecholamines [11, 20 21] the subsequent intracellular Ca^{2+} overload [22] and the drop of intracellular pH [3] create a milieu which facilitates the uncoupling of gap junctions and an increases cellular resistance [23, 8] resulting in non-uniform changes in impulse conduction. Coronel et al [24] demonstrated that increased wall stretch due to elevated ventricular filling pressure, as well as a second rise in extracellular K^+ [18], the latter by further increasing spatial heterogeneity, are also important contributory factors to the development of phase Ib arrhythmias.

1.2 Structure and regulation of gap junctions and their role in impulse propagation and arrhythmogenesis

Gap junctions are intercellular channels creating a “cytoplasmic bridge” between neighbouring cells. These channels facilitate metabolic and electrical communication among cells as they are permeable to molecules of molecular weight less than 1kDa, such as ions and second messengers [25, 26]. Gap junctions are formed by connexin protein subunits. Six connexins build up a connexon or a hemichannel, and two connexons of adjacent cells form a gap junction. In the myocardium, connexin formed intercellular channels aggregate at the longitudinal end-to-end connections of the myocytes in the intercalated discs. This cellular distribution of gap junction channels creates anisotropy, meaning that cardiac impulse is conducted preferably in longitudinal direction instead of transversal to myofibre orientation. In the ventricular myocardium the primary isoform is connexin 43 (Cx43) [27]. Connexin 43

has a rapid turnover of 1.5hrs, indicating that the total number of available channels can be regulated rapidly by affecting Cx43 expression [28, 29]. Furthermore, open probability of the channels can be regulated by the phosphorylation of Cx43 as a result of activation of various kinase pathways [30, 31]. There is evidence that under certain pathological conditions, such as heart failure [32, 33, 34] or myocardial infarction [35, 36], the expression of Cx43 is reduced and it is translocated from the intercalated discs to the lateral membrane [37]. These changes may contribute to impaired conduction and arrhythmogenesis under these pathological conditions.

McCallister et al [38] were the first to propose that gap junctions uncouple during ischaemia. The metabolic alterations i.e. low ATP [39] acidified intracellular pH [40, 41], Ca^{2+} overload [40] and the accumulation of amphiphylic metabolites [42], as well as dephosphorylation of Cx43 [43], together with an excessive release of catecholamines [44] facilitate the closure of gap junctions and trigger ventricular arrhythmias. In a seminal paper, Kléber et al [45] demonstrated that in rabbit papillary muscle subjected to ischaemia, tissue resistivity increased in a biphasic manner. The first rise occurred immediately after the onset of ischaemia and was associated with cell swelling and tissue edema. A second rise in resistivity occurred at about the 15^{th} minute of ischaemia and this was thought to be resulted from gap junctional uncoupling [45]. This finding was confirmed later in *in vivo* experiments by showing that in the porcine heart the second steep rise in tissue resistivity due the uncoupling of gap junctions was associated with the occurrence of severe ventricular arrhyhtmias and a high incidence of ventricular fibrillation [18]. Besides, it also turned out that the level and the rate of uncoupling are also important factors for arrhythmia generation. There is evidence that ventricular fibrillation most likely occurs, under conditions of a "moderate uncoupling" of gap junctions [19, 46] or when uncoupling speeds up. In contrast, a more advanced uncoupling attenuates the occurrence of arrhythmias, since the substrate for them disappears [8].

1.3 The cardioprotective effect of preconditioning and the role of Cx43 and gap junction channels in the protection

In 1986 Murry et al [47] described a new cardioprotective phenomenon termed as ischaemic preconditioning during which repeated sublethal ischaemic episodes provide protection against the otherwise lethal consequences of a more prolonged ischaemic insult. Since then it has become obvious that preconditioning provides protection not only against

ischaemia and reperfusion-induced cell death as well as tissue necrosis, but against ischaemia and reperfusion-induced severe ventricular arrhythmias [48] as well as contractile dysfunction [49]. It has also been well-established that the protective effect of PC occurs in two distinct phases: the early phase is apparent immediately after the stimulus but the protection is short lived; it persists only for 1-2 hours. However, the protection re-occurs 20-24 hours later and is called the delayed phase [50] or "second window" of the protection [51]. Since the first description of this phenomenon many laboratories have dealt with the exploration of the mechanisms underlying the PC-induced adaptive phenomenon. It has now become generally accepted that a number of endogenous protective substances are released from the myocardium during the PC stimulus [14, 52], e.g. adenosine [53], bradykinin [16], ROS [54, 55], prostanoids [55] and nitric oxide (NO) [17]. These mediators by acting on different receptors may stimulate various signalling pathways thus ultimately leading to cardioprotection [56].

More recent evidence suggest Cx43 and gap junctions as one of the major targets in this adaptive phenomenon. For example it was demonstrated that in Cx43 heterozygous mice, protection could not be induced by PC [57]. Furthermore, in Langendorff-perfused rat hearts ischaemic PC delayed the onset of cellular uncoupling, and reduced the ischaemia-induced dephosphorylation of Cx43, as well as the translocation of Cx43 from intercalated discs to other cellular compartments [58]. Similarly, Boengler et al reported that ischaemic preconditioning caused the translocation of Cx43 from the sarcolemma into the inner mitochondrial membrane [59], where it is supposed to modulate the mitoK_{ATP} channel function [60]. The mitochondria are considered as one of the major end-effectors of PC-induced cardioprotection [61]. There is also evidence that pharmacological closure of gap junctions in an *in vitro* model by heptanol mimicked PC's protection [62], however when heptanol was administered during the PC stimulus abrogated the PC-induced infarct limiting effect [63]. The first *in vivo* evidence came from a study of Cinca et al [64] who demonstrated in anaesthetised pigs that ischaemic PC delayed the uncoupling of gap junctions as well as the occurrence of ventricular arrhythmias without substantially reducing the number and severity of arrhythmias. In our own experiments in anaesthetized dogs we have found that ischaemic PC not only delayed but indeed markedly reduced the incidence and severity of ventricular arrhythmias during coronary artery occlusion and this effect was associated with a reduced uncoupling of gap junctions [65]. We have also demonstrated that the administration a selective gap junction uncoupler carbenoxolone prior to ischaemia resulted in a PC-like antiarrhythmic protection [65], however, when carbenoxolone was given prior to and during

the PC procedure, it abolished the protection [65]. Taken together, there seems to be no doubt that gap junction channels plays an important role in arrhythmia generation and also in the antiarrhythmic protection afforded by PC. However, the exact mechanisms by which PC modulate these channels remains to be elucidated.

1.4 Regulatory role of nitric oxide on gap junction channels

As mentioned above, there are a number of mediators, which are released in response to the preconditioning stimulus, among which NO has paramount importance. The first piece of evidence that NO participate in the antiarrhythmic effect of preconditioning came from those studies, which demonstrated that L-NAME, an inhibitor of the L-arginine-NO pathway, given either prior to preconditioning or just before the coronary artery occlusion, attenuated the protection against arrhythmias [17]. The second piece of evidence that NO most likely plays a role in this antiarrhythmic protection resulted from those studies which showed that the administration of NO donors, e.g. nicorandil [66] and isosorbide-2-mononitrate [67] exerts a preconditioning-like antiarrhythmic effect. Similarly, the intracoronary infusion of SNAP was able to reduce ischaemia-reperfusion injury in anaesthetized pigs [68].

A growing body of evidence shows that NO, among its several other actions, may modulate the expression of different connexin isoforms and the gating properties of gap junction channels. However, the majority of these results are mainly derived from *in vitro* studies in non-myocardial tissue. For example, Bolanos and Medina described that in astrocytes the induction of NOS or the administration of a NO donor reduced the permeability of gap junctions [69]. Also, in rat myometrial cells, the NO donor SNAP decreased the expression of Cx43 [70]. In an *in vivo* rat model, iNOS induction following myocardial infarction was shown to be responsible for the down regulation of Cx43 expression [35]. On the other hand, studies on mesangial cells revealed that NO has a positive regulatory effect on connexin expression and gap junction permeability [71]. Furthermore, NO increased *de novo* formation of Cx40 formed gap junctions in endothelial cells [72]. One of the proposed mechanisms through which NO is able to modulate gap junctions is the activation of protein kinase G, via the soluble guanylate cyclase – cGMP pathway [73]. This pathway is also involved in the antiarrhythmic effect of preconditioning [74] and of NO donors [75]. However, as to whether NO is able to modulate gap junction channels in cardiac myocytes and whether the antiarrhythmic effect of NO donors can be associated with the modulation of gap junction function, has still not been examined.

1.5 Delayed preconditioning and modulation of gap junctions by cardiac pacing

Preconditioning induces not only an early, but also a delayed phase of protection [49]. The mechanisms responsible for the induction and development of the delayed phase, differs in some aspects from those of the early phase. Whereas the early phase is primarily due to the release of endogenous substances [52], and the subsequent activation of their respective signal transduction cascades, the development of the delayed phase is attributed to gene and protein expression changes via activation of the early signalling events [76]. These proposed changes involve the activation of transcription factor NF-κ-B [77], the NO induced iNOS activation [78, 79], the upregulation of cyclooxygenase-2 [80], the induction of antioxidant enzymes [52] and heat shock proteins [81], etc.

It was an important recognition in the preconditioning research that both an early and a delayed protection can be induced by stimuli other than short ischaemic events. In this regard, high frequency right ventricular pacing [82] and heavy physical exercise [83] have been shown to serve as preconditioning stimuli. Moreover, of clinical importance is the finding that by repeating the stimulus, when the protection from the previous stimuli has already waned [84], the protection can be extended over a longer period [85].

A number of evidence supports the concept that cardiac pacing is able to modify gap junction function and expression. For example, Kontogeorgis et al [86] showed that pacing of mice hearts for 6 hours reduced endocardial Cx43 level and the redistribution of gap junctions. Similarly, Matsushita et al [87] demonstrated that cardiac pacing for 3 hours caused dephosphorylation of Cx43 at the vicinity of the pacing site. Furthermore, long-term cardiac pacing (more than 3 weeks) in order to induce heart failure, decreased Cx43 expression [88], increased the level of dephosphorylated Cx43 in the intercalated discs [89] and altered cellular distribution of Cx43 [89, 90]. It is interesting to note, that whereas long-term overdrive cardiac pacing induces maladaptive responses, like remodelling of cellular architecture, and contributes to the development of heart failure and arrhythmogenesis, the same stimulus when applied for short, intermittent periods, induces both early and delayed adaptive processes [82]. Despite the extensive research, whether gap junctions play a role in this pacing-induced delayed protection has not yet been examined.

2. AIMS OF THE STUDY

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

a) There is abundant evidence that NO plays an important trigger and mediator role in the antiarrhythmic effect of preconditioning and that the administration of NO donors mimic the preconditioning-induced protection. In the first series of experiments we have attempted to investigate whether this antiarrhythmic effect, at least in part, can be attributed to the modulation of gap junction function by NO.
   For this purpose in anaesthetised open-chest dogs sodium nitroprusside (SNP) was administered locally into a side branch of the left anterior descending coronary artery (LAD) in a dose of $0.2 \mu g \cdot kg^{-1} \cdot min^{-1}$. The infusion was started 20 minutes prior to and maintained over a 60 minute occlusion period.

b) The role of gap junctions in the early antiarrhythmic effect of preconditioning has already well-described, but as to whether they play a role in the delayed phase of the protection is still not elucidated. Thus we aimed to investigate whether gap junctions participate in the delayed antiarrhythmic of preconditioning induced by rapid right ventricular pacing. We also examined the effect of cardiac pacing on gene- and protein expression, as well as on the cellular distribution of the gap junction forming protein Cx43, at different time points after rapid right ventricular pacing. Since in this particular study we observed a significant reduction in the expression of Cx43 12 hours after cardiac pacing, we designed further studies in which the severity of arrhythmias was examined during a 25 min occlusion of the LAD 12 h after cardiac pacing.

3. MATERIALS AND METHODS

3.1. Experimental animals and ethical concerns

All experiments were performed in adult mongrel dogs of either sex with a mean body weight of 20 ± 2 kg. The origin and upkeep of the animals were in accord with the Hungarian Law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals, which confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised in 1996).

3.2. Surgical preparations

Animals were anaesthetised with pentobarbital (Euthanyl®, 0.5mg · kg^{-1}, Bimeda-MTC Animal Health Inc.). The right femoral vein was prepared and cannulated for the administration of a mixture of α-chloralose and urethane (60 and 200 mg · kg^{-1} respectively, Sigma, St Louis, MO, USA). Further doses of anaesthetic were administered, when it was necessary, to maintain anaesthesia. The right femoral artery and the left arteria carotis communis were prepared and catheterised in order to measure arterial (systolic: SABP, diastolic: DABP, and mean: MABP) and left ventricular blood pressures (systolic: LVSP, end-diastolic: LVEDP) respectively. The catheters were connected through Statham transducers (Statham P23XL) to a PLUGSYS haemodynamic apparatus (Hugo Sachs Electronics, Germany) and signals were recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany). Arterial blood gases and pH were frequently monitored (Radiometer ABL 505, Denmark) and ventilation (Harvard Apparatus, Natick, MA, USA) was adjusted to maintain these values within the physiological limits.

After thoracotomy the left anterior descending coronary artery (LAD) was prepared for occlusion distal to the first main diagonal branch. This site of occlusion usually results in approximately 35% area at risk, which was verified after the experiments by the infusion of methylene blue into the LAD at the occlusion site as described previously [82]. Coronary blood flows were measured on the left circumflex and the left anterior descending branches using a 2.0 mm electromagnetic flowmeter (Statham SP2202) and an ultrasonic Doppler flowmeter (Triton Technologies, USA) respectively.

3.3. Measurement of ischaemia severity

This was assessed by the measurement of changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment using two methods. In one series of the experiments a composite electrode was sutured onto the epicardial surface of the ischaemic myocardial region. This electrode gives a summarised R-wave of 24 bipolar epicardial points. In the adequately perfused myocardium, all sites under the electrode are activated simultaneously, resulting in a single large spike. However, during occlusion, fractionation and widening of this summarized R-wave occurs, indicating that adjacent fibers are not simultaneously activated. The inhomogeneity of electrical activation measured as the greatest delay in activation was expressed in ms. Additional two unipolar electrodes served for measuring changes in epicardial ST-segment, and was expressed in mV.

In the other series of experiments a mapping electrode was used consisting of 31 unipolar electrodes (interelectrode distance: 2mm). Signals from this electrode were collected together with a chest lead II ECG at a frequency of 1 kHz and stored on a personal computer. Data analysis was performed offline. Activation and ST maps were created by means of a custom made software. Local activation times at each electrode points were calculated, i.e. the time interval between the beginning of ventricular activation on the surface ECG and the maximal negative slope [$-dV/dt_{max}$] on the unipolar electrogram. Changes in epicardial activation were measured as a time delay between the first and the last point activated under the electrode and expressed as the total activation time (TAT) in ms. Changes in epicardial ST-segment, recorded from 31 unipolar electrode sites were averaged and expressed in mV.

3.4. Measurement of gap junctional electrical coupling

Electrical coupling of gap junctions was determined by changes in tissue impedance using the four-pinned electrode method [65, 91]. In brief, the electrode was fixed within the potentially ischaemic area parallel to myofibre orientation. High frequency subtreshold alternating current (10 μA, 8 kHz) was applied between the outer electrode pairs and voltage drop was measured between the inner electrode pairs with a lock-in amplifier (SR 830 DSP, Stanford Research Systems, CA, USA). Signals were recorded with an acquisition time of 4 sec on a personal computer with custom made software and analyzed offline. Relative changes in tissue resistivity (R; Ohm·cm) and in phase angle shift (°) were calculated and plotted at 1 min intervals. Before each experiment, the electrodes were calibrated in saline with a known resistivity.

3.5. Assessment of ventricular arrhythmias

The number and incidence of ventricular arrhythmias occurring during LAD occlusion was evaluated according to the Lambeth conventions [92] with the modifications outlined previously [48]; the total number of ventricular premature beats (VPBs), the number and incidence of ventricular tachycardiac episodes (VT) and the incidence of ventricular fibrillation (VF) during coronary artery occlusion were calculated. During reperfusion only VF was assessed. Dogs that were alive after 5 min of reperfusion were considered as survivors (S).

3.6. Assessment of gap junctional metabolic coupling

Gap junction permeability was assessed by double dye loading method [93]. Euthanasia was performed with an excess dose of anaesthetics at the end of the coronary artery occlusion and transmural tissue blocks (approximatetely 0.5x0.5x1.0cm) were excised from myocardial areas supplied by the LAD and the left circumflex coronary artery (LCX). The samples were immersed into saline containing Lucifer Yellow (LY 1.5mg/ml, Sigma) and TRITC-dextran (TD 3.5mg/ml, Sigma) for 15 min. Since LY is a small molecule (~500Da) it readily penetrates through gap junctions, whereas TD, which is a large molecule (~ 10kDa), it stains only the injured cells on the cutting edge of the tissue. Tissue blocks were then fixed in 4% paraformaldehyde, dehydrated in 30% sucrose solution containing 0.1% sodium-azide. Cryosections (20μm) were made with a Cryostat (Leica) and were mounted on gelatin coated slides and covered with PBS-glycerol. Images were taken with an Olympus fluorescent microscope (Olympus IX 70) attached to a CCD camera, and were analyzed with Image J software (NIH). Metabolic coupling of cells was assessed as the ratio of LY and TD stained areas. Permeability within the ischaemic (LAD) area was expressed as the percentage of permeability within the non ischaemic area (LCX).

3.7. Immunoblot analysis

To examine whether cardioprotective interventions cause alterations in Cx43 protein content and phosphorylation, Western blot analysis was performed. For this purpose, myocardial samples were taken either at the end of the occlusion, or, in the delayed experiments, at different time points after cardiac pacing and were stored at -80°C until further procession. Tissue samples were homogenized in ice-cold lysis buffer consisting of

250mM sucrose, 20mM Tris-HCl, 10mM β-mercaptoethanol, 10mM sodium-orthovanadate, 0.5% protease inhibitor cocktail (Sigma), at pH 7.4. For total protein isolation homogenates were centrifuged at 10000g for 15 min, whereas for membrane fraction isolation tissue homogenates were centrifuged at 2000g for 10 min. Then the supernatants were collected and centrifuged at 100000g for 45 min and the pellet was resuspended in lysis buffer. Protein concentrations were determined by the Lowry method. Total (40μg) and membrane protein (10-20 μg) samples were separated on 10-12% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% non-fat milk, membranes were labeled overnight at 4°C against Cx43 with a rabbit anti-Cx43 primary antibody (dilution: 1:1000 for total protein, 1:2000 for membrane fraction, Zymed) and subsequently HRP-conjugated goat secondary antibody (1:8000, Santa Cruz). Blots were developed with ECL Plus Kit on X-ray films or scanned with a Typhoon laser scanner (Amersham Biosciences). Band intensities were measured using ImageJ software (NIH). For the verification of equal loading, PVDF membranes were labelled against GAPDH for total protein content or stained with Coomassie Blue for membrane proteins.

3.8. Immunofluorescence analysis

In order to examine whether the various interventions cause changes in the cellular localisation and in the quantity of Cx43 in intercalated discs, immunofluorescence analysis was performed. Transmural tissue samples were collected, embedded in OCT compound and stored at -80°C. Longitudinal frozen tissue sections (8μm) were cut from the mid-myocardial layer and specimens were mounted on gelatine coated slides. Unspecific binding capacity of the tissue was blocked with 5% BSA, and the samples were labelled with rabbit anti-Cx43 primary antibody against Cx43 (1:800, Zymed) overnight at 4°C, then with FITC conjugated secondary antibody (1:1500) for 1 h at room temperature, and finally with WGA-conjugated Texas Red (1:500, Invitrogen) for 30 min at room temperature. Images were captured with a laser scanning confocal microscope (Olympus, FV1000, Olympus) using a 40x magnifying objective. Areas of well preserved cardiomyocites were selected for analysis, performed with ImageQuant 5.2 (Molecular Dynamics) or Image J (NIH). Relative amount of Cx43 was calculated as pixel intensities of intercalated discs with background correction. Samples from six individuals from 2 groups were mounted on the same slide at the same time. To compare differences between groups, data from each sample were normalized to the sample with the highest intensity on the same slide.

3.9. Gene expression analysis with RT-PCR

For the examination of changes in Cx43 gene expression level we performed by RT-PCR. Total RNA was isolated from tissue samples (25 mg of each heart) using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. Quantitative PCR was performed on an ABI7000 instrument (Applied Biosystems, Carlsbad, USA) with gene-specific primers using SybrGreen protocol to monitor gene expression as described earlier [94]. From each sample 2 μg of total RNA was reverse transcribed using ImPromII Reverse Transcription system (Promega, USA) in the presence of oligo(dT) at 42^{o}C for 2h. After this the cDNAs were diluted in 50 μl of water, and 1 μl of the reaction mix was used for qPCR. Reactions using SybrGreen protocol were performed with Power SYBR Green Master mix (Applied Biosystem, Carlsbad, USA) according to the manufacturer's instructions at a final primer concentration of 250 nM under the following conditions: 10 min at 95 ^{o}C, 40 cycles of 95 ^{o}C for 15s, 60 ^{o}C for 25s and 72 ^{o}C for 25s. Melting temperature analysis was performed after each reaction to check the quality of the reaction.

Primers were designed using Primer Express 2.0 software package. Relative expression ratios were calculated as normalized ratios to HPRT (hypoxanthine guanine phosphoribosyltransferase) and to tubulin. Samples were tested for genomic DNA contaminations by using non-template controls for each PCR reaction. The final relative gene expression changes were calculated as delta-delta Ct values. Genes with expression values lower than 0.5 or higher than 1.5 were considered to be down or up-regulated (the values correspond to an interval of log2=-1 and log2=0.6). All the PCRs were performed in triplicate.

3.10. Experimental protocols

3.10.1. Evaluation of the effect of sodium nitroprusside on gap junction function during coronary artery occlusion

This is illustrated in Fig. 1. Two groups of anaesthetised dogs were used. In the control group (C; n=11) saline was infused into a side-branch of the LAD 20 min prior to and during the 60 min occlusion of the LAD. In the other group sodium nitroprusside (SNP; n=10) was administered in a dose of 0.2μg·kg^{-1}·min^{-1} 20 min before and throughout the occlusion period. The syringes containing SNP solution were covered with foil to avoid

photodegradation of SNP. The dose of SNP was selected in preliminary experiments using increasing doses of SNP in order to find a dose, which caused only minor haemodynamic alterations (i.e. less than 5 mmHg reduction in arterial blood pressure).

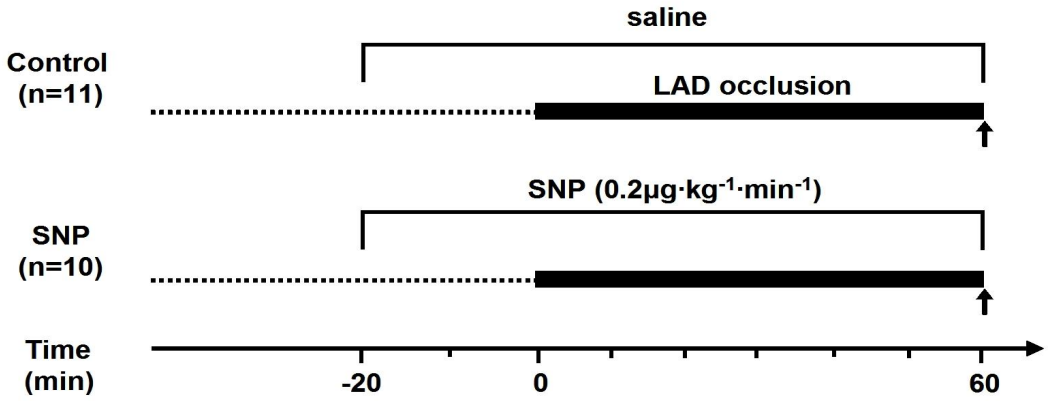

Figure 1. *Experimental protocol for the evaluation of the cardioprotective effect of sodium nitroprusside. Arrows indicate tissue sampling at the end of the 60 min ischaemia..*

3.10.2. Evaluation of the role of gap junctions in the delayed antiarrhythmic protection induced by cardiac pacing

This is illustrated in Fig 2. Under light pentoparbital anaesthesia (0.5mg·kg^{-1} Euthanyl, Bimeda-MTC Animal Health Inc.) a bipolar pacing catheter (Cordat F4, Johnson & Johnson) was inserted through the jugular vein into the right ventricle and the hearts were paced four times for 5 minutes, with 5 minute intervals, at a rate of 240 beats/min. Twenty-eight dogs were subjected to pacing (P), whereas in another group of animals (n=32) the pacing electrode was introduced into the right ventricle but the dogs were not paced. These sham-paced (SP) dogs served as controls. From both the paced and control groups 12 dogs were selected and divided into four further groups, each containing 3 animals. In these dogs euthanasia was induced by the administration of an intravenous overdose of the anaesthetic at various time intervals, i.e. immediately (P-0/SP-0), six (P-6/SP-6), twelve (P-12/SP-12) and twenty-four (P-24/SP-24) hours after the pacing or sham-pacing procedures and myocardial tissue samples were taken for biochemical analyses (Fig. 2). Dogs that were rendered to occlusion and reperfusion were allowed to recover from the surgical interventions and 24 h later were

subjected to a 25 min coronary artery occlusion and reperfusion (SP+O; n=20 and P+O; n=16)

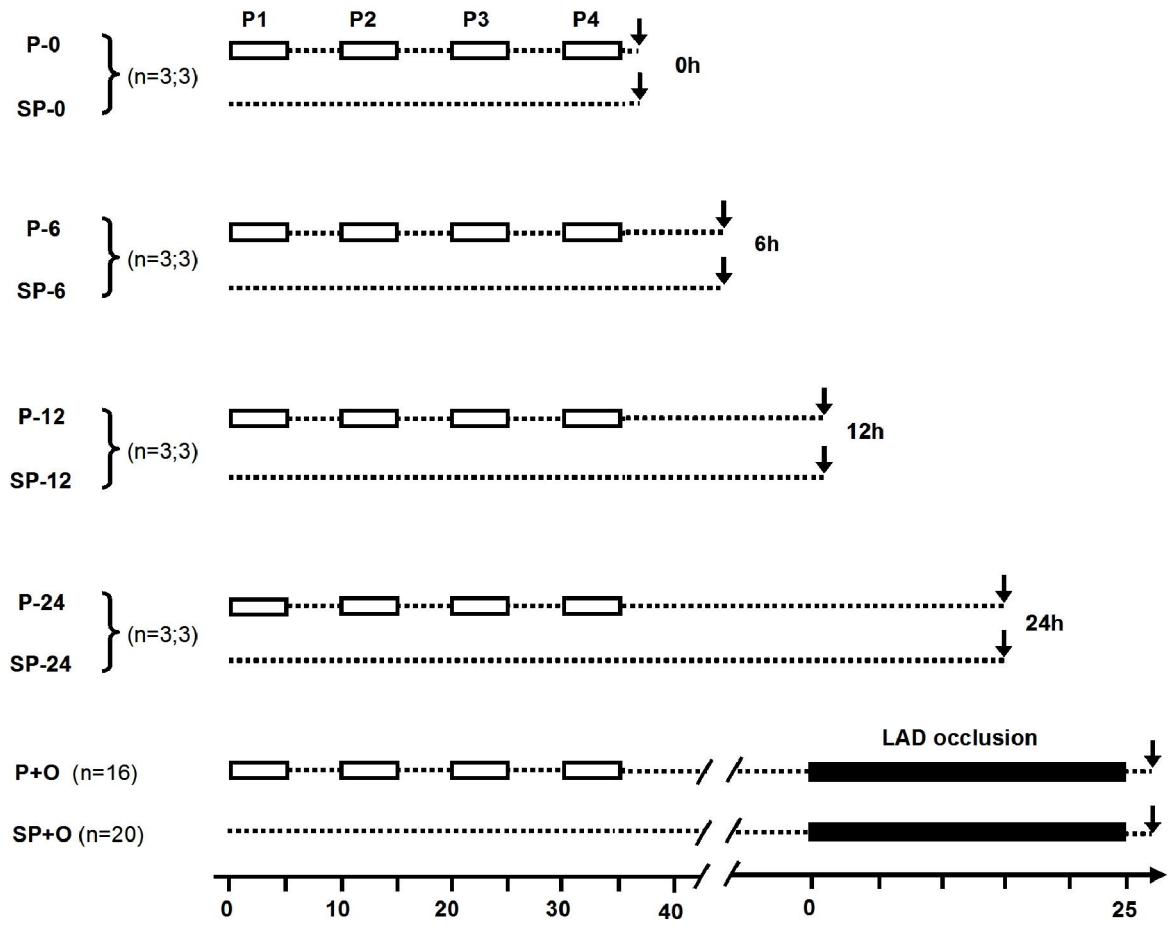

Figure 2. *Experimental protocol for the evaluation of the role of gap junctions in the delayed phase of preconditioning, induced by rapid cardiac pacing. Arrows indicate time points of tissue sampling. P1, P2, P3 and P4 indicate periods of cardiac pacing.*

3.11. Statistical analysis

All values are expressed as mean ± s.e.m. Differences between groups were compared with Student t-test or Mann Whitney U test, One Way ANOVA or Repeated measures ANOVA as appropriate. For analysing arrhythmia events as the number of VPBs, VT episodes, and the incidence of VT and VF, Manny-Whitney U test or Fisher exact test was used. Differences between groups were considered significant at level $p<0.05$.

4. RESULTS

4.1. Evaluation of the effect of sodium nitroprusside on gap junction function during coronary artery occlusion

4.1.1. Haemodynamic changes during SNP infusion and the subsequent coronary artery occlusion

This is illustrated in Table 1. Intracoronary administration of SNP caused slight but statistically significant reductions in arterial blood pressure, left ventricular end-diastolic pressure and +dP/dt. These changes were, however, returned to baseline by the beginning of the occlusion. LAD occlusion resulted in significant reductions in arterial blood pressure, left ventricular systolic pressure and +dP/dt as well as an increase in LVEDP. The heart rate remained unchanged. No difference was observed in these haemodynamic paremeters between the two groups. The infusion of SNP did not substantially affect the diastolic blood flow and resistance changes, measured in both the LAD and the LCX arteries. The increase in compensatory blood flow which occurs on the LCX when the LAD is occluded were also not substantially modified by the infusion of SNP.

4.1.2. The severity of ischaemia during coronary artery occlusion

This was evaluated by changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment (Fig. 3). In control animals, sudden occlusion of the LAD resulted in marked increases in the inhomogeneity of activation and epicardial ST-segment, reaching their maximum around the 5^{th} min of the occlusion. These changes remained elevated throughout the entire 60 min occlusion period. The intracoronary infusion of SNP significantly attenuated the increase of both indices of ischaemia severity.

4.1.3. The severity of ventricular arrhythmias during coronary artery occlusion

This is illustrated in Fig 4. In the controls, coronary artery occlusion resulted in high number of VPBs (666 ± 202), many separate episodes of VT (8.1±2.3) which occurred in 55% of the dogs. In those animals which received intracoronary SNP infusion 20 minutes prior to and during the occlusion, the ectopic activity (49±18) and the number and incidence of VT episodes were markedly suppressed; there were indeed only a few episodes of VT (0.2±0.1), which occurred in 2 out of 10 SNP treated dogs (20%).

Table 1. *Haemodynamic changes following saline and SNP infusion as well as coronary artery occlusion.*

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

SABP, systolic arterial blood pressure (mmHg); DABP, diastolic arterial blood pressure (mmHg); MABP, mean arterial blood pressure (mmHg); LVSP, left ventricular systolic pressure (mmHg); LVEDP, left ventricular end-diastolic pressure (mmHg); +dP/dt$_{max}$ (mm Hg·s^{-1}); -dP/dt$_{max}$ (mmHg·s^{-1}); HR, heart rate (beat·min^{-1}); LAD flow (cm·s^{-1}); LAD resistance (mmHg·cm·s^{-1}); LCX flow (mL·min^{-1}); LCX resistance (mmHg·mL·min^{-1}). Values are means ± s.e.m, *p<0.05 compared to baseline values or to pre-occlusion value.

The intracoronary infusion of SNP in the applied dose did not affect the incidence of ventricular fibrillation during coronary artery occlusion.

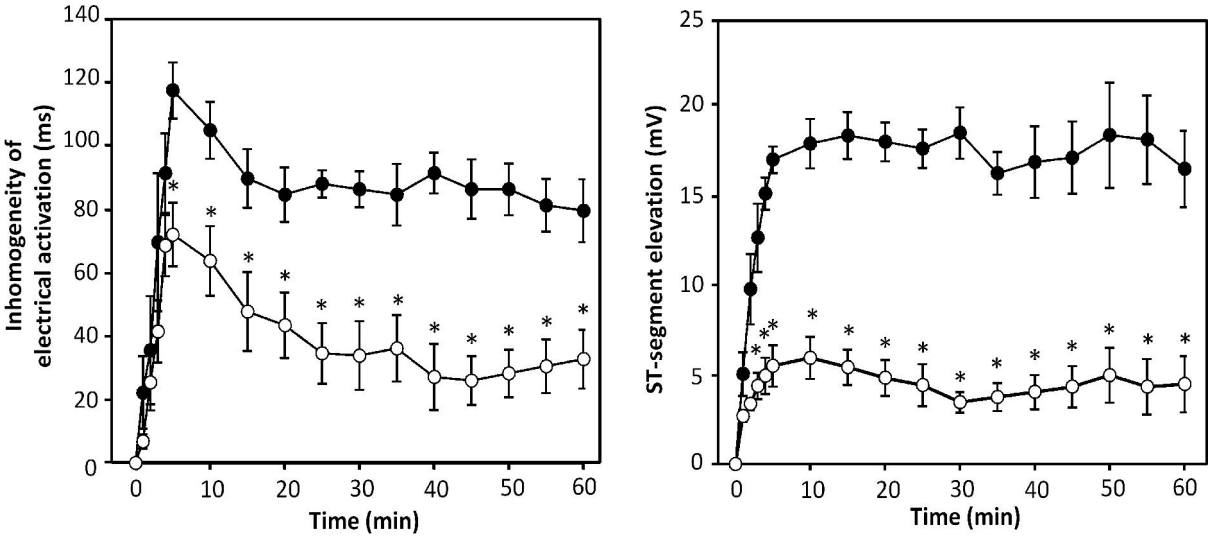

Figure 3. *Changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment during a 60 min coronary artery occlusion in control (black circles) and in SNP treated animals (empty circles). Values are means ± s.e.m.,* p<0.05 vs controls.*

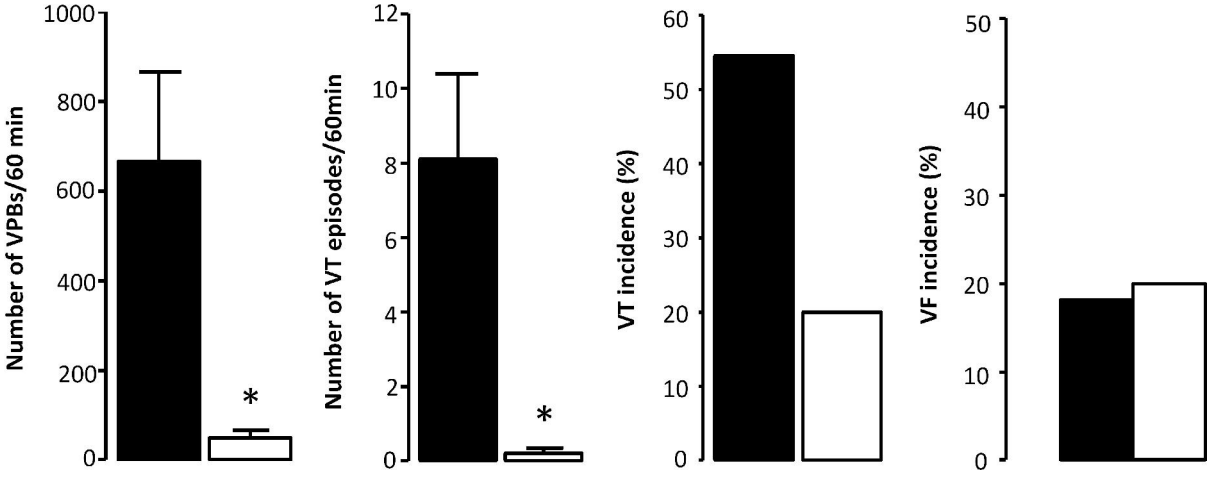

Figure 4. *Arrhythmia severity during a 60 min LAD occlusion in control (black bars) and SNP treated dogs (empty bars). Values are means ± s.e.m., *p<0.05 vs controls.*

4.1.4. The distribution of ventricular premature beats and changes in myocardial electrical coupling during coronary artery occlusion

In the control group, occlusion of the LAD resulted in a high number of VPBs which were distributed in two distinct phases; phase Ia arrhythmias occurred soon after the commencement of coronary artery occlusion and peaked between 3rd and 8th min of the ischaemia. During this period there was a sudden increase in tissue resistivity (R_{rel}) and a decrease in phase angle (φ_{rel}). After these immediate changes, ectopic activity started to decrease and changes in tissue impedance became slower. However, a second steep rise in tissue resistivity and a reduction in phase angle occurred at around the 15th min of ischaemia, accompanied by the appearance of phase Ib arrhythmias (Fig. 5.). These arrhythmias lasted until about the 30th min of ischaemia. Thereafter ectopic activity faded, but tissue impedance changes continued over the rest of the occlusion.

The intracoronary infusion of SNP alone did not cause any significant change in tissue impedance values. In these SNP treated dogs, occlusion of the LAD resulted in less marked tissue impedance changes, especially during phase Ib than the controls and ventricular ectopic beats were almost completely abolished.

4.1.5. Changes in gap junction permeability

The metabolic coupling of cells was examined by the measurement of gap junction permeability after 60 min ischaemia in controls and in SNP treated dogs. In control samples taken from the ischaemic myocardial wall, gap junction permeability reduced to 65±3 % of the permeability observed in the non-ischaemic samples (100±7 %). In contrast, in dogs given SNP infusion, gap junction permeability was reduced only to 95±5 % of the non-ischaemic samples, indicating a preservation of metabolic coupling of cells by SNP.

4.1.6. Changes in the phosphorylation status of Cx43

The phosphorylated and dephosphorylated isoforms of Cx43 were determined at the end of a 60 min ischaemia in control and in SNP treated dogs. The phospho-dephospho ratio of Cx43 is illustrated in Fig 6.b together with representative Western blot images (Fig 6.a). Connexin43 occurred in two distinct bands; the 41kDa band represents dephosphorylated Cx43 (dP-Cx43) while the 45kDa band indicates phosphorylated Cx43 (P-Cx43) isoform. In the non-ischaemic control samples, the P/dP-Cx43 ratio was almost equal (53/47% ± 1%). This was shifted towards dephosphorylation by the end of a 60 min coronary artery occusion in ischaemic samples, resulting in a 29/71% ± 4% P/dP ratio. In dogs infused with SNP, the

P/dP ratio was 58/42% ± 1 % in non-ischaemic samples, and it was 62/38% ± 1% in ischaemic samples at the end of the 60 min occlusion, indicating that SNP infusion prevented the ischaemia-induced dephoshorylation of Cx43.

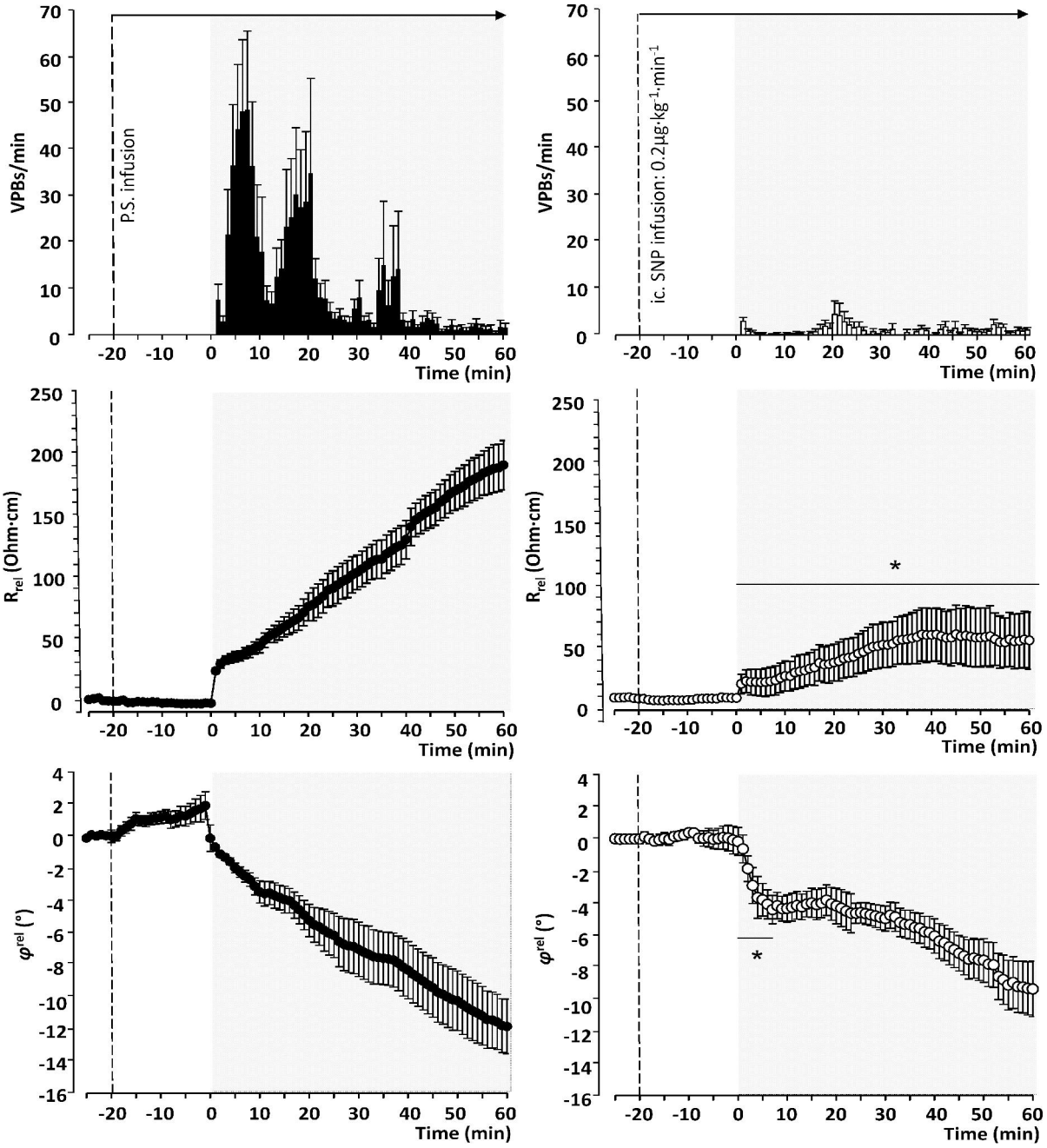

Figure 5. *Distribution of VPBs as well as changes in tissue electrical impedance in control (black) and SNP treated groups (empty). Values are means ± s.e.m. *p<0.05 vs controls.*

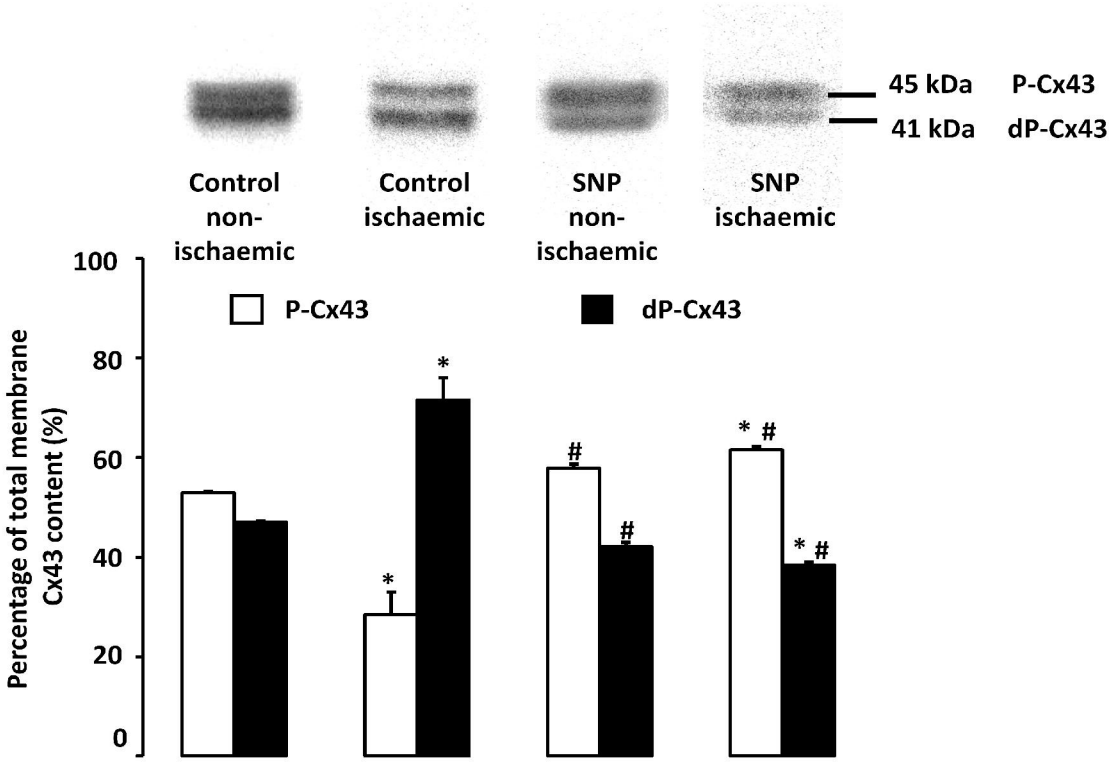

Figure 6. *Ratio of P-Cx43 and dP-Cx43 (expressed as the percentage of the total membrane Cx43 content) in ischaemic and non-ischaemic myocardial tissue samples, obtained from control and SNP treated dogs. A representative Western blot image is also shown. Values are means ± s.e.m. *p<0.05 vs control non-ischaemic, #p<0.05 vs control ischaemic.*

4.2. Evaluation of the role of gap junctions in the delayed antiarrhythmic protection induced by cardiac pacing

4.2.1. Haemodynamic changes during a 25 min LAD occlusion

These are summarized in Table 2. There were no significant differences between the control and paced dogs in the haemodynamic parameters determined at baseline. In control dogs, occlusion of the LAD resulted in significant decreases in arterial blood pressure, LVsystolic pressure, positive and negative LVdP/dt_{max} and increases in LVEDP and heart rate. These changes were similar in the paced dogs, except that the increase in LVEDP was somewhat less pronounced following coronary artery occlusion.

Haemodynamic parameters	**SPO**		**PO**	
	Baseline	Post occlusion max change	Baseline	Post occlusion max change
SABP	156 ± 5	-11 ± 2*	150 ± 4	-7 ± 4*
DABP	108 ± 4	-9 ± 1*	101 ± 4	-7 ± 4
MABP	123 ± 4	-9 ± 1*	118 ± 4	-5 ± 4
LVSP	139 ± 5	-11 ± 1*	134 ± 5	-4 ± 5
LVEDP	9 ± 1	4 ± 1*	9 ± 1	2 ± 1
+dP/dt_{max}	2937 ± 125	-536 ± 85*	2896 ± 186	-379 ± 193*
-dP/dt_{max}	2662 ± 137	-511 ± 119*	2662 ± 153	-386 ± 138*
HR	159 ± 6	5 ± 1*	149 ± 10	7 ± 1*

Table 2. *Haemodynamic changes during a 25 min LAD occlusion in sham-paced (SPO) and paced (PO) dogs. Values are means ± s.e.m, *p<0.05 compared to baseline values.*

4.2.2. Chages in epicardial ST-segment and TAT during a 25 min occlusion of the LAD

This is illustrated in Figure 7. These indices of ischaemia severity were assessed by recording epicardial unipolar ECG signals from 31 distinct epicardial sites. In sham-paced controls, occlusion of the LAD resulted in a sudden increase in TAT and similarly in epicardial ST-segment, indicating the rapid development of ischaemia following LAD occlusion. In contrast, in dogs subjected to cardiac pacing 24h previously, these ischaemia-induced changes were significantly less pronounced.

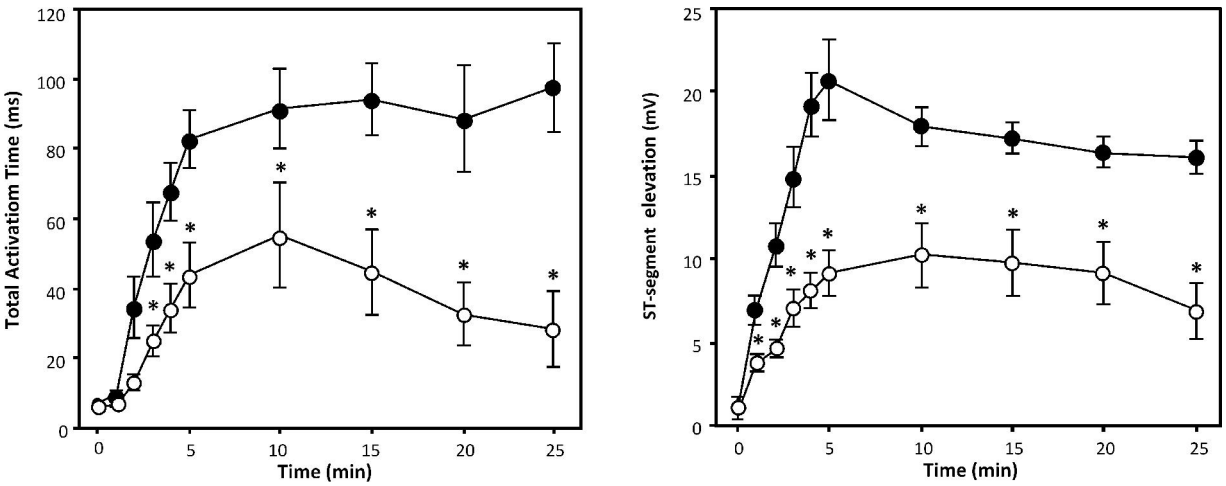

Figure 7. *Changes in ischaemia severity in SPO (black circles) and PO (empty circles) dogs during a 25 min coronary artery occlusion.. Values are means ± s.e.m., *p<0.05 vs SPO.*

4.2.3. The severity of ventricular arrhythmias during a 25 min occlusion and reperfusion of the LAD

This is shown in Fig 8. Compared to the sham-paced controls in which LAD occlusion resulted in high number of VPBs (294 ± 78) and many episodes of VT (7.4 ± 2.2) that occurred in 60% of animals, in dogs subjected to cardiac pacing 24h previously, the number of VPBs (63 ± 25) as well as the number (1.1 ± 0.6) and the incidence (25%) of episodes of VT were significantly reduced. Although cardiac pacing did not substantially modify the incidence of VF during occlusion, it markedly increased survival from the combined ischaemia-reperfusion insult (78% vs 20%).

4.2.4. Distribution of VPBs in relation to changes in myocardial electrical impedance during LAD occlusion

These are illustrated in Fig 9. In control dogs, immediately after the onset of ischaemia, a steep increase in tissue resistivity and a decrease in phase angle occurred, accompanied by the appearance of phase Ia arrhythmias. This was followed by a short, relatively arrhythmia-free interval, during which myocardial impedance changes were also attenuated (Fig 9a). However, at around the 13th min of the ischaemia a second rise in tissue resistivity and a decline in phase angle occurred, followed by the occurrence of severe ectopic activity. These arrhythmias, as well as the abrupt changes in tissue impedance were significantly attenuated especially during phase Ib in dogs subjected to cardiac pacing 24h previously (Fig 9b).

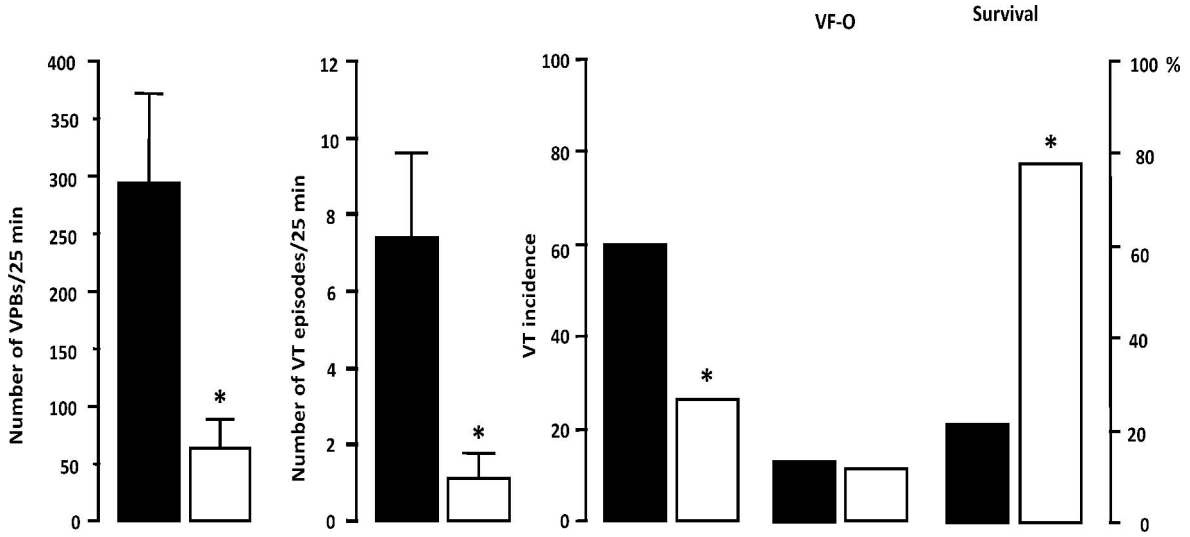

Figure 8. *The total number of VPBs, the number of episodes and the incidence of VT, the incidence VF during LAD occlusion, as well as survival after reperfusion in SPO (black bars) and in PO (empty bars) dogs. Values are means ± s.e.m. *p<0.05 vs SPO.*

4.2.5. Changes in myocardial Cx43 expression after rapid cardiac pacing

In order to determine whether intermittent periods of cardiac pacing modifies Cx43 expression in the canine myocardium, we performed immunoblot, immunohistochemical and RT-PCR analyses from myocardial samples taken at various time points after cardiac pacing over a period of 24h. The results are shown in Fig 10. Since data obtained from sham-paced animals at different time points were almost identical, these were pooled (n=12). Compared to the sham paced controls, in paced dogs there were no significant changes either in total (Fig 10a.) or in the Cx43 content of intercalated discs (Fig 10b and d) up to 12h after cardiac pacing. However, at this time point both total and membrane Cx43 contents were significantly decreased, preceded by a marked down regulation of Cx43 mRNA expression at 6h after pacing (Fig 10c). Nevertheless, mRNA expression was already up regulated at 12h and subsequently Cx43 protein content as well as mRNA level returned to normal by 24h, resulting in no significant differences between SP and P groups prior to the coronary artery occlusion.

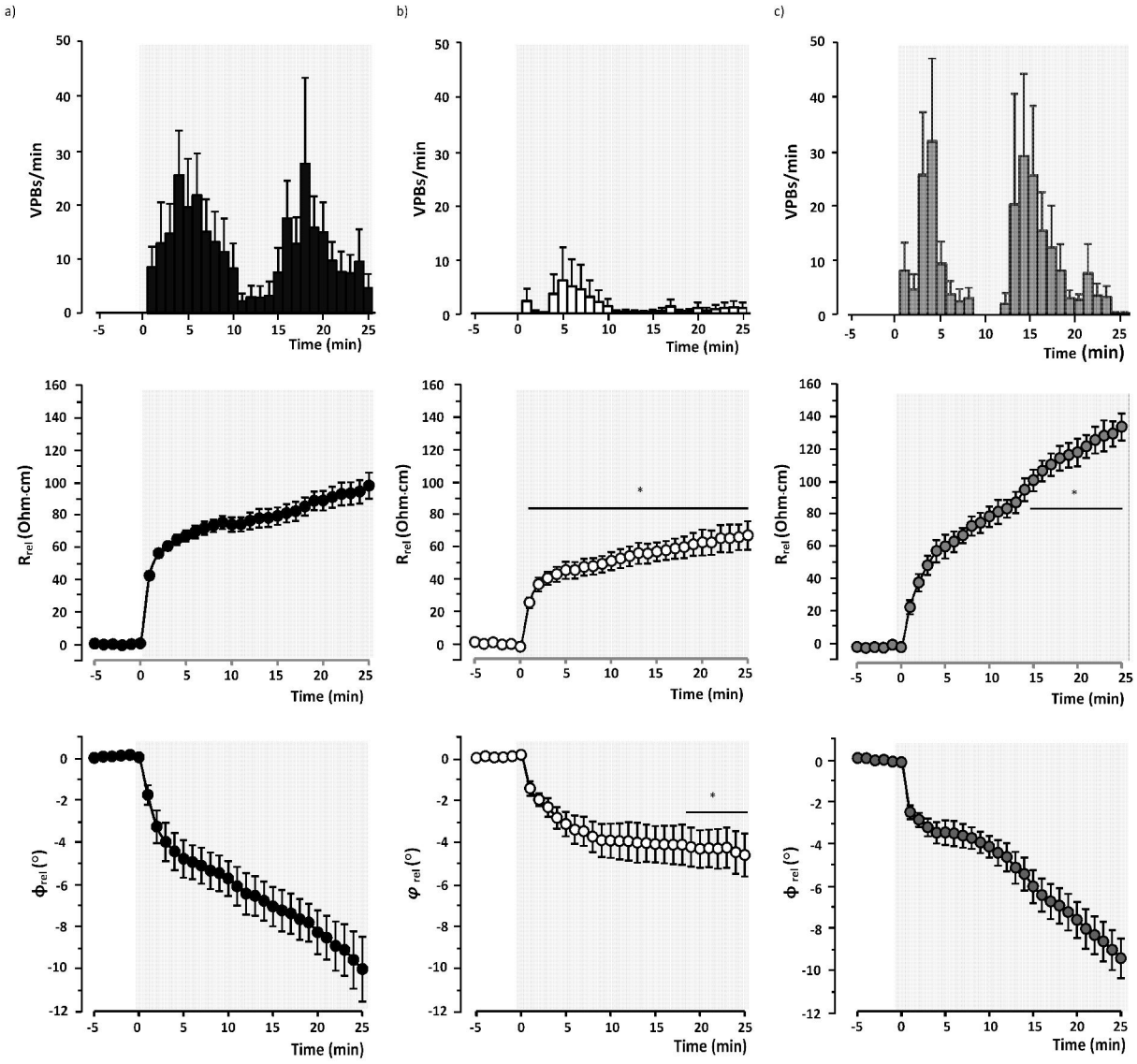

Figure 9. *Distribution of VPBs and changes in tissue impedance during a 25 min occlusion 24 h after sham-pacing (a), pacing (b) as well as when occlusion was performed 12h after cardiac pacing (c). Values are means ± s.e.m. *p<0.05 vs SPO.*

4.2.6. Changes in Cx43 expression during LAD occlusion 24hrs after cardiac pacing

These results are illustrated in Fig 11. Compared to the pre-occlusion Cx43 levels (SP and P24) occlusion of the LAD resulted in no significant alterations in total or in membrane Cx43 protein content in either of the groups (Fig11a and b). Cx43 mRNA level was also unchanged after coronary artery occlusion and reperfusion in both groups (Fig 10c). However, immunohistochemical images showed that in control dogs subjected only to occlusion or to a combined occlusion-reperfusion insult, the end-to-end connections became "blurred" (Fig 11. c), indicating a structural impairment of the intercalated discs. This alteration was particularly

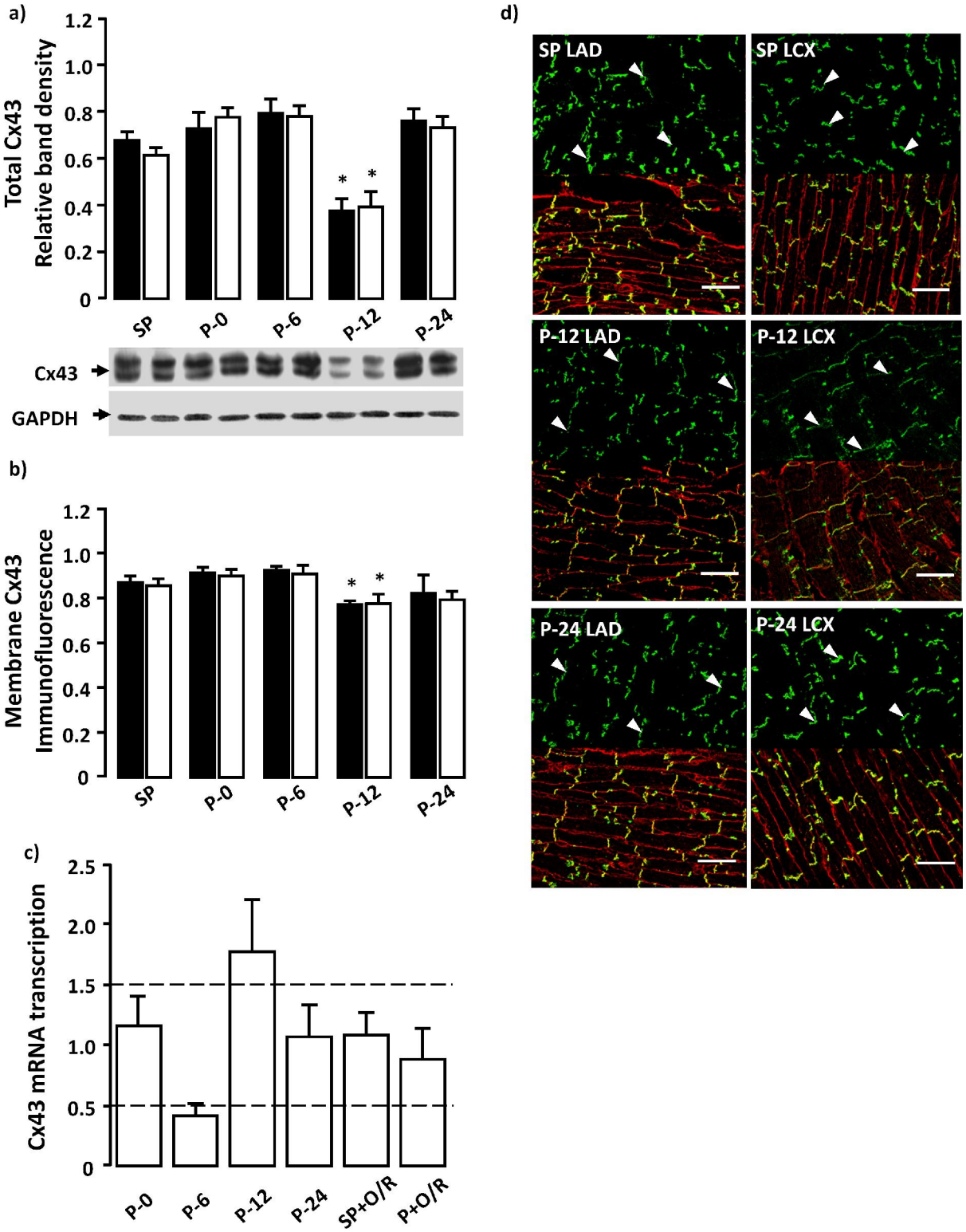

Figure 10. *Time-course changes in Cx43 total protein level (a), mRNA expression (c) and Cx43 immunosignal in intercalated discs (b) with representative confocal immunofluorescent images d) of end-to-end connections (green) with sarcolemmal staining (red) at different time points after cardiac pacing. Arrow heads point to intercalated discs. Scale bar=50μm. Values are means ± s.e.m. *p<0.05 vs SP.*

marked in samples, which were taken 5 min after reperfusion. In contrast, the structural integrity of end-to-end connections was well-preserved in paced dogs, following a 25 min ischaemia-reperfusion insult.

4.2.7. Changes in gap junction metabolic coupling and in the phosphorylation status of Cx43

These were assessed in samples taken from dogs that underwent a 25 min coronary artery occlusion without reperfusion. Gap junction permeability in the controls was significantly reduced within the ischaemic area to 68 ± 3 % compared to the non-ischaemic value (100±3%). In contrast, in dogs paced 24h previously, gap junction permeability within the ischaemic myocardial wall was only slightly reduced (97±5% of the permeability measured in non-ischaemic samples) by the end of the 25 min LAD occlusion.

The ischaemia-induced changes in the phosphorylation of Cx43 are illustrated in Fig 12. In control and paced dogs, the P/dP ratio within the non-ischaemic samples was 64/36±2%. In control dogs, occlusion of the LAD caused significant dephosphorylation of Cx43 resulting in a shift of P/dP ratio to 46/54±4%. In contrast, in dogs subjected to cardiac pacing 24h prior to the occlusion, the P/dP ratio was 61/39 ± 1% and 62/38 ± 1%, within the non-ischaemic and the ischaemic region, respectively. These results indicate that pacing prevented the ischaemia-induced dephosphorylation of Cx43.

4.2.8. Arrhythmia and ischaemia severity during LAD occlusion 12 hours after cardiac pacing

In order to determine ischaemia and arrhythmia severities at a time point when Cx43 contents were markedly reduced, other nine dogs were subjected to a 25 min coronary artery occlusion and reperfusion 12h after cardiac pacing. In three dogs out of these 9 dogs, occlusion of the LAD resulted in only few ectopic beats (50 ± 11) and no VT during the occlusion period, and two of these dogs even survived the rapid reperfusion. However, six dogs exhibited many VPBs (211 ± 61) and episodes of VT (5.2 ± 2.7) that occurred particularly during the later period of the occlusion i.e. between 13 and 25 min. These Ib phase arrhythmias were somewhat more marked than those obtained in the controls during the same occlusion period. Furthermore, two of these 6 dogs experienced VF during occlusion and none of them survived reperfusion. Similar to the sham paced controls, changes in tissue resistivity and phase angle were more pronounced in dogs paced 12h before occlusion compared to those which had been paced 24h prior to occlusion (Figure 9). Whereas there were no significant differences in epicardial ST segment changes between the controls and

dogs that had been paced 12h previously (at 5 min of the occlusion in dogs paced 12h before: 17 ± 1mV vs. 20 ± 2 mV, and at 20 min of the occlusion: 15 ± 3 vs. 16 ± 1 mV), the increases in TAT were substantially higher in these paced dogs (120 ± 17 and 113 ± 9 ms) than in the non-paced controls (83 ± 9 and 89 ± 11 ms).

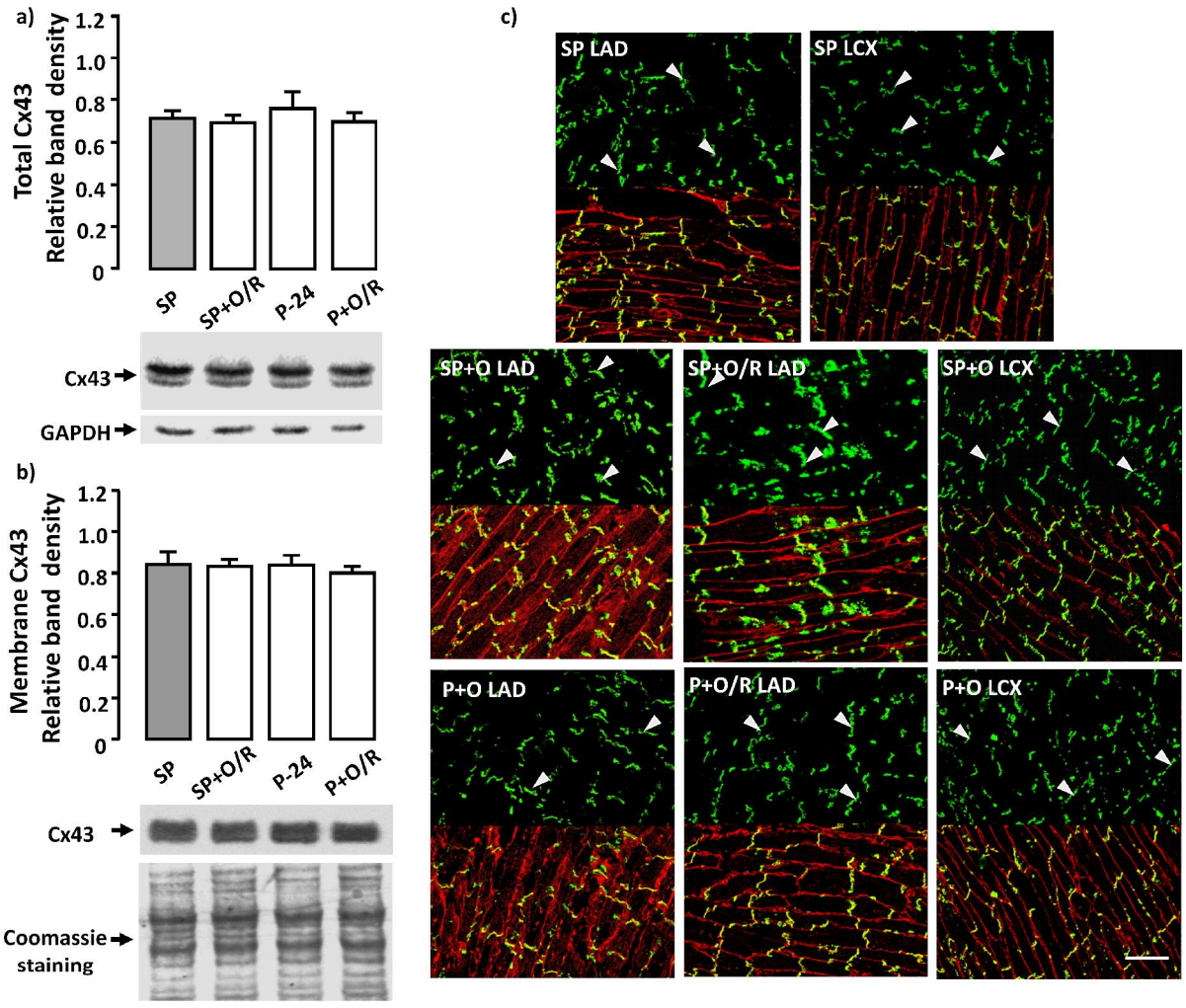

Figure 11. *Total (a) and membrane Cx43 content (b) in control and paced dogs after a 25 min coronary artery occlusion and reperfusion. c) Confocal images of end-to-end connections (green) in ischaemic (LAD) and non-ischaemic (LCX) myocardial sections. Arrow heads point to intercalated discs. Scale bar=50μm. Values are means ± s.e.m.*

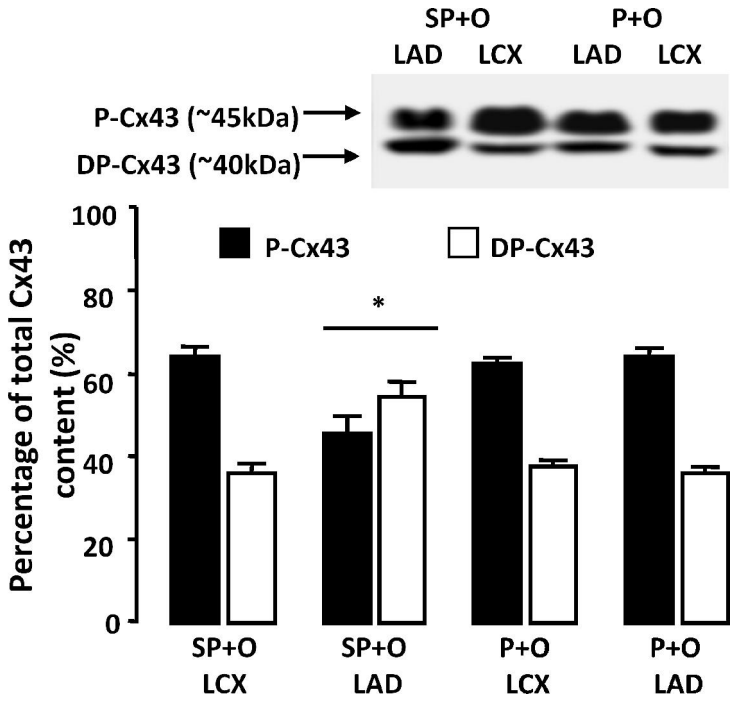

Figure 12. *Changes in the phosphorylated and dephosphorylated isoforms of Cx43 after a 25 min LAD occlusion in control (SP+O) and paced dogs (P+O). Tissue samples were taken from ischaemic (LAD) and non-ischaemic (LCX) myocardial wall regions. Values are means ± s.e.m. *p<0.0.5 vs SP+O LCX.*

5. DISCUSSION

5.1 New findings

1. We provided evidence that the intracoronary infusion of SNP in a dose of 0.2μg·kg^{-1}·min^{-1} markedly reduces the severity of ischaemia and of ventricular arrhythmias during a 60 min period of coronary artery occlusion. This protection may result from the ability of SNP to release NO. The present results propose that this NO, among its several other effects can inhibit uncoupling of gap junctions and preserve gap junction function during ischaemia. This effect may certainly play a role in the antiarrhythmic effect of SNP.

2. We have demonstrated that cardiac pacing results in a time-dependent change in Cx43 expression, which almost certainly contributes to the altered gap junction function following ischaemia and reperfusion, 24 h later. This effect is manifested in reduced uncoupling of gap junctions, preserved phosphorylation of Cx43 and gap junction permeability, as well as the maintenance of the structural integrity of intercalated discs; all these pacing-induced changes at gap junction level may account for the protection against arrhythmias resulting from ischaemia and reperfusion. This assumption is supported by the finding that when Cx43 content is considerably reduced, more severe gap junctional uncoupling occurs and the myocardium becomes more susceptible to arrhythmias. This observation may have a clinical relevance, by raising the possibility that there is a critical time-window after a preconditioning stimulus, when the heart becomes more vulnerable to arrhythmias in case of an ischaemic event.

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

In a previous study, we have demonstrated that the marked antiarrhythmic effect of ischaemic preconditioning is associated with the preservation of gap junction electrical and metabolic coupling and with the prevention of the ischaemia-induced dephosphorylation of the main gap junction protein Cx43 during myocardial ischaemia [65]. We have also shown that this protective effect of preconditioning is NO-dependent [17], and that drugs which are able to donate NO exert similar antiarrhythmic effect to that of preconditioning [67]. Although there is increasing evidence coming mainly from non-cardiac tissues [70, 71, 95, 96] and also from the vasculature [97] that NO is an important regulator of gap junction channels and is able to modulate the expression of different connexins, still such an

information for the a role of NO in the myocardium is lacking. Thus, we designed studies to investigate whether the antiarrthythmic effect of an NO donor can be attributed to the modification of gap junction channels by NO. For this purpose, we used the NO donor SNP in our anaesthetised dog model of ischaemia and reperfusion. The results confirmed our previous findings that drugs, which are able to release NO, protect the myocardium against ishaemia and reperfusion-induced ventricular arrhythmias. However, the protective effect of SNP was less pronounced than that obtained either with nicorandil [66], or isosorbide-2-mononitrate [67]. Although SNP almost completely abolished the ectopic activity, it failed to reduce the incidence of more severe arrhythmias such as VF. There are conflicting results regarding the effect of NO donors on ischaemia-induced arrhythmias. For example, in anaesthetised rats [98, 99] different NO donors (nitroglycerine, SNP, SIN-1 and isosorbide-dinitrate) failed to influence arrhythmia severity, whereas in anaesthetised pigs [100, 101] and also in human patients [102, 103] organic nitrates were effective antiarrhythmic agents. The reason for these conflicting results can be attributed to the differences in the models used, the dose and route of the administration of NO donors, as well as the ability of different donor molecules to release NO [104]. However, another explanation might be that in the present study the average body weight of the dogs involved was smaller (between 15-25 kg) than in a previous study with isosorbide-2-monomitrate [67]. As it has been pointed out body weight can be one of the factors, which may determine the susceptibility of the heart to arrhythmias [17, 105]. Although the precise mechanism by which NO donors protect against arrhythmias is still not fully understood, other beneficial effects, such as anti-platelet aggregatory effect, the favourable haemodynamic effects, as well as coronary vasodilatation may also account for the antiarrhythmic protection [100, 101]. In our present study, the latter seems unlikely to be responsible for the antiarrhythmic protection, since the SNP in the applied dose did not affect either baseline coronary blood flow or the compensatory coronary flow (Table 1).

The modulation of gap junction function following SNP administration was assessed under both *in vivo* and *in vitro* conditions by measuring changes in tissue electrical impedance, gap junction permeability and Cx43 phosphorylation. We demonstrated that in contrast to the controls, SNP administration significantly attenuated the ischaemia-induced increase in tissue resistivity without considerably affecting phase angle shift. Although in SNP treated animals there was a sudden reduction of phase angle during the first 5 min of ischaemia, interestingly, these abrupt changes were not accompanied by an increased ectopic activity (Fig 5.). This phenomenon deserves further investigation. Nevertheless, the second decline in phase angle which occurred before the appearance of phase 1b arrhythmias in the

controls, in the presence of SNP remained almost unchanged. This supports the hypothesis that the attenuation of gap junctional uncoupling during this “critical” phase of ischaemia is indeed associated with the suppression of arrhythmias [65]. It is well-established that this is the period of ischaemia when catecholamines are released [11] and further increase the electrical instability of the heart [106]. There is also evidence that NO is able to inhibit noradrenaline release from the sympathetic nerve endings [107] and facilitate the release of acetylcholine [108, 109]. Both effects may account for the antiarrhythmic effect and the improvement of cellular coupling perhaps by the prevention of the iscahemia-induced dephosphorylation and degradation of Cx43 [110, 111].

Although the present study provided the first functional evidence that NO is able to modulate myocardial gap junctions, the exact regulatory mechanisms remains to be unanswered. Also, we do not know, whether NO directly or indirectly affects gap junctions and whether NO opens or closes these intercellular channels in the myocardium. For example, NO by reducing intracellular Ca^{2+} overload – a key signal for gap junctional uncoupling [112] - through the phosphorylation of L-type Ca^{2+} channel via PKG [113] and by the activation of SERCA2a [114] may indirectly inhibit the ischaemia-induced gap junctional uncoupling. Several, sometimes diverse signalling pathways are proposed to play a role in the regulation of gap junctions [30]. We think that one possible way might be the stimulation of sGC-cGMP pathway by NO and the subsequent activation of PKG [115], which by phosphorylating Cx43 is able to modify gap junction function [116]. On the other hand, S-nitrosylation of Cx43 might also be a possible mode of action by which NO is able to directly affect channel function [117, 118]. Apart from these, there may be other possible pathways whereby NO can indirectly influence gap junction coupling.

In summary, this study provided the first evidence that the antiarrhythmic effect of SNP, at least in part, can be attributed to the effect of NO on gap junctions, as their function is largely preserved in the presence of SNP during coronary artery occlusion. Besides the favourable haemodynamic and anti-ischaemic effects of NO, the activation of more specific signalling pathways directed to gap junctions are most likely involved in the antiarrhythmic effect of SNP, nevertheless to explore the exact mechanism of this protection warrants further investigations.

5.3. The role of gap junctions in the delayed phase of preconditioning induced by rapid cardiac pacing

In a previous study we have demonstrated that repeated, brief periods of rapid cardiac pacing through the right ventricle markedly reduces the severity of ventricular arrhythmias occurring during a subsequent coronary artery occlusion and reperfusion 24 h later [82]. Although the precise mechanism of this protection is still not fully understood, it was proposed that the global ischaemic changes - caused by rapid right ventricular pacing - induces the release of protective substances [119], via the activation of gene expression [76] evoke the delayed protection. In a more recent study we have demonstrated that gap junctions play a role in the early antiarrhythmic effect of ischaemic preconditioning [65]. Furthermore we showed that NO, which is a key mediator of both the early [17] and the delayed [120, 121] protection may also modulate gap junction channels (see the first part of the present thesis) thus influencing arrhythmia generation during an acute coronary artery occlusion. There is also increasing evidence that cardic pacing – depending on the duration of the stimulus – can induce adaptive (short-term pacing) and maladaptive (long-term pacing) processes by influencing the expression, cellular localization and phosphorylation of the main structural protein of gap junctions, Cx43 [86-90].

On the basis of these previous results we designed studies to examine whether gap junctions also play a role in the delayed antiarrhythmic effect afforded by cardiac. This was examined in our anaesthetised canine model using various *in vivo* and *in vitro* measurements. The results of the *in vivo* experiments demonstrated that the marked reduction in arrhythmia severity and mortality during a 25 min LAD occlusion 24 h after cardiac pacing was associated with attenuated electrical uncoupling of gap junctions; the changes in tissue impedance were significantly less pronounced especially during phase Ib in the paced dogs than in the sham-paced controls. This preserved cell-to-cell electrical coupling in the paced group was also reflected by less pronounced increase of TAT indicating better impulse conduction within the ischaemic area. This, together with the less marked elevation of epicardial ST-segment in paced dogs supports those previous findings that cardiac pacing reduces ischaemia severity 24 later [82].

These results have been confirmed by the *in vitro* measurements, performed in myocardial samples taken at the end of the experiments. These showed that rapid cardiac pacing 24 h prior to occlusion preserved Cx43 phosphorylation as well as gap junction permeability after a 25 min period of occlusion. These findings are in accord with previous

results showing that ischaemic preconditioning prevents Cx43 dephosphorylation and preserves metabolic coupling of myocytes during myocardial ischaemia when it is performed soon after the preconditioning stimulus [58, 65]. More recently, the preservation of Cx43 phosphorylation and cellular coupling during the delayed phase of the protection have been found by the activation of cardiac muscarinic M3 receptors [122]. However, we could not find difference in Cx43 phoshporylation between the paced and the control group, when reperfusion had been performed. A possible explanation for this might be that Cx43 dephosphorylation rapidly recovers after a short period of ischaemia (less than 30 min) when the myocardium is reperfused [87]. We have also failed to demonstrate substantial changes in total and membrane Cx43 protein content after a 25 min ischaemia/reperfusion insult. This was in contrast with other studies, which showed a significant decrease in Cx43 protein level in the epicardial border zone of dogs after 30 min of ischaemia [123] or even a 20 min ischaemia [124]. Nevertheless, other studies suggest that a fairly prolonged period of ischaemia is required (i.e. more than 1h) to observe significant Cx43 degradation [125]. In this respect, our results are in accordance with those studies, which demonstrated that Cx43 dephosphorylation may occur, without a net loss of protein level after a 40 min period of ischaemia [43].

We have found however, considerable changes during the analysis of Cx43 immunofluorescence signal of intercalated discs. In control dogs, there were apparent signs of structural impairment at the longitudinal cell-to-cell connections both at the end of the occlusion period and particularly when the myocardium was reperfused. This faint or dim Cx43 signal density in the intercalated discs may indicate ultrastructural changes, such as membrane disruption or internalization of Cx43 [43, 124] at the end-to-end connections during the ischaemic period. It is also possible that these structural changes resulted from the ischaemia-induced altered interactions between Cx43 and other structural proteins, such as c-Src and ZO-1 [123, 126, 127]. Whatever the explanation for this phenomenon is, it seems that cardiac pacing interferes with these processes, since cardiac pacing preserved the structure of the intercalated discs both during occlusion, as well as after reperfusion. However, in contrast to others, using more prolonged period of cardiac pacing [86, 87], we did not observe any sign of Cx43 lateralization at any time point after cardiac pacing.

Interestingly, cardiac pacing itself resulted in time-dependent changes in Cx43 mRNA and protein expressions. We observed almost a 50% reduction in total Cx43 protein content, 12 h after pacing and this was preceded 6 h earlier by a significant down regulation of Cx43 mRNA. A similar effect was observed in an *in vivo* mice model with 6h of overdrive pacing

[86]. It is also interesting to note that in our model, pacing the heart from the right ventricle caused global changes affecting the left ventricle as well, where these expression changes in Cx43 protein and mRNA were observed. Although we do not know how cardiac pacing modifies Cx43 gene and protein expression, it most likely interferes somehow with Cx43 turnover [86, 124, 128]. A plausible explanation might be that endogenous substances released during the pacing stimulus may play a role. One such substance might be NO, which is a key mediator of both the early and the delayed antiarrhythmic protection [17, 82, 120, 121], and as we (see the previous section of the present thesis) and others [36, 70, 71] proposed that NO is able to modulate gap junctions. There is evidence that NO, derived from iNOS activation, reduces myocardial Cx43 protein expression in anaesthetised rats [129] and in mice [36]. Furthermore, pacing the heart from the right ventricle – apart from causing transient ischaemia – interferes with normal activation sequence of the ventricles and increases wall stretch. This via the activation of the renin-angiotensin aldosterone system can lead to the redistribution gap junctions and to a reduction in Cx43 protein level [130]. Whatever the mechanism is cardiac pacing results in transient down regulation of Cx43 mRNA at 6h and protein expression at 12 h after pacing and these changes are definitely restored to normal level within another 12 h, when the delayed effect of a preconditioning stimulus is usually apparent (Fig 10).

In this particular study we have raised the question: if gap junctions through the modification of Cx43 are indeed involved in the delayed phase of the protection, then what would happen to the arrhythmias, when the expression of Cx43 was markedly reduced (i.e. 12h after pacing). This was important to know because we had previous evidence that the antiarrhythmic protection afforded by cardiac pacing wanes 1 and 6 h after the pacing stimulus [82], but in that particular study the severity of arrhythmias has not been examined 12h after pacing. Therefore we performed a series of experiments in which nine dogs were subjected to LAD occlusion and reperfusion 12 h after cardiac pacing and the severity of ischaemia and ventricular arrhythmias in relation to tissue impedance changes were assessed. Three out of these dogs exhibited only few ectopic beats and two of them survived, but in six dogs more severe ischaemic changes occurred compared with the controls. In these 6 dogs there were a large number of VPBs, and many episodes of VTs particularly during Ib phase and none of them survived the combined ischaemia-reperfusion insult. Tissue impedance changes were also more marked in these dogs than in the controls, indicating more severe gap junctional uncoupling. Although some studies suggest that almost a 90% reduction of myocardial Cx43 protein level is needed to increase the susceptibility of the heart to

arrhythmias [131, 132], we suppose that under certain circumstances such as myocardial ischaemia, a less marked reduction (around 50%) is already sufficient to promote arrhythmia generation. This assumption is supported by the findings which showed that myocardial conductivity is unaltered at reduced myocardial Cx43 level under normal conditions [133] but it is definitely associated with higher arrhythmia vulnerability in case of an ischaemic event [134]. From our findings, it is tempting to conclude that the reason for the increased propensity for arrhythmias 12h after pacing was due to the reduction of Cx43 and the impairment of electrical cell-to-cell coupling. We think that our results might also have clinical importance by pointing out that after a preconditioning-like stimulus, such as cardiac pacing or heavy physical exercise [83, 135, 136], there is a critical time interval, when an ischaemic condition may result in severe, or even fatal cardiac event.

In summary we have demonstrated that cardiac pacing results in time-dependent changes in Cx43 gene and protein expression. The reduced Cx43 protein level 12h after the pacing stimulus is associated with higher susceptibility for arrhythmias if an ischaemic challenge occurs during this period. Cardiac pacing also preserves gap junction function during a coronary artery occlusion 24h later by preventing the structural impairment of end-to-end connections during ischaemia and upon reperfusion, thus reducing the occurrence of severe ventricular arrhyhtmias. Our results suggest that gap junction channels are certainly play a role in the cardiac pacing induced delayed antiarrhythmic protection.

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8. ANNEX

Reprints of full papers

Gönczi M, Papp R, Kovács M, Seprényi Gy, Végh Á. Modulation of gap junctions by nitric oxide contributes to the antiarrhythmic effect of sodium nitroprusside? *Br J Pharmacol* **2009**; 156:786-93.

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