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

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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

INTRODUCTION

Myocardial ischaemia often leads to the generation of severe ventricular tachyarrhythmias and ultimately to sudden cardiac death. 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.

Ischaemia induced ventricular arrhythmias occur in a characteristic biphasic distribution. The first phase of these arrhythmias - termed as phase Ia –lasts from the 2^{nd} to the 10^{th} minute of ischaemia. The proposed mechanisms of these phase Ia arrhythmias implicate rapid metabolic changes and a concomitant ionic imbalance across the cell membrane leading to dramatic changes in action potential morphology and duration as well as impulse generation and conduction. The first phase is generally followed by a relatively arrhythmia free interval 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. 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. This phase generally occurs between the 15^{th} and the 30^{th} min of ischaemia and often terminates in ventricular fibrillation. Recent studies propose that among other factors uncoupling of gap junctions play a pivotal role in the generation of Ib phase of arrhythmias.

Gap junctions are intercellular channels facilitating metabolic and electrical communication among cells as they are permeable to molecules of molecular weight less than 1kDa, such as ions and second messengers. Gap junctions are formed by connexin protein subunits. These channels aggregate at the longitudinal end-to-end connections of the myocytes in the intercalated discs. In the ventricular myocardium the primary connexin isoform is connexin 43 (Cx43). The metabolic alterations during ischaemia i.e. low ATP and pH, Ca^{2+} overload and the accumulation of amphiphylic metabolites, as well as dephosphorylation of Cx43, together with an excessive release of catecholamines facilitate the closure of gap junctions and trigger ventricular arrhythmias.

In 1986 a new cardioprotective phenomenon was described and termed as ischaemic preconditioning: repeated sublethal ischaemic episodes provide protection against the otherwise lethal consequences (e.g. arrhythmias) of a more prolonged ischaemic insult. More recent evidence suggests Cx43 and gap junctions as one of the major targets in this adaptive phenomenon.

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, attenuated the protection against arrhythmias. The second piece of evidence resulted from those studies which showed that the administration of NO donors exerts a preconditioning-like antiarrhythmic effect.

Research of recent years demonstrated that NO, among its several other actions, may modulate the expression of different connexin isoforms and the gating properties of gap junction channels. Although, the majority of these results are mainly derived from *in vitro* studies in non-myocardial tissue, 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.

Preconditioning induces not only an early, but also a delayed phase of protection. 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, 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. 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. for example by high frequency right ventricular pacing or heavy physical exercise. A number of evidence supports the concept that cardiac pacing is able to modify gap junction function and expression. It is interesting, 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. Despite the extensive research, whether gap junctions play a role in this pacing-induced delayed protection has not yet been examined.

AIMS OF THE STUDY

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

I. 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.

II. 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.

MATERIALS AND METHODS

Surgical preparations

Animals were anaesthetised with pentobarbital ($0.5mg \cdot kg^{-1}$). The right femoral vein was prepared and cannulated for the administration of a mixture of α-chloralose and urethane (60 and $200\ mg \cdot kg^{-1}$). The right femoral artery and the left arteria carotis communis were prepared and catheterised in order to measure arterial and left ventricular blood pressures. Arterial blood gases and pH were frequently monitored and ventilation 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. Coronary blood flows were measured on the left circumflex and the left anterior descending branches.

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. The inhomogeneity of electrical activation measured as the greatest delay in activation. Additional two unipolar electrodes served for measuring changes in epicardial ST-segment.

In the other series of experiments a mapping electrode was used consisting of 31 unipolar electrodes. Local activation times at each electrode points were calculated. 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). Changes in epicardial ST-segment, recorded from 31 unipolar electrode sites were averaged.

Measurement of gap junctional electrical coupling

Electrical coupling of gap junctions was determined by changes in tissue impedance using the four-pinned electrode method. The electrode was fixed within the potentially ischaemic area parallel to myofibre orientation. High frequency subtreshold alternating current was applied between the outer electrode pairs and voltage drop was measured between the inner electrode pairs with a lock-in amplifier. Relative changes in tissue resistivity and in phase angle shift were calculated during offline analysis.

Assessment of ventricular arrhythmias

The number and incidence of ventricular arrhythmias occurring during LAD occlusion was evaluated according to the Lambeth conventions. The total numbers of ventricular premature beats (VPB), the number and incidence of ventricular tachycardiac episodes (VT) as well as 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.

Assessment of gap junctional metabolic coupling

Gap junction permeability was assessed by double dye loading method. transmural tissue blocks 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 and TRITC-dextran for 15 min. Lucifer Yellow is a small molecule, thus it readily penetrates through gap junctions, whereas TRITC-dextran, being a large molecule, stains only the injured cells on the cutting edge of the tissue. Tissue blocks were then fixed in 4% paraformaldehyde. Cryosections were mounted on gelatin coated slides. Images were taken with a fluorescent microscope attached to a CCD camera. Permeability within the ischaemic area was expressed as the percentage of permeability within the non- ischaemic area.

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 and were homogenized in ice-cold lysis buffer. 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. Protein samples were separated on 10-12% SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% non-fat milk, membranes were labeled against Cx43 with a rabbit anti-Cx43 primary antibody and subsequently HRP-conjugated goat secondary antibody. Blots were developed with ECL Plus Kit on X-ray films or scanned with a Typhoon laser scanner. Band intensities were measured using ImageJ software.

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. Longitudinal frozen tissue sections (8μm) were cut from the mid-myocardial layer and specimens were mounted on gelatine coated slides. Sections were blocked with 5% BSA, and were labelled with rabbit anti-Cx43 primary antibody against Cx43 then with FITC conjugated secondary antibody, and finally with WGA-conjugated Texas Red. Images were

captured with a laser scanning confocal microscope. Relative amount of Cx43 was calculated as pixel intensities of intercalated discs with background correction.

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 using the RNeasy Fibrous Tissue Mini Kit according to the manufacturer's instruction. Quantitative PCR was performed with gene-specific primers using SybrGreen protocol to monitor gene expression. From each sample 2 μg of total RNA was reverse transcribed using ImPromII Reverse Transcription system in the presence of oligo(dT) at 42^{o}C for 2h. Reactions using SybrGreen protocol were performed with Power SYBR Green Master mix at a final primer concentration of 250 nM. Melting temperature analysis was performed after each reaction to check the quality of the reaction.

Relative expression ratios were calculated as normalized ratios to HPRT (hypoxanthine guanine phosphoribosyltransferase) and to tubulin. 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.

Experimental protocols

I. In order to evaluate the possible modulatory role of NO on cardiac gap junctions, two groups of anaesthetised dogs were used. In the control group 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 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).

II. The following protocol was used to evaluate the role of gap junctions in the pacing-induced delayed cardioprotection: **u**nder light pentoparbital anaesthesia (0.5mg·kg^{-1}) a bipolar pacing catheter was inserted through the jugular vein into the right ventricle and the hearts were paced four times for 5 minutes, at a rate of 240 beats/min. In sham-paced animals the pacing electrode was introduced into the right ventricle but the dogs were not paced. From both the paced and control groups 12 dogs were selected and divided into four further groups.

In these dogs euthanasia was induced at various time intervals, i.e. immediately, six, twelve and twenty-four hours after the pacing or sham-pacing procedures and myocardial tissue samples were taken. 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

RESULTS

I/1. Intracoronary administration of SNP caused slight but statistically significant reductions in the haemodynamic parameters. These changes returned to baseline by the beginning of the occlusion. LAD occlusion resulted in significant reductions in haemodynamic parameters in both groups. Infusion of SNP did not substantially affect either the diastolic blood or the increase in compensatory blood flow upon occlusion. In the presence of SNP, the marked elevation of epicardial ST segment and the degree of inhomogeneity of electrical activation were significantly reduced.

I/2. In the control group, occlusion of the LAD resulted in a high number of VPBs which were distributed in two distinct phases. Tissue resistivity and phase angle changes showed steep changes during occlusion reflecting severe uncoupling of gap junctions. The intracoronary infusion of SNP markedly attenuated ectopic activity during both arrhythmia phases. Although SNP itself did not cause any significant alteration in tissue impedance values in these dogs, occlusion of the LAD resulted in less marked tissue impedance changes indicating a preservation of electrical coupling of gap junctions. The maintained coupling of these channels was also proven by the measurement of metabolic coupling of myocytes and the phosphorylation of Cx43. These experiments showed that the reduction of metabolic coupling assessed by double fluorescent dye method and the marked ischaemia-induced dephosphorylation of Cx43 was prevented in the presence of SNP.

II/1. In order to determine whether intermittent periods of cardiac pacing modify Cx43 expression in the canine myocardium, we performed immunoblot, immunohistochemical and RT-PCR.. Compared to the sham paced controls, in paced dogs there were no significant changes either in total or in the Cx43 content of intercalated discs 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 and subsequently Cx43 protein content as well as mRNA level returned to normal by 24h.

II/2. The 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 in epicardial ST-segment, indicating the rapid development of ischaemia following LAD occlusion. In contrast cardiac pacing 24h previously attenuated these changes. Also, compared to the sham-paced controls in which LAD occlusion resulted in high number of VPBs and many episodes of VT cardiac pacing significantly reduced arrhythmia activity. Tissue impedance measurements revealed that in contrast to the sham-paced dogs, where after the onset of ischaemia, a steep increase in tissue resistivity and a decrease in phase angle occurred, the abrupt changes in these parameters were significantly attenuated especially during phase Ib in dogs subjected to cardiac pacing 24h previously, indicating a better electrical coupling of myocytes during ischaemia in these dogs. Also, measurement of metabolic coupling and the phospho-dephospho ratio of Cx43 demonstrated improved coupling through gap junction channels. In addition coronary occlusion did not result in any significant alterations in Cx43 protein content and mRNA level in either groups after 25 min of ischaemia. However, immunohistochemical images showed that in control sham-paced dogs after the combined occlusion-reperfusion insult, the end-to-end connections became “blurred”, referring to a structural impairment of the intercalated discs. In contrast, the structural integrity of end-to-end connections was well-preserved in paced dogs, following a 25 min ischaemia-reperfusion insult.

II/3. In order to determine ischaemia and arrhythmia severities at a time point when Cx43 contents were markedly reduced, a group of dogs were subjected to a 25 min coronary artery occlusion and reperfusion 12h after cardiac pacing. In a few of them, occlusion of the LAD resulted in only few ectopic beats and no VT during the occlusion period. However, the majority exhibited greater arrhythmia activity especially during Ib phase. Moreover, 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.

5. DISCUSSION

I. 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. We have also shown that this protective effect of preconditioning is NO-dependent, and that drugs which are able to donate NO exert similar antiarrhythmic effect to that of preconditioning. Although there is increasing evidence coming mainly from non-cardiac tissues 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-induced ventricular arrhythmias. However, the protective effect of SNP was less pronounced than other NO donors used earlier. Although SNP almost completely abolished the ectopic activity, it failed to reduce the incidence of more severe arrhythmias such as VF. 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.

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. It is well-established that this is the period of ischaemia when catecholamines are released further increasing the electrical instability of the heart. There is also evidence that NO is able to inhibit noradrenaline release from the sympathetic nerve endings and facilitate the release of acetylcholine; both effects may account for the antiarrhythmic effect and the improvement of cellular coupling. 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 prevents uncoupling of gap junctions or S-nitrosylation of Cx43 might also be a possible mode of action by which NO is able to affect channel function. 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 favorable 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.

II. Previously 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. 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 via the activation of gene expression evoke the delayed protection. 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.

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. Our results 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.

These results have been confirmed by the *in vitro* measurements. 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. We have failed to demonstrate substantial changes in total and membrane Cx43 protein content after a 25 min ischaemia/reperfusion

insult. Some studies suggest that a fairly prolonged period of ischaemia is required (i.e. more than 1h) to observe significant Cx43 degradation.

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 at the end-to-end connections during the ischaemic period. Whatever the explanation for this phenomenon is, it seems that cardiac pacing interferes with these processes.

Interestingly, cardiac pacing itself resulted in time-dependent changes in Cx43 mRNA and protein expressions. Although we do not know how cardiac pacing modifies Cx43 gene and protein expression, it most likely interferes somehow with Cx43 turnover. A plausible explanation might be that endogenous substances released during the pacing stimulus may play a role, for example NO, which is a key mediator of both the early and the delayed antiarrhythmic protection and it is able to modulate gap junctions. Furthermore, pacing the heart from the right ventricle 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.

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, but in that particular study the severity of arrhythmias has not been examined 12h after pacing. The results indicate that during this period arrhythmia activity is increased and tissue impedance changes refer to more severe gap junctional uncoupling.

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. 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. 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 most likely play a role in the cardiac pacing induced delayed antiarrhythmic protection.

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