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

PhD Thesis

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LIST OF PUBLICATIONS

Full papers

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

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

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

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

1. Introduction

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

Following a sudden complete occlusion of one of the main branches of a coronary artery ventricular arrhythmias occur in two distinct phases, termed as phase 1a and phase 1b. Regarding the distribution of the acute arrhythmias it has been proposed that the phase 1a arrhythmias can be detected between 2 and 8 min of the occlusion, whereas the phase 1b arrhythmias occur between the 15 and 30 min of the ischaemia. These are usually more severe than the arrhythmias during phase 1a, and often terminating in sudden ventricular fibrillation. Recent studies propose that, among many other factors, uncoupling of gap junctions (GJs) plays a role in the generation of the 1b phase arrhythmias.

Gap junctions are specific channels, which directly connect the cytoplasm of the adjacent cells, thus providing fast electrical and metabolic coupling between the cells. The GJ channels can transport molecules smaller than 1kDa, such as ATP and Ca^{2+} (metabolic coupling) between the cells, most probably by a twisting motion resulting in an opened and closed state of the channel. In myocardial tissues, these channels are accumulated in clusters, located in the intercalated disks, and they represent low resistance pathways between the neighbouring cells, allowing fast spread of impulse from the one cell to the other (electrical coupling). Gap junctions are formed by connexin protein subunits; six connexin form a connexon or a half channel, and two channels form a GJ. In the ventricular myocardium the primary connexin isoform is connexin 43 (Cx43). Under ischaemic conditions the rapid metabolic changes (loss of ATP, decrease of pH, increase in intracellular Ca^{2+} and release of catecholamines) promote the uncoupling of GJs. Recent studies have shown that the closure of GJs contributes to the generation of phase 1b arrhythmias. A number of drugs have been developed to modify GJ function and arrhythmias either by facilitating (e.g. rotigaptide) or inhibiting (e.g. carbenoxolone; CBX) the opening of GJs.

We have previous evidence that GJs play an important role in the delayed antiarrhythmic effect of preconditioning, induced, for example, by rapid cardiac pacing. Pacing the dog

hearts 24h prior to a 25 min period of ischaemia and reperfusion (I/R) insult, markedly reduced the severity of ventricular arrhythmias, and, in parallel, the changes in tissue impedance, preserved the structure and function of GJs, which manifested in better metabolic and electrical coupling of cells during ischaemia, most probably through the maintenance of the phosphorylated form of Cx43. This study also showed that pacing not only results in changes in Cx43 phosphorylation, but it interferes with the connexin turnover by modifying the Cx43 gene and protein expression.

A more direct evidence for the role of GJs in the delayed antiarrhythmic effect of pacing would be the use of pharmacological modulators of GJs. Hypothetically, if pacing induces delayed protection against arrhythmias through the modulation of GJs, then closing of GJs by the administration of a GJ uncoupler prior to and during ischaemia would attenuate these protective effects of pacing. Therefore, **we designed studies in which we examined whether the GJ uncoupler carbenoxolone (CBX) modifies the antiarrhythmic effect of cardiac pacing.**

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

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

AIMS

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

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

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

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

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

MATERIALS AND METHODS

Surgical preparations

Cardiac pacing protocol

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

Coronary artery occlusion

The anaesthesia was introduced with intravenous pentobarbital (0.5 mg kg^{-1} of body weight). The right femoral vein was prepared and cannulated for the further administration of the anaesthetic (mixture of chloralose and urethane [60 and 200 mg kg^{-1} of body weight respectively]). The dogs were intubated and ventilated with room air using a Harvard respirator at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits. The right femoral artery was prepared and a polyethylene catheter was introduced to measure arterial blood pressure. The Millar tip catheter, which had been

inserted previously into the carotid artery, was now pushed into the left ventricle to detect changes in LVSP and end LVEDP pressures, as well as in changes in positive and negative dP/dt_{max}. A thoracotomy was performed at the left fifth intercostal space and the anterior descending branch of the LAD was prepared for occlusion just proximal to the first main diagonal branch. Myocardial ischaemia was induced by a 25 min period LAD occlusion, followed by rapid reperfusion.

Measurement of ischaemia severity

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

Measurement of gap junction electrical coupling

Assessment of GJ electrical coupling was similar to that described in detail previously. In brief, four stainless steel electrodes mounted on a non-conductive panel, were calibrated in saline (0.9%, resistivity: 71 Ω cm), and were inserted into the left ventricular wall within the proposed ischaemic myocardial area. A sub-threshold alternating current (10 μA, 8 kHz) was applied through the outer pair of electrodes, and voltage was measured between the inner electrode pair using a lock-in amplifier. A current frequency of 8 kHz was selected in order to detect maximal changes in phase angle without impairing the assessment of resistivity. Changes in resistivity (in Ω cm) and in phase angle (in °) were recorded by a computer with an acquisition time of 4 s and plotted at 1 min intervals. To eliminate small oscillations, resulting from ventilation, 5 consecutive 4 s measurements were averaged at each minute.

Assessment of ventricular arrhythmias

Ventricular arrhythmias during a 25 min coronary artery occlusion and following reperfusion were assessed according to the Lambeth Conventions with modification as has been outlined previously. In brief, the total number of VPBs, the incidence and the number of episodes of VT, and the incidence of VF were assessed during the occlusion period. During

the reperfusion only the incidence of VF, which is a fatal event in this species, was determined. Dogs that were alive 5 min after reperfusion were considered to be survivors. At the end of the experiments the animals were euthanized with an overdose of the anaesthetic. In those dogs from which myocardial tissue samples were taken for the *in vitro* measurements, the animals were euthanized at the end of the occlusion period. The hearts were rapidly excised and placed in ice-cold saline. Tissue samples were taken from the area supplied by the occluded LAD and from the non-ischaemic area supplied by the LCX.

Determination of changes in the cellular distribution of Cx43

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

Determination of changes in Cx43 protein expression and phosphorylation

Immunoblot analysis was used to determine Cx43 protein expression and phosphorylation as described in detail elsewhere. In brief, freshly excised tissue samples were immersed in liquid nitrogen and stored at −70°C. Membrane protein fraction was prepared, and the protein concentration was determined by the method of Bredford. From each sample 10 μg protein was separated on 10% polyacrylamide gels, and transferred to PVDF membranes. The blots were blocked with 5% non-fat milk, dissolved in TBST for 1 h, and labelled overnight with a rabbit polyclonal anti-Cx43 antibody, and then incubated for 1 h with HRP-conjugated anti-rabbit goat secondary antibody. Blots were visualized with the ECL Plus kit using x-ray film. Band intensities were determined by the ImageJ software, and the relative amount of

phosphorylated and dephosphorylated Cx43 isoforms were expressed as a percentage of the total sarcolemmal connexin content.

Determination of gap junctional metabolic coupling

Gap junctional metabolic coupling was evaluated by measuring tissue permeability using the 'double-dye loading' method, described previously. In brief, freshly excised transmural tissue blocks from both the ischaemic and non-ischaemic ventricular walls were submerged in a mixture of LY and TRITC–dextran, fixed in paraformaldehyde and cryosections prepared.. The ratio of LY and TD stained areas was calculated using the ImageJ software. GJ permeability within the ischaemic (LAD) area was expressed as a percentage of permeability measured within the non-ischaemic (LCX) wall region.

Experimental protocols

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

Under light pentobarbital anaesthesia 24 dogs were subjected to rapid right ventricular pacing (4 x 5 min, at a rate of 240 beats min^{-1}) and, 24 hours later, to a 25 min occlusion of the LAD. Ten paced dogs remained without treatment (PO group), whereas in 14 paced dogs CBX, an inhibitor of GJ opening, was infused locally into a small branch of the LAD in doses of 50 μmol L^{-1} (n = 7) and in 100 μmol L^{-1} (n = 7), starting the infusion 20 min prior to, and maintained over the entire occlusion period. Another 10 dogs were instrumented but not paced, only subjected to a 25 min occlusion (SPO group). These dogs served as controls. At the end of the occlusion period the hearts were stopped by an excess of anaesthetic and myocardial tissue samples were taken from both the LAD and LCX areas for further biochemical analyses.

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

In this series of experiments a total of 42 dogs were used and randomly divided into three groups. Each animal was subjected to a 25 min LAD occlusion followed by rapid reperfusion. Control dogs (n = 15) were infused with saline, commencing 10 min prior to the end of the coronary artery occlusion. In the other two groups $NaNO_2$ was administered by intravenous infusion at a concentration of 0.2 μmol kg^{-1} min^{-1} either 10 min prior to and throughout the occlusion (n = 14) or 10 min prior to reperfusion (n = 13), as has been described previously. In dogs that survived the combined ischaemia and reperfusion insult, the hearts were stopped with an overdose of the anaesthetic either within 2 min (where myocardial tissue samples

were taken) or 5 min (where the area at risk was determined). In dogs that suddenly fibrillated on reperfusion, the heart was quickly excised when the fibrillation had been observed. In 7 control and 7 nitrite treated dogs, after excising the heart, the risk area was assessed by injecting Patent Blue V dye into the re-occluded artery using the same method that has been described in detail elsewhere.

RESULTS

I. Evidence for the involvement of gap junctions in the delayed antiarrhythmic effect of cardiac pacing

Haemodynamic effects of carbenoxolone and of coronary artery occlusion

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

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

Compared with the sham-paced dogs, in dogs subjected to cardiac pacing 24h previously, the number of VPBs, the number of episodes of VT, and the incidences of VT and VF were markedly reduced during coronary artery occlusion. The infusion of CBX in both concentrations (50 and 100 μmol L^{-1}) attenuated or even abolished the protective effect of pacing against arrhythmias.

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

In the non-paced control dogs occlusion of the LAD resulted in marked changes in tissue electrical resistance that occurred in two phases. There was a marked increase in resistivity immediately after the commencement of the coronary artery occlusion, and then, a second steep rise could be observed around the 15 min of the occlusion. In parallel, the ectopic activity occurred also in two phases; there was an increase in the number of VPBs between 3 and 10 min (peaked around 5 min) of the occlusion that is called as phase 1a arrhythmias, which was followed by a second arrhythmia burst, termed as phase 1b, occurring between 16 and 25 min (peaked around 18 min) of the ischaemia. Both the resistivity changes and the number of VPBs over the entire occlusion period were significantly less in dogs that had been

subjected to cardiac pacing 24h before the coronary artery occlusion. In the presence of both doses of CBX the resistivity changes were similar to that occurred in the non-paced controls and the number of VPBs was also significantly increased. Interestingly, CBX in 50 μmol L^{-1} concentration ($PO+CBX_{50}$ group) increased the number of VPBs during phase 1b, whereas in 100 μmol L^{-1} concentration ($PO+CBX_{100}$ group) the number of ectopic beats was significantly increased during both phase 1a and phase 1b.

Changes in ischaemia severity during coronary artery occlusion

The severity of ischaemia was assessed by two indices; i.e. changes in the epicardial ST-segment and in the TAT, recorded from the epicardial surface of the left ventricular wall, supplied by the occluded LAD. In the non-paced dogs, occlusion of the LAD resulted in immediate and marked increase in both epicardial ST-segment and TAT. These ischaemia-induced electrophysiological changes were significantly less marked in dogs subjected to cardiac pacing, 24h previously. In the presence of CBX these indices of ischaemia severity were again increased.

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

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

Changes in gap junctional permeability following cardiac pacing and CBX

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

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

The structural alterations occurring at the end of a 25 min occlusion of the LAD in non-paced and paced dogs, with and without CBX administration using a series of representative confocal immunofluorescence images obtained from tissue samples collected from both the

ischaemic (supplied by the LAD) and the non-ischaemic (supplied by the LCX) myocardial wall. In the non-paced controls (SPO), occlusion of the LAD resulted in, within its supplied region, signs of structural impairment of the intercalated discs; i.e. the contour of the end-to-end connections became "blurred" compared with those images which were obtained in samples taken from the non-ischaemic (LCX) myocardial region. Cardiac pacing, 24h previously, prevented these ischaemia-induced structural changes, and there were no visible differences between the ischaemic and the normal areas. Carbenoxolone, given in 50 and 100 μmol L^{-1} concentrations to paced dogs prior to and during the occlusion, resulted in again perceivable alterations (dim membrane density, 'blurred' contour) around the disc areas, but, interestingly, these changes occurred in both areas, supplied either by the occluded LAD or the patent LCX arteries, suggesting that CBX itself may cause re-arrangements in the structure of GJs.

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

Haemodynamic effects of sodium nitrite and coronary artery occlusion

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

The severity of ventricular arrhythmias during coronary artery occlusion and reperfusion

In control dogs, occlusion of the LAD resulted in a high number of VPBs and episodes of VT. In this group 38% of the dogs fibrillated during occlusion and no control dog survived reperfusion. Compared to these controls, the intravenous administration of $NaNO_2$, prior to and during the occlusion ($NaNO_2$-PO group) significantly reduced these arrhythmias during occlusion, and increased survival. Similarly, when $NaNO_2$ was administered 10 min prior to reperfusion ($NaNO_2$-PR), the number and incidence of arrhythmias were significantly reduced.

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

In control dogs occlusion of the LAD resulted in an immediate increase in tissue resistivity and a decrease in phase angle accompanied by the occurrence of many ectopic beats. These changes were apparent during the first 10 min of ischaemia (phase 1a), after which the impedance changes became slower and the number of VPBs was significantly decreased. A second steeper rise in resistivity and decline in phase angle started around 13 min of the occlusion, followed immediately by the re-appearance of the phase 1b arrhythmias. In dogs that had received $NaNO_2$ prior to and during the occlusion, the steep impedance changes were markedly reduced, and in parallel, the ectopic activity over the entire occlusion period was attenuated. In dogs, in which $NaNO_2$ was infused 10 min prior to reperfusion, the ischaemia-induced impedance changes, as well as the ectopic activity, were reduced only during the second phase of the occlusion (i.e. almost immediately after the commencement of the nitrite infusion), indicating a rapid effect of $NaNO_2$ on GJs.

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

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

DISCUSSION

New findings

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

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

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

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

parallel with the alterations in electrical impedance, an indirect measure of gap junction function.

We have found that CBX infused in paced dogs 24 h after the pacing stimulus, but prior to and during the entire occlusion period, attenuated the antiarrhythmic effect of cardiac pacing. In the presence of CBX there was a marked increase in the number and severity of arrhythmias resulted from a 25 min occlusion of the LAD. This effect was dose- dependent; CBX in a concentration of 100 μmol L^{-1} increased, interestingly, not only the phase 1b, but the phase 1a arrhythmias as well. This result suggests that CBX in higher concentrations may not selective for gap junctions. We have evidence that CBX in a concentration of 50 μmol L^{-1} does not influence the sodium, potassium and calcium channels, however, the higher concentrations (100 μmol L^{-1}) of CBX has been found to increase the calcium oscillations during diastole, resulting in delayed after-depolarisations and the subsequent generation of ventricular arrhythmias. In order to clarify this question, in some *in vitro* experiments, using canine right ventricular papillary muscle preparations, we have determined the dose-dependent effects of CBX on the maximum upstroke velocity (dV_{max}), and on the duration of the action potential (APD). The results suggest that the higher concentration of CBX may modify the ion channels involved in impulse generation and conduction, and in the development of phase 1a arrhythmias.

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

We have found that the administration of CBX increased the dephospho ratio of Cx43 compared with the paced dogs, but it did not completely abolish the effect of pacing on Cx43 phosphorylation. This result suggests that the gap junction uncoupling effect of CBX is, perhaps, not entirely due to a change in Cx43 phosphorylation.

Carbenoxolone, however, almost completely abolished the pacing-induced preservation of gap junction permeability, independently from the doses applied. Furthermore, CBX resulted in substantial structural alterations in the intercalated discs (ID).

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

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

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

We have found that sodium nitrite administered either before and during coronary artery occlusion the steep increase in resistivity and the decline in phase angle that usually occur around the 14-15 min of ischaemia in the control animals were abrogated and the number of ectopic beats during phase 1b was markedly suppressed. There were also significant

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

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

Second, the short period of nitrite administration during ischaemia but prior to reperfusion can effectively increase NO bioavailability resulting in significant reduction in the ischaemic changes and its severe consequences, such as the occurrence of the life-threatening ventricular arrhythmias. The increased NO bioavailability resulting from a short period of sodium nitrite infusion prior to reperfusion, may favourably affect the reperfusion-induced severe ventricular arrhythmias, such as ventricular fibrillation, which is common in this canine model when the coronary artery is suddenly reopened. We have found that only one nitrite-treated dog out of the remaining 12 dogs fibrillated during reperfusion in contrast with the controls, in which all the dogs that survived the occlusion died in VF on reperfusion.

Third, the results of the present study confirm our hypothesis that NO, among its several other effects, may influence cardiac gap junctions and thereby modify the generation of the ischaemia and reperfusion-induced severe ventricular arrhythmias. The effect of NO derived from sodium nitrite on gap junction channels is also immediate, since after the commencement of the infusion the second steep rise in resistivity and decline in phase angle were absent, and the number of VPBs during phase 1b was considerable suppressed. This finding suggests that the infusion sodium nitrite just at a time of the occlusion period, when the serious, often life-threatening phase 1b arrhythmias start to appear, is able to inhibit the generation of these arrhythmias, and this effect can be associated with the preservation of gap junction by NO. Although it is still not known whether opening or closing of gap junctions by NO leads to the antiarrhythmic protection, our electrical impedance measurements suggests, by providing indirect evidence that $NaNO_2$ inhibits the further uncoupling of gap junctions, since it abolished those steep impedance changes that had preceded the occurrence of the phase 1b arrhythmias.

An interesting, and perhaps, clinically useful aspect of the rapid release of NO from nitrite during ischaemia the marked protection against the reperfusion-induced severe ventricular arrhythmias. The reduction in VF during reperfusion is particularly pronounced, resulting in a high rate of survival (85%) in the nitrite treated group. As to whether the attenuation of the reperfusion-induced VF in the nitrite infused dogs can be associated with a direct effect of NO on gap junctions during reperfusion we do not know, since the impedance changes is difficult to assess during reperfusion, particularly in control dogs, where VF commonly occurs soon after the reperfusion. Since, it is more than likely that the outcome of reperfusion, and thus the severity of reperfusion-induced arrhythmias, largely depends on the severity of the ischaemia, we may assume that the nitrite-induced less severe ischaemic changes and the suppression of arrhythmias during phase 1b, via the modulation of gap junctions, may contribute to the better survival of these animals from the combined ischaemia and reperfusion insult.

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

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