Cardioprotection with inorganic nitrites; potential mechanisms with particular reference to changes in mitochondrial morphology and function

Summary of the PhD Thesis

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LIST OF PUBLICATIONS

Full papers related to the Thesis


Other studies


IV. Juhasz Laszlo, Haludka Vivien Demeter, Seprenyi Gyorgy, Kaszaki Jozsef, Gardi Janos, Vegh Agnes Acute inhibition of monoamine oxidase with pargyline does not modify the severity of ischemia and reperfusion-induced ventricular arrhythmias in dogs EXPERIMENTAL AND CLINICAL CARDIOLOGY 19:(1) pp. 1-7.-2013


LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-NT</td>
<td>3-nitrotyrosine</td>
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<tr>
<td>ABP</td>
<td>Arterial blood pressure</td>
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<tr>
<td>CI</td>
<td>Mitochondrial respiratory chain complex I</td>
</tr>
<tr>
<td>CII</td>
<td>Mitochondrial respiratory chain complex II</td>
</tr>
<tr>
<td>CytC</td>
<td>Cytochrome c</td>
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<tr>
<td>DABP</td>
<td>Diastolic arterial blood pressure</td>
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<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
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<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<tr>
<td>ETS</td>
<td>Electron transport system</td>
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<tr>
<td>FCCP</td>
<td>Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone</td>
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<tr>
<td>HR</td>
<td>Heart rate</td>
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<tr>
<td>I/R</td>
<td>Ischaemia and reperfusion</td>
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<tr>
<td>IMF</td>
<td>Inter-myofibrilar</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>LAD</td>
<td>Left anterior descending coronary artery</td>
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<tr>
<td>LVEDP</td>
<td>Left ventricular end-diastolic pressure</td>
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<tr>
<td>LVSP</td>
<td>Left ventricular systolic pressure</td>
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<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition pore</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
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<tr>
<td>PN</td>
<td>Perinuclear</td>
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<tr>
<td>RCR</td>
<td>Respiratory control ratio</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SNO</td>
<td>S-nitrosylation</td>
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<tr>
<td>SSM</td>
<td>Sub-sarcolemmal</td>
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<tr>
<td>VF</td>
<td>Ventricular fibrillation</td>
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<tr>
<td>VPBs</td>
<td>Ventricular premature beats</td>
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<tr>
<td>VT</td>
<td>Ventricular tachycardia</td>
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INTRODUCTION

Coronary artery disease and its consequences, such as the occurrence of life-threatening ventricular tachyarrhythmias and sudden cardiac death are one of the main causes of mortality worldwide (Finegold et al., 2013), and compared to the world statistics, there is no substantial difference in the statistics in Hungary (IHME et al., 2018).

Organic nitrates and nitrates were the first drugs that had been introduced in the treatment of angina pectoris and their protective effects have been described in various experimental settings. These drugs, by releasing nitric oxide (NO), are able to increase NO bioavailability even under ischaemic conditions and protect the heart against the consequences of ischaemic injury, including the generation of severe ventricular arrhythmias.

An important discovery in nitrite biology was the recognition that inorganic nitrites and nitrates, the end-products of nitrite metabolism, which had been considered as biologically inert molecules, can also form NO (Lefer et al., 2006). It has been recognised that these natural oxidative metabolites of NO, are able to readily reduce back to NO, particularly in a reductive milieu, such as hypoxia and ischaemia. It is proposed that they can serve as stores of NO under ischaemic conditions, when the synthesis by nitric oxide synthase (NOS) enzymes has become limited, would provide NO and increase NO bioavailability in the ischaemic myocardium (Zweier et al., 1995).

In experimental studies, the most frequently used nitrite is the sodium nitrite (NaNO₂), which has been proved to possess cardioprotective effects in very low concentration range (Dejam et al., 2004). We have previous evidence that the infusion of sodium nitrite (NaNO₂) in low (micromolar) concentrations, prior to coronary artery occlusion, or just prior to reperfusion in anaesthetized dogs, results in marked protection against the I/R-induced arrhythmias (Kovács et al., 2015). Thus, the number of ventricular premature beats (VPBs), the number of episodes and the incidence of ventricular tachycardia (VT), as well as the incidence of ventricular fibrillation (VF) during occlusion were significantly decreased, and the survival rate in dogs infused with sodium nitrite 10 min prior to reperfusion was 92% compared to 0% in the controls. We have also shown that in this protection S-nitrosylation and/or glutathionylation of proteins may play a role (Kovács et al., 2015).

The aim of the experiments was to examine, whether sodium nitrite exerts a similar antiarrhythmic effect, when it is administered 24h before ischaemia and reperfusion. We have also examined the potential mechanisms, involved in this delayed antiarrhythmic protection.

As it is mentioned above, there is substantial evidence for the key role of NO both in the early and the late cardioprotection (Végh et al., 1992b, Végh and Parratt, 1996; Bolli et al., 1997). It is well accepted that in the delayed protection, the PC stimulus-induced eNOS activation resulted NO formation, and the subsequent increased NO-stimulated iNOS activation (Bolli et al., 1997, Kovács et al., 2013), which can further increase NO bioavailability during ischaemia, may play a mandatory role. Thus, we have examined whether changes in the inducible nitric oxide (iNOS) activity would play a major role in the late cardioprotective effect of NaNO₂.
The cardiomyocytes are abundant in mitochondria (approximately 30% of the total cell volume), which are responsible for vital functions such as ATP production. Several studies have been examined the role of mitochondria in the generation of arrhythmias. Two main underlying mechanisms are responsible for the generation of arrhythmias regarding the mitochondria: the sudden increase of reactive oxygen radicals (ROS) production and the changes in calcium handling. In the first few minutes of reperfusion a burst of ROS can be observed which is able to provoke arrhythmias (Manning et al., 1988). The attempts which targeted the suppression of this ROS burst or the use of mitochondria specific antioxidants are proven to be antiarrhythmic (Kónya et al., 1992; Cho et al., 2007).

There is little previous evidence that sodium nitrite results in delayed cardioprotection in various experimental models (Shiva et al., 2007a). These studies suggested that nitrite, following its administration nitrosylates mitochondrial proteins, which remains stable during the next 24h. This S-nitrosylation (SNO) process then preserves mitochondrial function, when the heart is subjected to ischaemia (Shiva et al., 1997a,b). Since SNO involves respiratory complexes of the mitochondria, responsible for free radical formation, the proposed mechanism of protection is the limitation of ROS production during I/R, by nitrite (Shiva et al., 2007a). We have examined whether alterations in mitochondrial morphology and function would play a major role in the late cardioprotective effect of NaNO₂.

AIMS

I. Examination of the potential delayed antiarrhythmic effect of sodium nitrite against ischaemia and reperfusion-induced severe ventricular arrhythmias in anaesthetized dogs. If nitrite induces delayed cardioprotection, it was obvious to examine, whether the mechanism involves the NO/iNOS/NO pathway, as had been seen with PC.

Therefore, in this series of experiments in anaesthetizes dogs, sodium nitrite was infused 24h before a coronary artery occlusion and reperfusion in a concentration of 0.2 µmol kg⁻¹ min⁻¹ (this concentration does not significantly modify coronary blood flow and blood pressure) over a period of 20 min. 24 hours later the left anterior descending coronary artery (LAD) was occluded for 25 min, followed by sudden reperfusion. In order to assess the role of iNOS activation in the effect of nitrite, the inducible nitric oxide synthase (iNOS) inhibitor S-(2-aminoethyl)-isothiourea (iv., AEST, 2.0 mg kg⁻¹) was given prior to the infusion of nitrate and, again, before the coronary artery occlusion. The severity of ventricular arrhythmias and of ischaemia was assessed. We also determined the activity of iNOS enzyme and the plasma concentrations of nitrite/nitrate (NOₓ).

II. Examination of the role of mitochondria in the delayed antiarrhythmic effect of sodium nitrite. In this series of experiments, we have attempted to examine, whether 24h after the administration of sodium nitrite changes can be detected in mitochondrial structure and function in the absence and presence of I/R.

Thus, in these experiments we have analysed the effect of NaNO₂ on those structural and functional (mainly respiratory) alterations of the mitochondria that occur following a 25 min period of I/R. Therefore, 24h after the administration of NaNO₂ (0.2 µmol kg⁻¹ min⁻¹) the hearts were either stopped and removed, or the animals were subjected to a 25 min occlusion and 2 min reperfusion insult, after which the hearts were also stopped, and myocardial tissue samples were
taken. Changes in mitochondrial morphology and in various respiratory parameters (e.g., oxidative phosphorylation, respiratory control and P/E ratios), as well as in the rate of ATP production, superoxide and peroxynitrite productions were assessed.

MATERIALS AND METHODS

3.1 Ethics

All the experiments were carried out in accordance with the Hungarian law 40/2013 (II. 14.). Approval number: I-74-5-2012 (I.) and 4657/2016 (II.).

3.2 Animals

In the first study, 33 adult mongrel dogs of either sex were used. The mean body weight of the dogs was: 21±4 kg. In the second study, 30 adult dogs of both sexes with a mean body weight of 22±4 kg were used.

3.3 Surgical interventions and in vivo measurements

The surgical interventions were carried out as described previously (Végh et al., 1992; Végh et al. 1994). On day one, the dogs were lightly anaesthetized with sodium pentobarbitone (30 mg kg⁻¹, i.v., Euthasol 40%, Produlab Pharma B.V., Netherlands). A polyethylene catheter was introduced into the jugular vein for the injection of the drugs (saline, NaNO2 or AEST). Twenty-four hours later (day 2) the dogs were re-anaesthetized with a bolus injection of sodium pentobarbitone (30 mg kg⁻¹, i.v.; Euthasol 40%, Produlab Pharma B.V., Netherlands) and the anaesthesia was maintained with the mixture of α-chloralose and urethane (60 and 200 mg kg⁻¹, i.v.; Sigma, USA). Arterial blood pressure (ABP), left ventricular (LV) systolic (LVS) and end-diastolic pressure (LVEDP), as well as the LV positive and negative dP/dt max were measured using a Miller tip catheter (5F, Millar Instruments Inc., USA). A thoracotomy was performed in the fifth intercostal space. The pericardium was opened, and the heart was explored. The anterior descending branch of the left coronary artery (LAD) was prepared proximal to the first diagonal branch. Myocardial ischaemia was induced by a 25 min occlusion of the LAD, followed by a 2 min period of reperfusion (Végh et al., 1992). At the end of the experiments the animals were euthanized with an excess dose of sodium pentobarbitone. The severity of ischaemia was assessed by measuring changes in the epicardial ST-segment (mV) and in the degree of inhomogeneity of electrical activation (ms) using a composite electrode. Ventricular arrhythmias and the heart rate (HR) were assessed from chest lead II electrocardiogram. The arrhythmias were evaluated according to the Lambeth Conventions (Walker et al., 1998; Végh et al., 1992). The dogs were considered survivors, when sinus rhythm was shown on the ECG two minutes after reperfusion. All parameters were recorded on a Plugsys Hemodynamic Apparatus (Hugo Sachs Electronik, Germany) and evaluated by LabChart 7 software (AD Instruments, Australia).

3.4 Determination of the area at risk

The risk area was measured as described previously (Végh et al., 1992).
3.5 *In vitro* measurements

3.5.1 Determination of iNOS enzyme activity

The assessment of iNOS enzyme activity was performed using the radio immunoassay method.

3.5.2. Determination of plasma nitrite/nitrate (NO<sub>x</sub>) concentrations

Plasma NO<sub>x</sub> levels were determined by Griess reaction as described previously (Kiss et al., 2010; Kisvári et al., 2014).

3.5.3. Assessment of changes in mitochondrial morphology

Three different regions: sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and perinuclear (PN) were captured by transmission electron microscopy. Changes in the area (µm<sup>2</sup>), perimeter (µm), Feret diameter (µm) and roundness (0-1) ([4x[Area]/(πx[Major axis]<sup>2</sup>)) were calculated. Five images were evaluated and averaged per dog and within a certain group also averaged. This average was used for the comparison among the groups.

3.5.4. Assessment of the mitochondrial respiration

The respiration of freshly isolated mitochondria was measured by a Clarke-type oxygen electrode. The mitochondrial respiratory measurements were carried out according to the SUIT protocol (Gnaiger, 2014). Mitochondrial complex I (CI) and complex II (CII) respiration were induced by either glutamate (final concentration: 10 mM) and malate (CI, 1 mM) or rotenone (0.5 µM) and succinate (CII, 10 mM). The following were measured to assess the different states: oxidative phosphorylation: ADP (State 3, 5 mM); State 4: oligomycin (Omy, 2.5 µM, Sigma, USA); electron transport system (ETS): carbonyl-cyanide-p-(trifluoro-methoxy) phenyl-hydrazone (FCCP, 0.5 µM, Sigma, USA); residual oxygen consumption: Antimycin A (5 µM). From the measured parameters respiratory control ratio (RCR=OXPHOS/State 4) and P/E (OXPHOS/ETS) ratios were calculated.

3.5.5. Measurement of ATP production

The mitochondrial ATP production was measured by a bioluminescent assay (ATP Determination Kit, Invitrogen, USA) according to the manufacturer’s suggestion. The values were expressed as relative luminescence unit (RLU). Three parallel measurements were averaged per dogs and then averaged within a group. These means were used to compare among groups.

3.5.6. Determination of the superoxide levels

This was performed by confocal microscopy using the dihydroethidine (DHE) dye method (1 µM DHE (Sigma, USA)). Ten pictures per dog were captured. The intensity of randomly chosen four pictures was averaged, and data obtained from dogs within a group were also averaged.
3.5.7. Assessment of the peroxynitrite formation

Peroxynitrite production was measured by assessing 3-nitrotyrosine (3-NT) production using Western blot. Left ventricular samples were excised, snap frozen in liquid nitrogen, and stored at -80 °C. Total protein extracts (25 µg) were resolved using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking in 5% milk-TBS-T, the membranes were immunolabeled with the mouse monoclonal anti-nitrotyrosine (3-NT), as the primary antibody (Chemicon, Millipore, USA; overnight, at 4 °C; dilution: 1:3000). Horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dakocytomation, Denmark; 1h, room temperature, 1:1000) was used as the secondary antibody. The blots were developed with an ECL kit (Western Bright ECL, Advansta, USA) and exposed to X-ray film and scanned. Equal loading was provided by determining the protein concentration by Bradford protein assay and was verified by Coomassie Brilliant Blue staining and the samples were normalized to total protein. Parallel western blots were used for the statistical analysis using Welch-ANOVA and Bonferroni-Holm post hoc tests. Integrated optical density values (sum of each band corrected to the background) was assessed using Image J (Fiji; NIH, Bethesda, MD).

3.6 Statistical Analysis

Data were expressed as mean ± standard error of mean. Kruskal-Wallis test was used to compare the differences between the means, regarding the number of VPBs and number of episodes of VT. The incidence of VT, VF and survival were compared using Fisher exact test. For the statistical analysis one-way ANOVA/Bonferroni post hoc tests and Welch-ANOVA/Bonferroni-Holm post-hoc tests were used. Differences between groups were considered significant at P<0.05.

3.7 Experimental Protocol

Protocol I.

Thirty-three dogs were divided into three groups. In the control group (n=12) the dogs were infused with saline (1 ml min⁻¹), whereas in the treated group (n=21) NaNO₂ (Merck, USA) was administered in a concentration of 0.2 µmol kg⁻¹ min⁻¹ for 20 minutes. In nine dogs out of the twenty-one NaNO₂-treated animals, the iNOS enzyme inhibitor S-(2-aminoethyl)-isothiourea (AEST, 2.0 mg kg⁻¹) was given over a 5 min period, 5 min prior to the administration of NaNO₂. AEST was given again, 24h later, over a period of 30 min, just prior to the coronary artery occlusion. In all groups, 24h after saline or drug administration the dogs were subjected to a 25 min of LAD occlusion, followed by 2 min reperfusion interval. Three dogs served as sham-operated controls (SO group); these dogs underwent the same surgical intervention as the other groups.
Protocol II.

In this study, four groups of anaesthetized dogs were used. In two groups out of the four groups, each containing 5 animals, either saline (SC group) or sodium nitrite (NaNO₂ group, 0.2 µmol kg⁻¹ min⁻¹; Merck) were administered intravenously over a period of 20 min. From these dogs, heart samples were taken 24h later without subjecting them to I/R. Another two groups of dogs (IC, n=5; NaNO₂+I/R, n=5), 24h after the administration of saline or NaNO₂ the animals were subjected to a 25 min occlusion and 2 min reperfusion. In these dogs, myocardial tissue samples were taken 2 min after reperfusion for in vitro analyses.

RESULTS

4.1. Examination of the delayed antiarrhythmic effects of sodium nitrite; the role of iNOS activation (Study I)

4.1.1. Haemodynamic effects of saline, NaNO₂, AEST, NaNO₂+AEST and coronary artery occlusion

Compared to the saline treated controls, the intravenous infusion of NaNO₂ reduced the arterial blood pressure and slightly increased the heart rate. AEST itself, administered either on day one or day two, did not significantly modify the haemodynamic parameters, but when it was given in NaNO₂ treated dogs, AEST attenuated the haemodynamic effects of NaNO₂. Occlusion of the LAD resulted in significant reductions in the arterial blood pressure, LVSP, positive and negative dP/dt max and an increase in LVEDP in all the examined groups, whereas the HR remained virtually unchanged. These ischaemia-induced haemodynamic changes were less marked in dogs infused with NaNO₂ 24h previously. The administration of AEST in the NaNO₂ treated dogs did not modify the occlusion-induced haemodynamic changes compared to the controls or the NaNO₂ treated dogs.

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

In control dogs (infused with saline) a 25 min coronary artery occlusion resulted in a high number of VPBs (379 ± 89), VT episodes (11.2 ± 3.2) and incidence of VT (100%) and VF (40%) during occlusion. Furthermore, all the remaining dogs fibrillated on reperfusion, thus in this group no dog survived the combined the occlusion and reperfusion insult. The administration of NaNO₂ significantly decreased the severity of the ischaemia-induced ventricular arrhythmias (VPBs: 47 ± 15; VT: 0.2 ± 0.2; VT%: 22%; VF%: 0%), and increased survival (0% in the controls vs. 50% in the nitrite treated group). When AEST was administered in the NaNO₂ treated animals, the NaNO₂-induced protection against the arrhythmias significantly attenuated, but it did not completely abolish. Thus, in the presence of AEST the number of VPBs (170 ± 43) and VT episodes (3.7 ± 1.1), as well as the incidence of VT (67%) during occlusion were again
significantly increased, whereas the incidence of the occlusion-induced VF (11%) and survival (11%) did not significantly differ from the nitrite treated dogs.

4.1.3. Changes in the severity of ischaemia during a 25 min occlusion of the LAD

In control dogs following the occlusion of the LAD both the epicardial ST-segment and the degree of inhomogeneity of electrical activation rapidly increased and reached their maximum at around the 5 min of the ischaemia. In contrast in dogs infused with NaNO₂, 24h previously, these ischaemic changes were significantly attenuated; i.e. both the elevation of epicardial ST-segment and the degree of inhomogeneity were less marked than in the controls. The administration of AEST almost completely abolished the anti-ischaemic effects of NaNO₂.

4.1.4. The effect of NaNO₂ on the area at risk

There were no significant differences in the area at risk among the groups. The area at risk was 36±3% in the controls, 37±3% in the NaNO₂, and 35±4% in the AEST+NaNO₂ treated groups.

4.1.5. Changes in plasma nitrate/nitrite (NOₓ) levels

These were assessed at 4 time points during the experiments. Twenty minutes after the infusion of NaNO₂ the NOₓ levels were significantly increased, and still remained elevated 24h later. Interestingly both in the NaNO₂ and the AEST+NaNO₂ groups this increase in the NOₓ resulted primarily from the marked elevation in the nitrate levels (the concentration of nitrite in all groups was almost the same as the baseline concentrations of nitrite on day one). During occlusion the NOₓ levels were significantly higher in the nitrite treated dogs than in the controls, and this effect of nitrite was not substantially modified by the administration of AEST.

4.1.6. The effect of nitrite on iNOS activity

Compared to the sham-operated controls, a 25 min ischaemia resulted in no significant changes in iNOS activity. However, the administration of NaNO₂ caused an increase in iNOS activation, but this change proved statistically not significant. AEST completely abolished the NaNO₂-induced activation of iNOS.

4.2 Examination of the role of mitochondria in the delayed cardioprotective effect of sodium nitrite (Study II)

4.2.1. Alterations in the mitochondrial morphology following I/R and nitrite administration

The analysis of TEM images showed that compared to the SC dogs, in dogs of the IC group a substantial swelling, a greater distance between the contractile units and disorganization of cristae of the mitochondrial matrix occurred. These alterations were less marked with NaNO₂. Furthermore, data obtained following the quantitative analysis of mitochondria localized in the sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and perinuclear (PN) areas showed that compared to the SC dogs, a 25 min I/R resulted in a significant reduction in the mitochondrial area, perimeter and Feret diameter, and a significant increase in mitochondrial
roundness. These alterations were significantly less marked in the nitrite treated dogs. Sodium nitrite itself did not significantly change the morphological parameters.

4.2.2. Changes in mitochondrial respiration following I/R and nitrite administration

Mitochondrial respiration was assessed by various measured (State 2, OXPHOS, State 4, ETS) and calculated (RCR and P/E) CI and CII-dependent respiratory parameters. There were no significant differences in the basal (State 2) respiration among the groups. However, following a 25 min I/R insult the CI-dependent OXPHOS, ETS and the RCR were markedly decreased compared with the SC dogs, whereas the P/E control coupling ratio was almost identical in the SC and in the IC dogs. These changes were less marked in case of the CII-dependent respiration. Compared to the SC dogs, nitrite alone significantly reduced the CI-dependent OXPHOS, ETS and RCR, without substantially modifying State 4 and the P/E coupling ratio. Moreover, in dogs infused with NaNO₂ and then subjected to I/R, further significant decreases occurred both in the CI and CII-dependent OXPHOS and RCR, as well as an increase in State 4, compared with the IC dogs. Since, in the NaNO₂+I/R group the OXPHOS was markedly reduced, but the ETS was unchanged compared with the IC group, the calculated P/E ratio in the nitrite treated dogs was markedly reduced. This result indicates that under ischaemic conditions, nitrite limits OXPHOS by changing the phosphorylation system.

4.2.3. The effect of sodium nitrite on the mitochondrial ATP production

Changes in the rate of ATP production are expressed in RLU (over 30 sec/mg protein). Compared with the SC group, a 25 min period of I/R significantly decreased the mitochondrial ATP production (12232 ± 1291 cp. 7213 ± 1117 RLU/30s/mg protein). The administration of nitrite alone (13001 ± 3109 RLU/30s/mg protein vs. SC group) and under ischaemic conditions (7130 ± 1560 RLU/30s/mg protein vs. IC group) did not significantly modify the rate of ATP production.

4.2.4. The effect of NaNO₂ on the superoxide and peroxynitrite production during reperfusion

Compared to the SC dogs, the generation of superoxide was markedly increased in the IC dogs. This I/R-induced increase in the superoxide production was attenuated by the administration of nitrite. Similarly, compared to the SC dogs, a 25 min occlusion and reperfusion resulted in a significant increase in peroxynitrite production. This increase in 3-NT formation was markedly reduced in the NaNO₂ treated dogs.
DISCUSSION

The aims of the presented studies were to examine the potential delayed antiarrhythmic effect of the inorganic sodium nitrite, and to explore mechanism(s) that might be involved in this protection.

We have now evidence that sodium nitrite can evoke a marked delayed antiarrhythmic effect, in a large animal model of I/R. Thus, compared with the untreated controls, if dogs had infused with sodium nitrite 24h before a 25 min occlusion and reperfusion insult, the number of VPBs, the number and incidence of VT, the incidence of VF during occlusion were markedly reduced. Furthermore, in contrast to the ischaemic control group, in which no dog survived reperfusion, in the nitrite treated group 50% of the dogs survived the combined I/R insult. Similarly, the infusion of nitrite significantly attenuated the ischaemic changes, assessed by measuring epicardial ST-segment and the degree of inhomogeneity of electrical activation.

It seems that it is unlikely that the delayed antiarrhythmic effect of sodium nitrite would be identical with the late protective effect of preconditioning. The evidence for this comes from the experiments, in which we used the partially selective iNOS enzyme inhibitor AEST. We have found that in contrast to preconditioning, where the administration of AEST completely abolished the antiarrhythmic effect of PC (Kis et al., 1999b), the nitrite-induced protection against arrhythmias was only partially diminished by AEST. Thus, in the presence of AEST there was an increase in the number of ectopic beats and of episodes of VT during occlusion, but these were still significantly less than in the controls. Furthermore, AEST did not substantially modify the protective effect of nitrite against the occlusion-induced VF, but it abolished the protection against the reperfusion-induced VF. AEST reversed the anti-ischaemic effects of nitrite.

Furthermore, the results of the measurement of iNOS activity showed that the infusion of nitrite only slightly increased the activity of iNOS, which also indicates that in contrast to PC, the stimulation of iNOS plays a less important role in the nitrite-induced protection. The fact that the nitrite-induced increase in iNOS activation was completely abolished in the presence of AEST; confirms the previous findings that AEST is able to effectively block the activation of iNOS, and subsequently the iNOS-derived generation of NO, to which we attribute a key role in the delayed cardioprotection (Végh et al., 1994; Kis et al., 1999b). It might well be that a difference between the PC and the nitrite-induced late protection can be associated with the difference, regarding the source of NO during ischaemia. Whereas in the preconditioning-induced delayed protection the iNOS-induced NO generation seems to have a mandatory role (Végh and Parratt, 1996; Bolli et al., 1997; Dawn and Bolli, 2002), since the inhibition of iNOS activation abolishes the protection (Végh et al., 1994; Kis et al., 1999a,b), in case of the nitrite evoked delayed protection the activation of iNOS to produce NO is probably less important, since after the complete inhibition of iNOS activity, the protection, at least against the occlusion-induced arrhythmias, is still present.

In order to examine the source of NO 24h after the administration of sodium nitrite, we have measured plasma nitrate and nitrite levels before and after the 20 min infusion of nitrite in the systemic blood, and again 24h later before and after a 25 min coronary artery occlusion in the blood of the coronary sinus. We have found that there were no significant differences among the groups in the baseline (pre-infusion) nitrate, nitrite and NOx levels. However, in dogs infused
with nitrite, irrespective of the presence of AEST, the concentration of NO metabolites were markedly increased by the end of the infusion period compared with the saline infused controls. Twenty-four hours later, when the level of these NO metabolites were assessed again, now in the blood of the coronary sinus, we have found that in all groups the nitrite levels were almost the same as the basal plasma nitrite concentrations, 24h previously, whereas the nitrate concentrations were highly elevated in the nitrite treated dogs compared with the controls. Thus, we concluded that soon after its administration, nitrite converts to nitrate, and over the next 24h nitrate circulate in the blood, since in the absence of food intake, the NOx is only affected by the renal function (Lauer et al., 2001). If at this time the dogs were subjected to coronary artery occlusion, probably nitrate was used, as a substrate, to produce NO. This is supported by the results that following the LAD occlusion, although the total nitrate/nitrite (NOx) concentrations were reduced in all groups compared to their corresponding pre-occlusion values, but in the nitrite treated dogs the NOx levels were significantly higher than in the untreated controls. Furthermore, the fact that the reduction of NOx in the nitrite infused animals resulted mainly from a decrease in nitrate concentrations, whereas the nitrite levels were rather increased, we may speculate that nitrate reduced to nitrite, and then possible to NO. In contrast, in the control dogs the marked reduction in NOx, determined at the end of the occlusion period, resulted from a significant decrease in both the nitrate and nitrite concentrations.

What we may propose as a mechanism from the abovementioned results is that under physiological conditions the majority of the infused nitrite converts to nitrate, and it is stored in this form over the next 24h. This is supported by the fact that the nitrate, but not the nitrite levels were markedly increased 24h after the infusion of nitrite. The results of the measurement of iNOS activity, however, suggest that a part of nitrite is most probably converted to NO, and this was sufficient to stimulate iNOS. The fact that AEST, although completely blocked the activity of iNOS, it did not modify the concentration of NO metabolites and the occlusion-induced arrhythmias suggests that iNOS has only a minor contribution to NO formation and the protection in the nitrite treated dogs. We propose that dogs treated with nitrite, use nitrate as a primary source of NO during ischaemia. This is converted back first to nitrite and then to NO, when reductive conditions attain, such as during coronary artery occlusion, when we could observe a marked reduction in the nitrate, and an increase in the nitrite concentrations.

In conclusion, the results of the present study provided evidence that, in contrast with preconditioning, the activation of iNOS does not play a mandatory role in the nitrite-induced delayed antiarrhythmic protection, since the blockade of iNOS activation only attenuated but not completely abolished the protection.

To explore mechanisms, which may also contribute to the marked delayed antiarrhythmic effect of sodium nitrite has prompted us to design studies in which the role of mitochondria in the delayed antiarrhythmic effect of nitrite has been examined.

There had been some previous evidence that the mitochondria play a central role in the late cardioprotection induced either by preconditioning or by nitrite administration (Shiva et al., 2007). One of the common mechanisms would be the NO regulated ROS formation (Kiss et al., 2010). For example, Shiva and colleagues (Shiva et al., 2007) proposed that NO derived from nitrite S-nitrosylates the mitochondrial respiratory complexes, mainly complex I (Couchani et
al., 2013) which has a significant role both in the acute and the delayed cardioprotective effect of nitrite. We have also evidence that protein S-nitrosylation and S-glutathionylation plays a role in the acute antiarrhythmic effect of nitrite (Kovács et al., 2015). It has been proposed that the redox-modification of the respiratory chain complexes by S-nitrosylation modifies the activity of the complexes, and thereby alter the ROS production (Dröse et al., 2014). Since in cardiac myocytes mitochondria CI and CIII are the main source of ROS production (Turrens et al., 2003), although, there is some evidence for the role of CII as well (Dröse et al., 2014), the modification of these complexes would certainly affect ROS formation, and thereby would be a part of the protective mechanisms. This hypothesis is supported that a decrease in CI and CII activity leads to an attenuated electron transfer to CIII and a subsequent reduction in the electron leakage and ROS production (Chen et al., 2006; Stewart et al., 2009).

Considering the abovementioned information, we have designed studies in order to examine whether in the nitrite-induced late antiarrhythmic effect changes in the I/R-induced mitochondrial structural and functional alterations would play a role.

We have found that the administration of nitrite prevented the I/R-induced morphological alterations of the mitochondria. Thus, the swelling, the change in the normal elongated shape of the mitochondria, the disorganized cristae, the large, empty blebs and the disruption of the membrane that had resulted from I/R, were significantly less marked in the nitrite-treated dogs. Also, the measured basic morphological parameters, such as the mitochondrial area, perimeter, Feret diameter and roundness, which had been substantially altered by I/R, were significantly less in the NaNO₂ treated animals. The best of our knowledge this is the first evidence that nitrite effects the I/R-induced structural changes of the mitochondria.

The question arises, whether the preservation of mitochondrial morphology would reflect in the mitochondrial function. We have found that nitrite depressed the CI (and in a smaller degree the CII)-dependent OXPHOS and had an effect on the members of the phosphorylation system. We measured the changes in mitochondrial respiration following I/R and nitrite administration. We have found that a 25 min ischaemia and 2 min reperfusion depressed mitochondrial respiration; i.e. both the CI and CII-dependent OXPHOS were significantly decreased, and there were also reductions in RCR (OXPHOS/state4) and in the ETS. Since, the P/E control coupling ratio was similar in the ischaemic and in the non-ischaemic control groups, we suppose that the reduced mitochondrial respiration resulted primary from the depression of the respiratory complexes (mainly CI) of the ETS.

Interestingly, nitrite alone reduced the mitochondrial respiration 24 h later, and this was even further decreased, when the nitrite-treated dogs had been subjected to ischaemia and reperfusion. Thus, in the nitrite treated dogs both the CI and CII-dependent OXPHOS, the RCR and the P/E coupling control ratio were significantly lower than in the ischaemic controls (IC). Furthermore, nitrite significantly reduced the superoxide and the 3-NT productions, resulted from a 25 min period of occlusion and reperfusion insult.

The results suggest that nitrite substantially modifies mitochondrial respiration. Moreover, the fact that nitrite decreased the P/E control coupling ratio raises the possibility that nitrite (NO) affects the phosphorylation system, and that the reduction in the CI-dependent OXPHOS would result from the modification of the phosphorylation system rather than of the proximal
complexes. Interestingly, despite the marked reduction in OXPHOS, the ATP production in the nitrite treated dogs was as the same as in the ischaemic, untreated controls. In contrast, the administration of nitrite significantly attenuated the ischaemia-induced increase in superoxide and 3-NT productions. This latter might be associated with the observation that the State 4 respiration was increased in the NaNO2+I/R dogs, indicating an increase in proton leakage in the inner membrane, which can result in a reduction in ROS production (Brand et al., 1999; Divakaruni and Brand, 2011).

There are, of course, a number of ways by which NO may regulate ROS formation. For example, NO inhibits the activities of xanthine/xanthine oxidase (Ichimori et al., 1999) and the NADPH oxidase (Fujii et al., 1997), which are the major sources of ROS production. The other potential source of ROS is the mitochondrial respiratory chain, especially in the heart, where the myocytes are abundant in mitochondria. Thus, the mitochondrial electron transport might become an important sub-cellular source of ROS, and a contributor to the reperfusion-induced injury (Ambrosio et al., 1993). There is evidence that NO reduces mitochondrial superoxide production by acting directly on the ETS or the uncoupling proteins (Burwell and Brookes, 2008), but the precise mechanisms are still not clarified. Recently, it has been suggested that the redox-modification of specific cysteine-thiol groups of proteins in the subunits of the respiratory chain complexes with S-nitrosylation influences the respiratory chain activity, and modifies ROS production (Dröse et al., 2014). Indeed, the reversible S-nitrosylation of CI was protective against myocardial I/R damage (Couchani et al., 2013). Although in the present study we did not measure protein SNO, our previous results have revealed that following acute administration (just prior to ischaemia or reperfusion) nitrite protects the myocardium by S-nitrosylation, and perhaps by glutathionylation (Kovács et al., 2015). As to whether in our model SNO may play a role in the delayed antiarrhythmic effect of nitrite warrants further investigations.

It seems well accepted that CI and, especially in cardiac myocytes, complex III (CIII) are the main sources of superoxide production (Turrens, 2003), but more recently, CII has also been considered as an important generator of ROS, under certain circumstances (Turrens, 2003; Dröse et al., 2014). The contribution of these sites for the overall ROS production depends on the organ, the milieu of substrates and redox conditions, as well as on the intactness of the respiratory chain activity (Turrens, 2003; Dröse et al., 2014). As the respiratory chain becomes reduced, such as during ischaemia and reperfusion or following a defect of mitochondrial complexes, electrons leak from the defective complex, resulting in the univalent reduction of oxygen to form superoxide. More recently, however, it is turned out that the inhibition of CI and CII activity attenuates the electron transfer to CIII, diminishes CIII reduction and decreases the electron leakage and the formation of ROS at CIII (Chen et al., 2003, 2006; Stewart et al., 2009), thereby protecting the myocardium against the reperfusion injury (Chen et al., 2006; Stewart et al., 2009).

Our proposal is that besides the involvement of the ROS producing complexes in the cardioprotective effects of nitrite, our results clearly show the importance of the phosphorylation system in the nitrite-induced protection. Although the evidence for the involvement of the phosphorylation system in the nitrite effect is mainly indirect, only the different change of the P/E ratio in the control and nitrite animals indicate this, we assume that the nitrite derived NO is able to act on some of the members of the phosphorylation system, such as on the ATP synthase (CV), the phosphate transporter or the ADP/ATP translocator (ANT). There is some evidence
that nitrite inhibits the interaction of ATP synthase and cyclophilin D (Halestrap and Richardson, 2015). This interaction might play a role in the formation and opening of mitochondrial permeability transition pores (MPTP), and subsequently in the increased ROS formation during I/R. There is also evidence that the activation of the cysteine 203 residue of cyclophilin D, which plays a role in the opening of MPTP (Nguyen et al., 2011), readily undergoes protein SNO (Kohr et al., 2011), and thereby protects the protein from the I/R-induced irreversible oxidation (Sun et al., 2006).

NEW FINDINGS

1. We have provided evidence that sodium nitrite (NaNO₂) infused 24h before a 25 min period of coronary artery occlusion and reperfusion in anaesthetized dogs, results in significant protection against the severe ventricular arrhythmias. This protection is manifested in a marked reduction in the ischaemia-induced arrhythmias and increase in survival upon reperfusion.

2. The nitrite-induced delayed antiarrhythmic effect is not, or only partially, mediated through the NO-induced activation of iNOS, since in the presence of the iNOS inhibitor AEST, the nitrite evoked antiarrhythmic effect was only attenuated, but not completely abolished, while the activity of iNOS enzyme was completely blocked. This study also points out the difference in the mechanisms between preconditioning and nitrite-induced delayed cardioprotection.

3. To the best of our knowledge, we have provided the first evidence that NaNO₂ is able to reduce the morphological changes of the mitochondria, resulting from I/R, and thereby preserves mitochondrial structure during ischaemia.

4. We have demonstrated that NaNO₂ suppresses mitochondrial respiration by influencing the mitochondrial respiratory complexes and the phosphorylation system in a way that the mitochondria produce less superoxide and peroxynitrite radicals, which certainly play roles in the arrhythmogenesis during I/R.

REFERENCES


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