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

Summary of the PhD Thesis

Vivien Demeter-Haludka

Department of Pharmacology and Pharmacotherapy

Albert Szent-Györgyi Medical Centre

Doctoral School of Multidisciplinary Medical Science

Supervisor: Prof. Dr. Ágnes Végh

University of Szeged

Hungary

2019

LIST OF PUBLICATIONS

Full papers related to the Thesis

I. Demeter-Haludka V, Juhasz L, Kovacs M, Gardi J, Vegh A Is there a role of iNOS activation in the delayed antiarrhythmic effect of sodium nitrite? CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 95:(4) pp. 447-454.-2017 IF: 2.21

II. Demeter-Haludka V, Kovács M, Petrus A, Patai R, Muntean DM, Siklós L, Végh Á Examination of the role of mitochondrial morphology and function in the cardioprotective effect of sodium nitrite administered 24 h before Ischemia/reperfusion injury FRONTIERS IN PHARMACOLOGY 9:(MAR) Paper 286.-2018 IF: 3.83

Other studies

III. Végh Á, Demeter-Haludka V, Kovács M, Miskolczi G Szívvédelem szervetlen nitrátokkal CARDIOLOGIA HUNGARICA 47:(Suppl.G) pp. G56-G63.-2017

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

V. Demeter-Haludka V, Kovács M, Petrus A, Patai R, Siklós L, Muntean D, Végh Á A nátrium-nitrit mitokondriális morfológiára és kálcium szintre gyakorolt késői hatása altatott kutyamodellben. CARDIOLOGIA HUNGARICA 47:(Suppl.C) pp. C36-C37. A Magyar Kardiológusok Társasága 2017. Évi Tudományos Kongresszusa. Balatonfüred-2017

VI. Demeter-Haludka V, Kovács M, Petrus A, Patai R, Siklós L, Muntean D, Végh Á A nátrium-nitrit késői hatása a mitokondriális funkcióra és morfológiára altatott kutya iszkémia/reperfúzió modellben. CARDIOLOGIA HUNGARICA 46:(Suppl.F) pp. F35-F46. Magyar Kardiológusok Társaságának Kongresszusa. Balatonfüred-2016

VII. Demeter-Haludka V, Juhász L, Seprényi Gy, Végh Á A nátrium-nitrit késői hatásának vizsgálata a koszorúér-okklúzió és reperfúzió kiváltotta korai kamrai ritmuszavarokra altatott kutyamodellben CARDIOLOGIA HUNGARICA 45:(Suppl. D) p. D30.-2015

VIII. Demeter-Haludka V, Kovács M, Petrus A, Muntean D, Nagy N, Varró A, Végh Á The effect of sodium nitrite on the calcium homeostasis of the cell In: Ravingerova T, Farkasova V, Slezak J (szerk.) Advances in Cardiovascular Research: From basic mechanisms to therapeutic strategies. Konferenciahelye, ideje: Smolenice, Szlovákia, Bratislava :2018. p. 72. 5th European Section Meeting of the International Academy of Cardiovascular Sciences (IACS-ES)-2018

IX. Demeter-Haludka V, Kovács M, Petrus A, Patai R, Siklós L, Muntean D, Végh Á Altered mitochondrial morphology and function involved in the delayed antiarrhythmic effect of sodium nitrite in anaesthetized dogs. CURRENT RESEARCH: CARDIOLOGY - EXPERIMENTAL CLINICAL 3:(3) p. 106. 3rd European Section Meeting of the International Academy of Cardiovascular Sciences (IACS-ES). Marseille, Franciaország-2016

X. Gazdag P, Hartai T, Demeter-Haludka V, Ördög B, Oravecz K, Nagy N, Acsai K, Barta B, Oláh A, Radovits T, Merkely B, Papp J Gy, Varró A, Prorok J Characterization of changes in Ca2+ handling and contractile function in a rat model of exercise-induced cardiac hypertrophy In: Ravingerova T, Farkasova V, Slezak J (szerk.) Advances in Cardiovascular Research: From basic mechanisms to therapeutic strategies. Konferenciahelye, ideje: Smolenice, Szlovákia, Bratislava: 2018. p. 78. 5th European Section Meeting of the International Academy of Cardiovascular Sciences (IACS-ES)-2018

XI. Prorok J, Gazdag P, Oravecz K, Hartai T, Demeter-Haludka V, Ördög B, Acsai K, Barta BA, Oláh A, Radovits T, Merkely B, Papp J Gy, Nagy N, Varró A Az intenzív edzés hatására kialakult változások jellezmése szív hipertrófiás patkánymodell Ca2+ háztartásában és kontraktilis funkciójában. CARDIOLOGIA HUNGARICA 48:(Suppl.C) p. C43. (2018) Magyar Kardiológusok Társasága 2018. Évi Tudományos Kongresszusa. Balatonfüred-2018

LIST OF ABBREVIATIONS

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

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 nitrites 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 ($NaNO_2$), 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 ($NaNO_2$) in low (micro-molar) 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 $NaNO_2$.

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 $NaNO_2$.

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 (NO_x).

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 $NaNO_2$ 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 $NaNO_2$ (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 (LVSP) 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_x) concentrations

Plasma NO_x 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 (µm2), perimeter (µm), Feret diameter (µm) and roundness (0-1) ([4x[Area]/(πx[Major axis]2)] 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 dihidroethidine (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 ℃. 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) $NaNO_2$ (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 $NaNO_2$-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 $NaNO_2$. 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 ($NaNO_2$ 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; $NaNO_2$+I/R, n=5), 24h after the administration of saline or $NaNO_2$ 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, $NaNO_2$, AEST, $NaNO_2$+AEST and coronary artery occlusion

Compared to the saline treated controls, the intravenous infusion of $NaNO_2$ 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 $NaNO_2$ treated dogs, AEST attenuated the haemodynamic effects of $NaNO_2$. 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 $NaNO_2$ 24h previously. The administration of AEST in the $NaNO_2$ treated dogs did not modify the occlusion-induced haemodynamic changes compared to the controls or the $NaNO_2$ 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 $NaNO_2$ 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 $NaNO_2$ treated animals, the $NaNO_2$-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 $NaNO_2$, 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 $NaNO_2$.

4.1.4. The effect of $NaNO_2$ 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 $NaNO_2$, and 35±4% in the AEST+$NaNO_2$ treated groups.

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

These were assessed at 4 time points during the experiments. Twenty minutes after the infusion of $NaNO_2$ the NO_x levels were significantly increased, and still remained elevated 24h later. Interestingly both in the $NaNO_2$ and the AEST+$NaNO_2$ groups this increase in the NO_x 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 NO_x 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 $NaNO_2$ caused an increase in iNOS activation, but this change proved statistically not significant. AEST completely abolished the $NaNO_2$-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 in dogs infused with $NaNO_2$. 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 $NaNO_2$ 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 $NaNO_2$+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 $NaNO_2$ 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 $NaNO_2$ 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 NO_x 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 NO_x 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 (NO_x) concentrations were reduced in all groups compared to their corresponding pre-occlusion values, but in the nitrite treated dogs the NO_x levels were significantly higher than in the untreated controls. Furthermore, the fact that the reduction of NO_x 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 NO_x, 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 $NaNO_2$ 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 $NaNO_2$+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 ($NaNO_2$) 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. The best of our knowledge, we have provided the first evidence that $NaNO_2$ 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 $NaNO_2$ 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

Ambrosio, G., Zweier, J.L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P.P. 1993. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J. Biol. Chem. 268: 18532-8541.

Bolli, R., Manchikalapudi, S., Tang, X.L., Takano, H., Qiu, Y., Guo, Y., Zhang, Q., Jadoon, A.K. 1997. The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. Circ. Res. 81: 1094-107. DOI: 10.1161/01.RES.81.6.1094

Brand, M.D., Brindle, K.M., Buckingham, J.A., Harper, J.A., Rolfe, D.F.S., Stuart, J.A. 1999. The significance and mechanism of mitochondrial proton conductance. Int. J. Obesity. 23: S4-S11.

Burwell, L.S., Brookes P.S. 2008. Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. Antioxid. Redox Signal 10: 579-599. doi: 10.1089/ars.2007.1845

Chen, Q., Moghaddas, S., Hoppel, C.L., Lesnefsky, E. J. 2006. Reversible blockade of electron transport during ischemia protects mitochondria and decreases myocardial injury following reperfusion. J. Pharmacol. Exp. Ther. 319: 1405-1412.doi.org/10.1124/jpet.106.110262

Chen, Q., Vazquez, E.J., Moghaddas, S., Hoppel, C. L. and Lesnefsky, E.J. 2003. Production of reactive oxygen species by mitochondria: central role of complex III. J. Biol. Chem. 278: 36027-36031. doi: 10.1074/jbc.M304854200

Cho, J., Won, K., Wu, D., Soong, Y., Liu, S., Szeto, H.H. 2007. Potent mitochondria-targeted peptides reduce myocardial infarction in rats. Coron. Artery. Dis. 18:215–220.

Dejam, A., Hunter, C.J., Schechter, A.N., Gladwin, M.T. 2004. Emerging role of nitrite in human biology. Blood Cells Molecules and Diseases 232: 423-429. DOI:10.1016/j.bcmd.2004.02.002

Dröse, S., Brandt, U., Wittig, I. 2014. Mitochondrial respiratory chain complexes as sources and targets of thiol-based redox-regulation. Biochim. Biophys. Acta. 1844: 1344-1354. doi: 10.1016/j.bbapap.2014.02.006.

Finegold, J.A., Asaria, P., Darrel P., F. 2013. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organisation and United Nations. Int. J. Cardiol. 168(2): 934–945. DOI:10.1016/j.ijcard.2012.10.046

Global Burden of Disease Collaborative Network. Global Burden of Disease Study 2016 (GBD 2016) Results. Seattle, United States: Institute for Health Metrics and Evaluation (IHME), 2017.

Gnaiger, E., Kuznetsov, A.V., Schneeberger, S., Seiler, R., Brandacher, G., Steurer, W., Margreiter, R. Mitochondria in the cold. 2014. Mitochondrial Physiology Network Mitochondrial Pathways and Respiratory Control. An Introduction to OXPHOS Analysis Springer. 2000: 431-442.

Halestrap, A.P., Richardson, A.P. 2015. The mitochondrial permeability transition: A current perspective on its identity and role in ischaemia/ reperfusion injury. J. Mol. Cell. Cardiol. 78: 129-141. doi: 10.1016/j.yjmcc.2014.08.018

Kis, A., Végh, Á., Papp, J.Gy., Parratt, J.R. 1999b. Repeated cardiac pacing extends the time during which canine hearts are protected against ischaemia-induced arrhythmias: role of nitric oxide. J. Mol. Cell. Cardiol. 31: 1229-1241. DOI:10.1006/jmcc.1999.0955

Kiss, A., Juhász, L., Seprényi, Gy., Kupai, K., Kaszaki, J., Végh Á. 2010. The role of nitric oxide, superoxide and peroxynitrite in the anti-arrhythmic effects of preconditioning and

peroxynitrite infusion in anaesthetized dogs. Br. J. Pharmacol. 160: 1263-1272. DOI: 10.1111/j.1476-5381.2010.00774.

Kisvári, G., Kovács, M., Gardi, J., Seprényi, Gy., Kaszaki, J., Végh, Á., 2014. The effect of acute simvastatin administration on the severity of ventricular arrhythmias resulting from ischaemia and reperfusion in the canine: Is there a role for nitric oxide? Eur. J. Pharmacol. 732: 96-104. DOI: 10.1016/j.ejphar.2014.03.021.

Kléber, A.G. 1983. Resting membrane potential, extracellular potassium activity and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig heart. Circ. Res. 42: 603-613.

Kohr, M.J., Apote, A.M., Sun, J., Wang, G., Murphy, E., Gucek, M., Steenbergen, C. 2011. Characterisation of potential S-nitrosylation sites in the myocardium. Am. J. Pysiol. 300: H1327-H1335. doi: 10.1152/ajpheart.00997.

Konya, L., Kekesi, V., Juhasz-Nagy, S., Feher, J. 1992. The effect of superoxide dismutase in the myocardium during reperfusion in the dog. Free. Radic. Biol. Med. 13:527–532.

Kovács, M., Gönczi, M., Kovács, E., Végh Á. 2013. Time course analysis of cardiac pacing-induced gene expression changes in the canine heart. Mol. Cell. Biochem. 372: 257-66. DOI: 10.1007/s11010-012-1467-8

Kovács, M., Kiss, A., Gönczi, M., Miskolczi, G., Seprényi, G., Kaszaki, J., Kohr, M.J., Murphy, E. Végh, Á. 2015. Effect of sodium nitrite on ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs: is protein S-nitrosylation involved? PLoS One 24:10(4): e0122243. doi: 10.1371/journal.pone.0122243.

Lauer, T., Preik, M, Rassaf, T., Strauer, B.E., Deuson, A., Feelish, M., Kelm, M. 2001. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc. Natl. Acad. Sci. 98: 12814-12819. DOI: 10.1073/pnas.221381098

Lefer, D.J. 2006. Nitrite therapy for protection against ischemia-reperfusion injury. Am. J. Physiol. Renal Physiol. 290: F777-F778. DOI: 10.1152/ajprenal.00470.2005

Manning, A., Bernier, M., Crome, R., Little, S., Hearse, D. 1988. Reperfusion-induced arrhythmias: a study of the role of xanthine oxidase-derived free radicals in the rat heart. J Mol. Cell. Cardiol. 20:35–45.

Miller, M.R., Megson I.L 2007 Recent developments in nitric oxide donor drugs. Br. J. Pharmacol. 151: 305-321.

Murphy, E., Eisner, D.A. 2009. Regulation of intracellular and mitochondrial sodium in health and disease. Circ. Res. 104:292–303.

Murry, C.E., Jennings, R.B., Reimer, K.A. 1986. Preconditioning with ischaemia: a delay of lethal cell injury in ischaemic myocardium. Circulation. 74: 1124-1136.

Nguyen, T.T., Stevens, M.V., Kohr, M.J., Steenbergen, C., Sack, M.N., Murphy, E. 2011. Cysteine 203 of cyclophilin D is critical for cyclophilin D activation of the mitochondrial permeability transition pore. J. Biol. Chem. 46: 40184-40192. doi: 10.1074/jbc.M111.243469.

Sasaki, N., Sato, T., Marban, E., O'Rourke, B. 2001. ATP consumption by uncoupled mitochondria activates sarcolemmal K(ATP) channels in cardiac myocytes. Am. J. Physiol. Heart Circ. Physiol. 280:H1882–H1888.

Shiva, S., Huang, Z., Grubina, R., Sun, J., Ringwood, L.A., MacArthur, P.H., Xu, X., Murphy, E., Darley-Usmar, V.M., Gladwin, M.T. 2007a. Deoxymyoglobin is a nitrate reductase that generates nitric oxide and regulates mitochondrial respiration. Circ. Res. 100: 654-661.DOI: 10.1161/01.RES.0000260171.52224.6b

Shiva, S., Sack, M.N., Greer, J.J., Duranski, M., Ringwood, L.A., Burwell, L., Wang, P.H. et al. 2007b. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J. Exp. Med. 204: 2089-2012. DOI: 10.1084/jem.20070198

Stewart, S., Lesnefsky, E.J., Chen, Q. 2009. Reversible blockade of electron transport with amobarbital at the onset of reperfusion attenuates cardiac injury. Transl. Res. 153: 224-231. doi: 10.1016/j.trsl.2009.02.003.

Sun, J., Steenbergen, C., Murphy, E. 2006. S-nitrosylation: NO-related redox signalling to protect against oxidative stress. Antioxid. Redox. Signal. 8: 1693-1705. doi: 10.1089/ars.2006.8.1693

Turrens, J.F. 2003. Mitochondrial formation of reactive oxygen species. J. Physiol. 552: 335-344. doi: 10.1113/jphysiol.2003.049478

Végh, Á., Parratt JR. 1996. Delayed ischaemic preconditioning induced by drugs and by cardiac pacing. in: Myocardial Preconditioning. Ed. C.L. Wainwright and J.R. Parratt (Springer, Berlin) 251-261.

Végh, Á., Komori, S., Szekeres, L., Parratt, J.R. 1992. Antiarrhythmic effects of preconditioning in anaesthetized dogs and rats. Cardiovasc. Res. 26: 487–495. https://doi.org/10.1093/cvr/26.5.487

Végh, Á., Komori, S., Szekeres, L., Parratt, J.R. 1992. Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. Cardiovasc. Res. 26: 487-495. DOI: http://dx.doi.org/10.1093/cvr/26.5.487

Végh, Á., Papp, J.Gy., Parratt, J.R. 1994. Prevention by dexamethasone of the marked antiarrhythmic effects of preconditioning induced 20 h after rapid cardiac pacing. Br. J. Pharmacol. 113: 1081-1082. DOI: 10.1111/j.1476-5381.1994.tb17104.x

Végh, Á., Szekeres, L., Parratt, J.R. 1992. Preconditioning of the ischaemic myocardium; involvement of the L-arginine - nitric oxide pathway. Br. J. Pharmacol. 107: 648-652. DOI: 10.1111/j.1476-5381.1992.tb14501.x

Walker, M.J., Curtis, M.J., Hearse, D.J., Campbell, R.W., Janse, M.J., Yellon, D.M. et al. 1998. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction, and reperfusion. Cardiovasc. Res. 22: 447–455. DOI: http://dx.doi.org/10.1093/cvr/22.7.447

Zweier, J.L., Wang, P., Kuppusamy, P. 1995a. Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. J. Biol. Chem. 270: 304-307.

ACKNOWLEDGEMENT

First, I would like to express my gratitude to my supervisor **Professor Ágnes Végh**, who with her knowledge, experience and support guided throughout my work and helped me to prepare the manuscripts for publication and write this thesis.

I would like to thank **Professor András Varró** for providing me the opportunity to carry out my experiments in the Department of Pharmacology and Pharmacotherapy, University of Szeged.

A special thank goes to **Dr. Mária Kovács** for helping me with her knowledge, experience and continuous support.

My precious thanks to **Erika Bakó** for her skilful and enthusiastic assistance during the *in vivo* and *in vitro* measurements.

I pleased to express my thanks to all co-authors of my publications for the valuable cooperation.

This work was supported by the GINOP 2.3.2-15-2016-00040 and by a special grant of the Medical Faculty of the Szeged University and the 'National Excellence Program' TÁMOP 4.2.4.A „Konvergencia-Eötvös Loránd Hallgatói Ösztöndíj-2012 (A2-ELMH-12)".