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

PhD Thesis

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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 (Administration of sodium nitrite produces delayed antiarrhythmic effects in anaesthetized dogs) CARDIOLOGIA HUNGARICA 45:(Suppl. D) p. D30.-2015

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LIST OF ABBREVIATIONS

3-NT	3-nitrotyrosine
ABP	Arterial blood pressure
ADP	Adenosine 5’-diphosphate
ATP	Adenosine 5’-triphosphate
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
LV	Left ventricle
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
RLU	Relative luminescence unit
ROS	Reactive oxygen species
SNO	S-nitrosylation
SSM	Sub-sarcolemmal
SUIT protocol	Substrate-uncoupler-inhibitor titration (SUIT) protocol
VF	Ventricular fibrillation
VPBs	Ventricular premature beats
VT	Ventricular tachycardia

SUMMARY

Ventricular tachyarrhythmias and sudden cardiac death (SCD), resulting from severe coronary artery disease, are one of the main causes of mortality worldwide. Over decades, attempts have been made on to reduce the ischaemia and reperfusion (I/R)-induced injury, and thereby its serious consequences. One of the first drugs that had been introduced to the therapy against anginal attacks was the organic nitrite and nitrates. 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. There is increasing evidence that a similar protection can be achieved by the inorganic nitrite and nitrate compounds. Nitrates and nitrites are the natural oxidative metabolites of NO, which are able to readily reduce back to NO, particularly under reductive conditions, such as hypoxia or ischaemia. It is proposed that they can serve as stores for NO, and increase the bioavailability of NO under ischaemic conditions, when the synthesis of NO by the nitric oxide synthase (NOS) enzymes has become limited.

We have previous evidence that the infusion of sodium nitrite ($NaNO_2$) in low (micro-molar) concentration, prior to coronary artery occlusion, or just prior to reperfusion in anaesthetized dogs, results in marked protection against the I/R-induced arrhythmias. The aim of the experiments, presented in the current Thesis, 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. Thus, we have examined whether (I) changes in the inducible nitric oxide (iNOS) activity, and/or whether (II) alterations in mitochondrial morphology and function would play a major role in the late cardioprotective effect of $NaNO_2$.

I. In this series of experiments $NaNO_2$ was infused in a concentration of 0.2 μmol kg^{-1} min^{-1} 24h before a 25 min coronary artery occlusion and reperfusion of the left anterior descending (LAD) coronary artery in anaesthetized dogs. We have shown that this concentration of $NaNO_2$ markedly reduced the number and severity of ventricular arrhythmias during occlusion, and increased survival (0% vs. 50%) from the combined I/R insult. Furthermore, nitrite markedly attenuated the I/R-induced increase of the indices of the ischaemia severity, such as the epicardial ST-segment and in the degree of inhomogeneity of electrical activation. The results of the measurements of iNOS enzyme activation showed that the inhibition of iNOS activity with S-(2-aminoethyl)-isothiourea

(AEST; 2.0 mg kg^{-1}, *i.v.*) completely abolished the nitrite-induced increase in iNOS activation, but it only attenuated the antiarrhythmic effect of $NaNO_2$. Thus our conclusion was that $NaNO_2$ protects the myocardium against the severe ventricular arrhythmias that results from a 25 min period of I/R, 24h later. Furthermore, it seems that in this antiarrhythmic effect, the activation of iNOS does not play a mandatory role, since the inhibition of iNOS by AEST only attenuates the protection against some types of I/R-induced arrhythmias.

II. In these experiments we have examined the role of changes in mitochondrial structure and function following nitrite administration in order to explore whether mitochondria are involved in the protective effect of nitrite. Therefore, we have determined the alterations in mitochondrial morphology, as well as of the changes in the respiratory parameters, ATP production and in the generation of reactive oxygen species (ROS) during I/R and following nitrite administration.

The results show that the administration of $NaNO_2$ prevented the I/R-induced changes in mitochondrial structure, 24h later. Furthermore, the infusion of sodium nitrite alone, and under conditions of ischaemia, depressed mitochondrial respiration (oxidative phosphorylation, ETC, respiratory control and P/E coupling ratios), without substantially modifying the rate of ATP production. Nitrite also reduced the generation of superoxide. We propose that nitrite has an effect on the mitochondria. It can preserve the structural integrity of the mitochondria, and by modifying the various elements of the mitochondrial respiratory chain that are responsible for ROS production, reduces the generation of ROS during I/R. Our hypothesis is that nitrite acts on the phosphorylation system, which is in turn suppresses the function of ROS producing complexes. This mechanism is certainly may contribute to the late antiarrhythmic effect of $NaNO_2$.

1. INTRODUCTION

Coronary artery diseases and its consequences, such as acute myocardial infarction, heart failure and the occurrence of serious, life-threatening ventricular arrhythmias, are the main causes of mortality worldwide (Finegold *et al.*, 2013). According to the World Health Organisation surveys in 2017, ischaemic heart disease (IHD) is the first morbidity, before stroke and lower respiratory infections that are responsible for death (Global Health Estimates, 2018), and its incidence continuously increases (Global Burden of Disease Collaborative Network, 2017). In Hungary, compared to the world statistics, there is no substantial difference; the main cause of death is IHD, before the cerebrovascular diseases and lung cancer (Global Burden of Disease Collaborative Network, 2017).

The high risk of IHD for mortality, and particularly for sudden cardiac death from fatal ventricular fibrillation, urged to develop new strategies for preventing and treating the severe consequences of IHD. In the last decades the development of the invasive and non-invasive surgical interventions, novel drugs and some other non-drug related treatments (e.g. ischaemic preconditioning) are promising approaches to reduce the generation of the ischaemia-induced life-threatening ventricular arrhythmias, and consequently to decrease the morbidity and mortality caused by IHD.

1.1 The pathomechaisms of the generation of ventricular arrhythmias after coronary artery occlusion

Following a sudden coronary artery occlusion, the acute arrhythmias occur in two phases, termed as phase 1A and phase 1B (Kaplinsky *et al.*, 1979). The phase 1A arrhythmias usually appear between the 3 and 8 min of the occlusion that is followed by an arrhythmia free period, whereas the phase 1B arrhythmias can be observed between the 15-30 min of the ischaemia. These 1B phase of arrhythmias are frequently terminate in ventricular fibrillation (deGroot *et al.*, 2001). The acute arrhythmia phase is difficult to assess under clinical conditions, since they usually appear and terminate before the patient reaches the hospital.

The mechanism of the two phases of the acute arrhythmias is different. It is proposed that sudden occlusion of a coronary artery leads to an imbalance between oxygen supply and demand of the myocardium distal to the occlusion site, and results in a rapid shift from aerobic to anaerobic metabolism. The impairment of glycolysis, the Szent-Györgyi-Crebs

cycle and the ATP production, as well as a drop in pH, modifies the function of ion channels, resulting in an increase in the intracellular Na^+ and in the efflux of K^+. These ionic alterations lead to changes in action potential morphology and the spread of impulse (Janse *et al.*, 1986; Kléber *et al.*, 2000), and can be served as underlying mechanisms of functional re-entry and the generation of the phase 1A arrhythmias (Janse *et al.*, 1986, Kléber *et al.*, 1987).

The mechanisms of the phase 1B arrhythmias are different. Among others, changes in the metabolic and electrical coupling between the cells may play an important role in the genesis of the 1B phase of the arrhythmias (Végh *et al.*, 2011). In this phase of arrhythmias preservation of gap junction (GJ) function upon the increase in calcium levels and catecholamines due to ischaemia, may have a protective role (Dekker *et al.*, 1996, Lameris *et al.*, 2000, Papp *et al.*, 2007). There are many endogenous and exogenous substances which can modify GJ function (Dhein *et al.*, 2004; Dhein *et al.*, 2010), and thereby influencing the generation of arrhythmias during phase 1B. Among these, the most important for us is nitric oxide (NO), which has been shown, in several previous studies, that plays an important role as a trigger and mediator of preconditioning-induced protection against arrhythmias (Végh *et al.*, 1992b, Végh and Parratt, 1996, Kis *et al.*, 1999b). We have also evidence that NO is able to modify GJ function and thereby influence arrhythmia generation (Gönczi *et al.*, 2009).

1.1.2. The role of mitochondria in the arrhythmogenesis

Mitochondria are abundant in the cardiomyocyte (approximately 30% of the cell volume) and 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 are 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 proved to be antiarrhythmic (Kónya *et al.*, 1992; Cho *et al.*, 2007). ROS are responsible for the impairment of cardiac excitability either directly via biochemical modification of the ion channels (Aggarwal *et al.*, 2013; Kawakami *et al.*, 1998) or indirectly through signal transduction (Shang *et al.*, 2008). ROS can modify the cellular cation levels (Murphy *et al.*, 2009; Williams *et al.*, 2013). Reactive oxygen radicals contribute to the appearance of an inward rectifying current during the first 10 minutes of ischaemia via modulating the energy

sensing ATP-sensitive potassium channels which can lead to action potential heterogeneity and arrhythmias (Sasaki *et al.*, 2001). ROS can mediate the partial dissipation or collapse in mitochondrial membrane potential via modifying the mitoK_{ATP} channels and sarcK_{ATP} current and can be arrhythmogenic (O'Rourke *et al.*, 2004; Aon *et al.*, 2003). There is evidence that superoxide can disrupt the mitochondrial membrane potential, resulted in a phenomenon called ROS-induced ROS release which leads to the whole depolarization of the cardiomyocyte in the myocardium (Zorov *et al.*, 2000; Zorov *et al.*, 2006). Mitochondrial permeability transition pore (MPTP) can also be modified during ischaemia/reperfusion and cause arrhythmias since MPTP is responsible for the spreading of death signal (Halestrap *et al.*, 2004). On the other hand, mitochondrial calcium overload can trigger ROS generation, open the MPTP, and release Cytochrome c which can be responsible for arrhythmia generation (Brookes *et al.*, 2004). Mitochondrial calcium uniporter (MCU) as well as the mitochondrial sodium–calcium exchanger (mNCX) play an important role in the regulation of calcium influx and efflux (Laurita *et al.*, 2008).

1.2 The role of nitrites and nitrates in the treatment of coronary artery diseases

Organic nitrites and nitrates were the first drugs that had been introduced in treatment of angina pectoris. Their discovery goes back to the 1870s, when people working in dynamite (glycerine-trinitrate) factory and suffering from the typical side-effects of nitro-glycerine (NTG), such as headache, flushing, vasodilatation, underwent medical investigation. As a result of this, it was recognized that the effects of NTG could be utilized for the prevention of symptoms of angina (Murrell, 1879). Since then, and still, NTG and the organic nitrates and nitrites are important part of the therapy of angina pectoris. The protective effects of nitrates and nitrites have been described in various experimental settings, moreover it was pointed out that the pharmacological effects are due to NO, which can release from these drugs undergoing both by enzymatic and non-enzymatic degradation (Miller *et al.*, 2007).

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, 2006). It has been recognised that these oxidative metabolites of NO can readily reduce back to NO in a reductive milieu, such as anoxia, hypoxia or myocardial ischaemia, and thereby can serve as stores for NO under pathophysiological conditions. This recognition might be an important therapeutic approach, since during ischaemia, when the oxygen supply to the myocardium is limited, and the NO

synthase enzymes are producing inadequate amount of NO, the natural metabolic products would provide NO and increase NO bioavailability in the ischaemic myocardium (Zweier *et al.*, 1995).

1.2.1. The role of nitric oxide in the antiarrhythmic effect of preconditioning

The phenomenon of ischaemic preconditioning (IPC) was first described by Murry and colleagues in 1986 (Murry *et al.*, 1986). They showed that brief periods of ischaemia can protect the myocardium against the severe consequences of a similar, but more prolonged ischaemic insult (Murry et al., 1986). Since then we have learnt much more about this remarkable protective phenomenon. For example, we know that preconditioning not only reduces the size of infarct, but it markedly attenuates the ischaemia and reperfusion-induced life-threatening ventricular arrhythmias (Shiki and Hearse, 1987; Végh *et al.*, 1990, 1992a) and improves myocardial contractility following reperfusion (Cave and Hearse, 1992). Furthermore, a preconditioning-like protection can be induced by other stimuli than acute coronary artery occlusion (Végh *et al.*, 1992a), such as cardiac pacing (Végh *et al.*, 1991a), heavy physical exercise (Babai *et al.*, 2002), heat stress (Cumming *et al.,* 1996) or various drugs (pharmacological preconditioning [Cohen *et al.*, 2000]). For example, we have ample of evidence that NO plays an important role in the early (Végh et al., 1992) and also in the delayed (Kiss *et al*. 1999a, 1999b) antiarrhythmic effect of coronary artery occlusion and cardiac pacing-induced PC. Furthermore, we have shown that the NO donor isosorbide mononitrate and the K_{ATP} channel opener and NO donor nicorandil (Végh *et al.*, 1996, György *et al.*, 2000), as well as sodium nitroprusside (Gönczi et al., 2009) are able to protect against arrhythmias. Moreover, statins through the stimulation of NO synthesis can protect the myocardium (Kisvári *et al.*, 2014; Kisvári *et al.*, 2015). Thus, there is strong evidence that an increase in NO bioavailability either by PC or by the administration of a NO donor molecule, under ischaemic conditions, is protective against arrhythmias and ischaemic damage (Végh *et al.*, 1992b; Parratt and Végh, 1997; Lochner *et al.*, 2000). One of the most accepted hypothesis for the mechanism of the delayed cardioprotective effect of PC is that the PC stimulus enhances NO synthesis via the stimulation of eNOS, which increased NO then stimulates the inducible form of NO synthase (iNOS), further enhancing NO production (Végh and Parratt, 1996; Bolli *et al.*, 1997).

1.2.2. The cardioprotective effect of the inorganic sodium nitrite

Inorganic nitrites and nitrates have been considered as biologically inert molecules until recently. There is now increasing evidence that not only the organic, but the inorganic nitrites and nitrates can release NO, and thereby may have cardioprotective effects. This recognition has opened new perspectives both for the research of NO biology and for clinical exploitation. The potential cardioprotective benefit of the inorganic nitrates and nitrites originated from the recognition that NO can be produced not only by the NOS enzymes, but through, a so-called, enzyme-independent manner as well (Zweier *et al.*, 1995a). They showed, using electron paramagnetic resonance measurements that in isolated rat hearts following I/R a marked increase in NO production could be observed, suggesting that under these conditions NO generated primary from nitrite rather than from synthesis by the NOS enzymes (Zweier *et al.*, 1995a).

Since then, many studies have revealed that inorganic nitrites and nitrates can transform to NO by a non-enzymatic way, when the surrounding milieu becomes hypoxic or anoxic, such as during ischaemia (Zweier *et al.*, 1995a; Zweier *et al.*, 1995b). Under these reductive conditions this way of the NO formation would be particularly important, since in the absence of oxygen the enzymatic generation of NO is compromised (Kevil *et al.*, 2011a; Kevil *et al.*, 2011b). Several studies have proved that under ischaemic conditions the transformation of nitrite to NO might be a way through which the NO bioavailability can be maintained or increased to the myocardium, thus nitrite can serve as a store for NO even under ischaemic conditions (Lefer, 2006; Lundberg *et al.*, 2011). 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 (Kevil *et al.*, 2011a; Lefer, 2006; Dejam *et al.*, 2004).

On the basis of these abovementioned findings, we have also examined the potential cardioprotective effects of nitrites. We wondered whether these drugs are able to protect against arrhythmias in our established canine model of ischaemia and reperfusion. We have shown that sodium nitrite, infused in a concentration of 0.2 μmol kg^{-1} min^{-1} either prior to ischaemia or just prior to reperfusion markedly reduced the severity of 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

survival increased. For example, the survival rate in dogs infused with $NaNO_2$ 10 min prior to reperfusion was 92%. This is, indeed, a remarkable protective effect. We have also shown that in this protection S-nitrosylation and/or glutathionylation of proteins may play a role (Kovács *et al.,* 2015).

As it 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 (eNOS) activation (Bolli et al., 1997, Kovács et al., 2013), which can further increase NO bioavailability during ischaemia, may play a mandatory role. The evidence for this comes from the direct measurement of NO production (Bolli *et al.*, 1997), and in our studies from the fact that the inhibition of iNOS activation markedly attenuates the antiarrhythmic protection (Végh *et al.*, 1994; Kis *et al.*, 1999a,b).

If an increase in NO bioavailability during I/R is an important factor for the induction of the delayed cardioprotection, it was obvious to examine, whether sodium nitrite can elicit a delayed antiarrhythmic effect, and if so, whether the mechanism involves the NO/iNOS/NO pathway, as could have been seen with PC.

There is a few previous evidence that $NaNO_2$ 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 for 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).

Taking account the potential involvement of mitochondria in the delayed antiarrhythmic effect if sodium nitrite, **we have examined whether 24h following the administration of sodium nitrite changes can be detected in mitochondrial structure and function in the absence and presence of I/R.**

2. AIMS

The objectives of studies presented in the Thesis were as the follows:

I. Examination of the potential delayed antiarrhythmic effect of sodium nitrite against ischaemia and reperfusion-induced severe ventricular arrhythmias in anaesthetized dogs. In the same experiments we wanted to explore the role of iNOS activation in the delayed effect of sodium nitrite.

This series of experiments were designed to study, whether sodium nitrite, administered 24h before a coronary artery occlusion and reperfusion, would result in protection against arrhythmias. Therefore, in anaesthetized dogs, $NaNO_2$ was infused 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 prior to a 25 min occlusion and reperfusion of the left anterior descending coronary artery (LAD). 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 nitrite 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. The role of mitochondria in the delayed antiarrhythmic effect of sodium nitrite, with particular reference on changes in mitochondrial morphology and function.

In this series of experiments we planned to examine the role of mitochondria in the delayed antiarrhythmic effect of $NaNO_2$. We have approached to this question by analysing 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, superoxide and peroxynitrite productions were assessed.

3. MATERIALS AND METHODS

3.1 Ethics

All the experiments were carried out in accordance with the Hungarian law 40/2013 (II. 14.) and were supervised and approved by the Ethical Committee for the Protection of Animals in Research of University of Szeged and the Csongrád County Governmental Office for Food Safety and Animal Health. Approval number: XIII./1211/2012 (file no. VI-I-01/1211-4/2012). (I.) and XIII./4657/2016 (file no. CSI/01/4657-6/2016) (II.).

3.2 Animals and housing

I. In the first study 36 adult mongrel dogs of either sexes (19 males and 14 females) were used. The mean body weight of dogs was: 21 ± 4 kg.

II. In the second study 30 adult dogs (17 males and 13 females) with a mean body weight of 22 ± 4 kg were used.

All the dogs were housed in an environmentally controlled room. Two animals were kept in one pen (house to kennel ratio 1:1). The temperature was set 10-20 ºC, humidity 40-70%, and lightening 12 hours per day, for two weeks before the experiment. The dogs were fed a standard diet and *ad libitum* access to water. Food was withdrawn 24h before the anaesthesia.

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) which allowed the animals to breathe spontaneously. A polyethylene catheter was introduced into the jugular vein for the injection of the drugs (saline, $NaNO_2$ or AEST). Arterial blood pressure was measured by a Millar tip catheter (F5, Millar Instruments Inc., USA), introduced into the left carotid artery.

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). The depth of the anaesthesia was monitored by the examination of cornea and pain reflexes, as well as the blood pressure, and further bolus injections of the anaesthetic were given, if was necessary.

The dogs were intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus, USA). The blood gas values were monitored during the experiment and were

maintained within physiological range during the whole experiment (Végh *et al.*, 1992). Body temperature was assessed from the mid-oesophagus and maintained between 36.5 and 37.5 using a heating pad. The right femoral artery was prepared, and a catheter was introduced to measure the systolic and diastolic arterial blood pressure. 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), positioned into the cavity of the left ventricle. Through the jugular vein a catheter was placed into the coronary sinus in order to collect blood samples for the determination of nitrite/nitrate (NO_x) levels.

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 (150 mg kg^{-1}, iv., Euthasol 40%, Produlab Pharma B.V., Netherlands).

The severity of ischaemia was assessed by measuring changes in the epicardial ST-segment and in the degree of inhomogeneity of electrical activation using a composite electrode. This electrode contains 24 bipolar and 2 unipolar electrodes, which collects the summarised recording of R waves, and records ST-segment changes, respectively, from the epicardial surface of the ischaemic myocardium (Végh *et al.*, 1992). In the normal, oxygenated myocardium all electrode sites are activated simultaneously, resulting in a single, large R spike. Following occlusion, however, widening and fractionation of this summarized R-wave occurs, indicating inhomogeneous fibres activation. Changes in inhomogeneity were expressed in milliseconds, whereas the alterations in epicardial ST-segment were expressed in mV.

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), modified by us previously (Végh *et al.*, 1992). Thus, the number of ventricular premature beats (VPBs), the number of episodes and the incidence of ventricular tachycardia (VT) and the incidence of VF during occlusion were evaluated. During reperfusion, only the incidence of ventricular fibrillation and survival were assessed. The dogs were considered survivors, if they were in sinus rhythm 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 (AAR)

The risk area was measured as described previously (Végh *et al.*, 1992). Briefly, at the end of experiments the hearts were removed, and either Patent Blue V Dye or saline was infused into the re-occluded LAD or into the circumflex branch, respectively, with a pressure equivalent to the mean arterial blood pressure. The area, stained by the dye, was separated, weighted and the AAR was expressed as the percentage of the left ventricle together with the septum.

3.5 *In vitro m*easurements

3.5.1 Determination of iNOS enzyme activity

The assessment of iNOS enzyme activity was performed using the radio immunoassay method. Tissue samples from the left ventricle were used for total protein isolation as described previously (Kisvári *et al.*, 2014). Protein concentrations were determined by Bradford protein assay. The measurements were carried out using a NOS activity assay kit (Cayman Chemical, USA), in the absence of calcium and calmodulin, based on the conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. The background NOS activity in the control samples was eliminated by the administration of 10 mmol L-N^{G}-Nitro-arginine [(L-NNA), a non-specific inhibitor of the NOS enzyme], to the reaction before the addition of the tissue sample. A liquid scintillation counter (Wizard, PerkinElmer, USA) was used to detect the amount of radio-labelled L-citrulline formation induced by iNOS and expressed as the percentage of the total counts corrected with the background counts per minute.

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). On day one plasma samples were collected from the jugular vein and on day two from the coronary sinus at different time points as shown in Figure 1. The method is based on measuring the absorbance of the azo-compound spectrophotometrically at 540 nm using a microplate reader (FLUOstar OPTIMA, BMG LABTECH GmbH, Germany). The total nitrate/nitrite (NO_x) concentration (μmol l^{-1}) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma, USA). The data were analysed by OPTIMA software (Control and Data Analyses; BMG LABTECH GmbH, Germany).

3.5.3. Assessment of changes in mitochondrial morphology

Tissue blocks (1 mm^3), excised from the ischaemic and non-ischaemic areas of the left ventricle, were immediately fixed in Karnovsky solution (Karnovsky *et al.*, 1965) for 4h, and then rinsed in glucose-containing buffer and post fixed in osmium-tetroxid (Millonig, 1961). After dehydration with ethanol the blocks were embedded into epoxy resin (Durcupan ACM, Sigma, USA) and polymerized at 56 ^oC for two days. Ultra-thin slices (50 nm) were cut and contrasted with uranyl-acetate and lead citrate. Three different regions: sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and perinuclear (PN) were captured by transmission electron microscopy in transmission mode (80 keV; TEM, Zeiss CEM 902, Germany) with a Spot RT 14.0 CCD camera (Diagnostic Instruments, USA). Changes in the area (μm^2), perimeter (µm), Feret diameter (µm) and roundness (0-1) ($[4x[Area]/(\pi x[Major\ axis]^2)]$ were calculated using the build-in applications of ImageJ 2 (NIH, Bethesda, USA). 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

Mitochondria were freshly isolated from phosphate buffer-perfused left ventricle segment, immediately after the removal of the heart. Left ventricular samples (0.75 g) were homogenized in a sucrose containing buffer, and centrifuged on 8000g, 700g and 8000g. Concentration of the isolated mitochondria was determined by Bradford protein assay and 0.1 mg ml^{-1} isolated mitochondrial was used in mir05 buffer (110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 20 mM HEPES, 0.5 mM EGTA, 3 mM $MgCl_2$, 10 mM KH_2PO_4,1 g l^{-1} BSA, pH 7.1 at 37 °C) (Gnaiger *et al.*, 2014). A Clarke-type oxygen electrode (Strathkelvin 782 oxygen system, Strathkelvin, Glasgow) was used to measure the mitochondrial respiration. In the closed electrode chamber the oxygen consumption reflects purely the mitochondrial oxygen consumption. The mitochondrial respiratory measurements were carried out according to the SUIT protocol (Gnaiger *et al.*, 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). To characterize the oxidative phosphorylation, ADP (State 3, 5 mM) was added to the chamber and the oxygen consumption rate was measured. State 4 respiration was assessed by the injection of the ATP synthase inhibitor, oligomycin (Omy, 2.5 µM, Sigma, USA). State 4 respiration refers to the proton leak and proton slip, when ATP synthase is blocked, and

protons accumulates in the inter-membrane space. Uncoupling of respiration was measured by adding carbonyl-cyanide-p-(trifluoro-methoxy) phenyl-hydrazone (FCCP, 0.5 µM, Sigma, USA). This protonophore was added to measure capacity of electron transport system (ETS). Antimycin A (5 µM), a complex III (CIII) ETS blocker was used to measure the residual oxygen consumption, which is independent from the mitochondria. From the measured parameters respiratory control ratio (RCR=OXPHOS/State 4) and P/E (OXPHOS/ETS) ratios were calculated. RCR refers to the efficiency of the coupling between the respiration and ATP production. P/E ratio combines the effect of coupling and limitation by the phosphorylation system (possible modification of the ATP synthase (CV), the phosphate transporter or the ADP/ATP translocator (ANT)).

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. Malate (100 mM, Sigma, USA) and pyruvate (100 mM, Sigma, USA) were used as substrates. The emitted light was detected with a luminescent optic using a plate reader (FLUOstar OPTIMA, Germany). 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 using the dihidroethidine (DHE) dye method, based on the oxidation of DHE to ethidine by superoxide. The amount of ethidine relates to the level of superoxide. Following reperfusion, tissue blocks (0.5cmx0.5cmx2.0cm) were excised from the ischaemic and non-ischaemic areas and embedded into a glycol and resin containing mounting media (Bio-Optica, Italy). Then snaps were frozen in liquid nitrogen, and the samples were stored at -80 ^{o}C and kept on -20 ^{o}C, 24h before the use. Longitudinal slices (20 µm) slices were prepared by cryo-sectioning (Leica, USA). The samples were incubated in dark at room temperature with 1 µM DHE (Sigma, USA) and washed with PBS two times for 5min. For the negative control, the antioxidant N-acetyl-cysteine (100 mM, Sigma, USA) was used. Confocal laser scanning microscope (Olympus FV 1000, Japan) was used to capture ten pictures per dog. The images were analysed by Image J2 (Fiji) and expressed in arbitrary units. 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 from the ischaemic and non-ischaemic area 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) $NaNO_2$ (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 $NaNO_2$. According to the previous study (Kis *et al.*, 1999), 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. In dogs that had survived this period of reperfusion, an excess dose of anaesthetics was given. Then the hearts were removed for further *in vitro* analyses. During experiments blood samples (BS) were collected from the jugular vein and from the coronary sinus at different time points as shown in Figure 1. Three dogs served as sham-operated controls (SO group); these dogs underwent the same surgical intervention as the other groups.

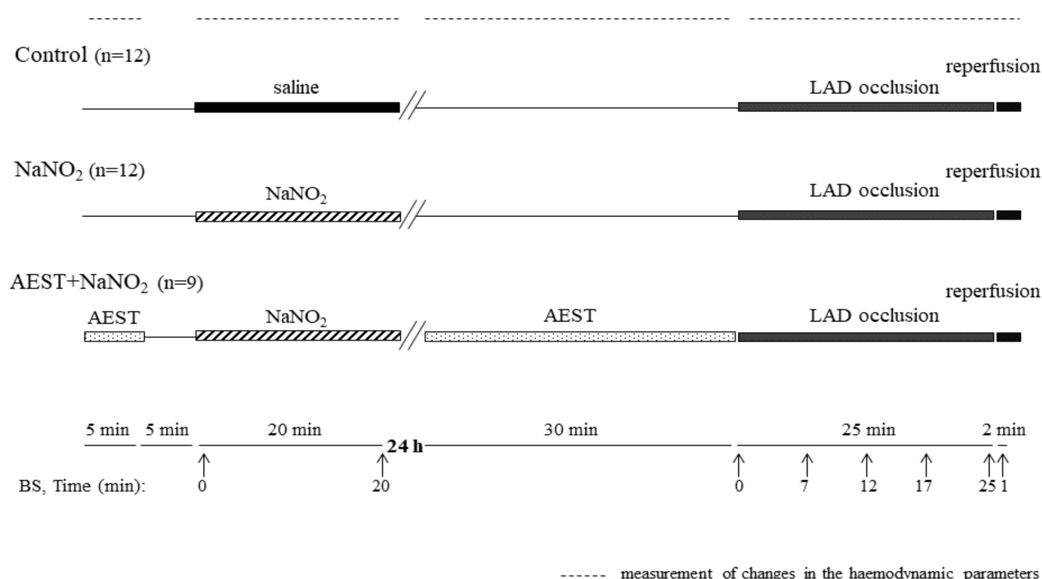

Figure 1. *Experimental protocol for the examination of the delayed antiarrhythmic effect of* $NaNO_2$ *and the role of iNOS enzyme activation*

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 and $NaNO_2$ the animals were subjected to a 25 min occlusion and reperfusion insult. In these dogs myocardial tissue samples were taken 2 min after reperfusion for *in vitro* analyses.

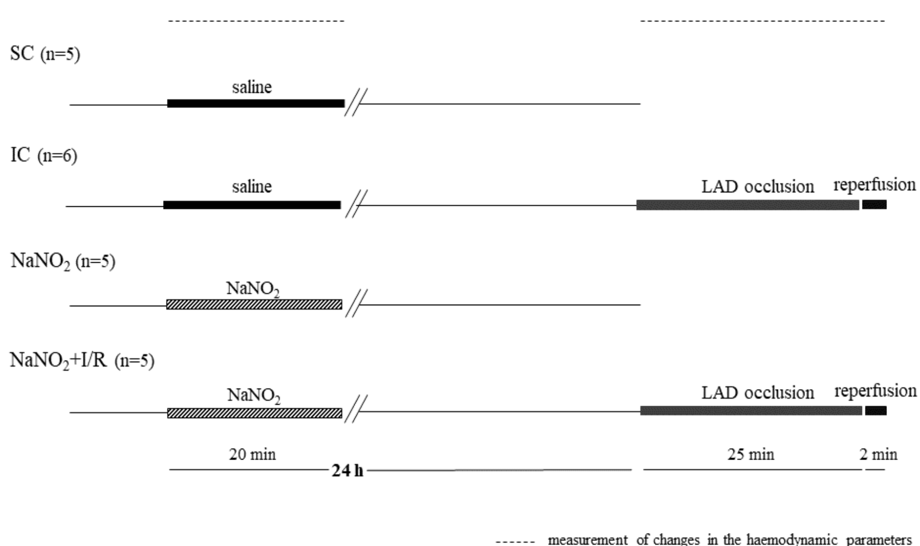

Figure 2. *Experimental protocol to examine the role of the mitochondria in the delayed antiarrhythmic effect of* $NaNO_2$

4. 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

These are shown in Table 1 and Table 2. 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 modified the haemodynamic parameters, but when it was given in $NaNO_2$ treated dogs, AEST attenuated the haemodynamic effects of $NaNO_2$ (Table 1).

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 (Table 2). These ischaemia-induced haemodynamic changes were less marked in dogs infused with $NaNO_2$ nitrite, 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.

Table 1. The haemodynamic effects of saline, $NaNO_2$, AEST and $NaNO_2$+AEST

	Saline		$NaNO_2$		AEST				AEST + $NaNO_2$	
					Day 1		Day 2			
	Baseline	Max change	Baseline	Max change	Baseline	Max change	Baseline	Max change	Baseline	Max change
SABP (mmHg)	148 ± 6	3 ± 2	155 ± 6	-11 ± 4*	161 ± 5	-1 ± 4	142 ± 4	5 ± 6*	153 ± 3	-3 ± 3†
DABP (mmHg)	100 ± 6	3 ± 2	122 ± 4	-7 ± 2*#	129 ± 4	-4 ± 2*#	101 ± 5	5 ± 6*	117 ± 3	-2 ± 2#†
MABP (mmHg)	116 ± 6	3 ± 2	133 ± 5	-8 ± 3*	140 ± 4	-3 ± 2*#	115 ± 4	4 ± 6*	136 ± 3	-3 ± 2#†
HR (beats/min)	159 ± 5	3 ± 1	166 ± 6	6 ± 2*#	187 ± 7	-13 ± 3*#	166 ± 2	-2 ± 2*	173 ± 4	-3 ± 3#†

*Values are means ± SEM, calculated from n=8 experiments. *$P<0.05$ vs. baseline value, #$P<0.05$ vs. control group, †$P<0.05$ vs. $NaNO_2$ group. Abbreviations: SABP: systolic arterial blood pressure, DABP: diastolic arterial blood pressure, MABP: mean arterial blood pressure, HR: heart rate*

Table 2. Changes in the haemodynamic parameters during a 25 min LAD occlusion

	Saline		$NaNO_2$		AEST+ $NaNO_2$	
	Baseline	Max change	Baseline	Max change	Baseline	Max change
SABP (mmHg)	140 ± 13	-17 ± 3*	146 ± 5	-11 ± 4*	146 ± 8	-14 ± 3*
DABP (mmHg)	99 ± 9	-18 ± 3*	98 ± 4	-11 ± 5*	105 ± 6	-12 ± 3*
MABP (mmHg)	113 ± 10	-17 ± 2*	114 ± 4	-11 ± 3*	119 ± 7	-13 ± 4*
LVSP (mmHg)	141 ± 9	-19 ± 5*	148 ± 12	-10 ± 5*	146 ± 9	-16 ± 2*
LVEDP (mmHg)	6.0 ± 1.1	7.3 ± 1.3*	3.0 ± 0.4	5.3 ± 0.6*#	2.7 ± 0.3	6.4 ± 0.6*
$+dP/dt_{max}$ (mmHg/s)	2792 ± 210	-720 ± 84*	2906 ± 136	-535 ± 130*	3431 ± 114	-710 ± 130*
$-dP/dt_{max}$ (mmHg/s)	2526 ±164	-583 ± 167*	2347 ± 75	-166 ± 112*#	2523 ± 149	-535 ± 62*
HR (beats/min)	167 ± 6	4 ± 4	161 ± 8	-4 ± 2	161 ± 4	4 ± 2

*Values are mean ± SEM, calculated from n=8 experiments. *P<0.05 vs. baseline value, #P<0.05 vs. saline treated control group. Abbreviations: SABP: systolic arterial blood pressure, DABP: diastolic arterial blood pressure, MABP: mean arterial blood pressure, LVSP: left ventricular systolic pressure, LVEDP: left ventricular end-diastolic pressure, HR: heart rate.*

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

The severity of ventricular arrhythmias occurring during occlusion and reperfusion is illustrated in Figure 3. 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 (Figure 3). 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 was significantly attenuated, but it was not completely abolished. Thus, the number of VPBs (170 ± 43) and episodes of VT (3.7 ± 1.1), as well as the incidence of VT (67%) were again significantly increased during occlusion. The incidence of the occlusion-induced VF (11%) and survival (11%), however, did not significantly differ from the nitrite treated dogs.

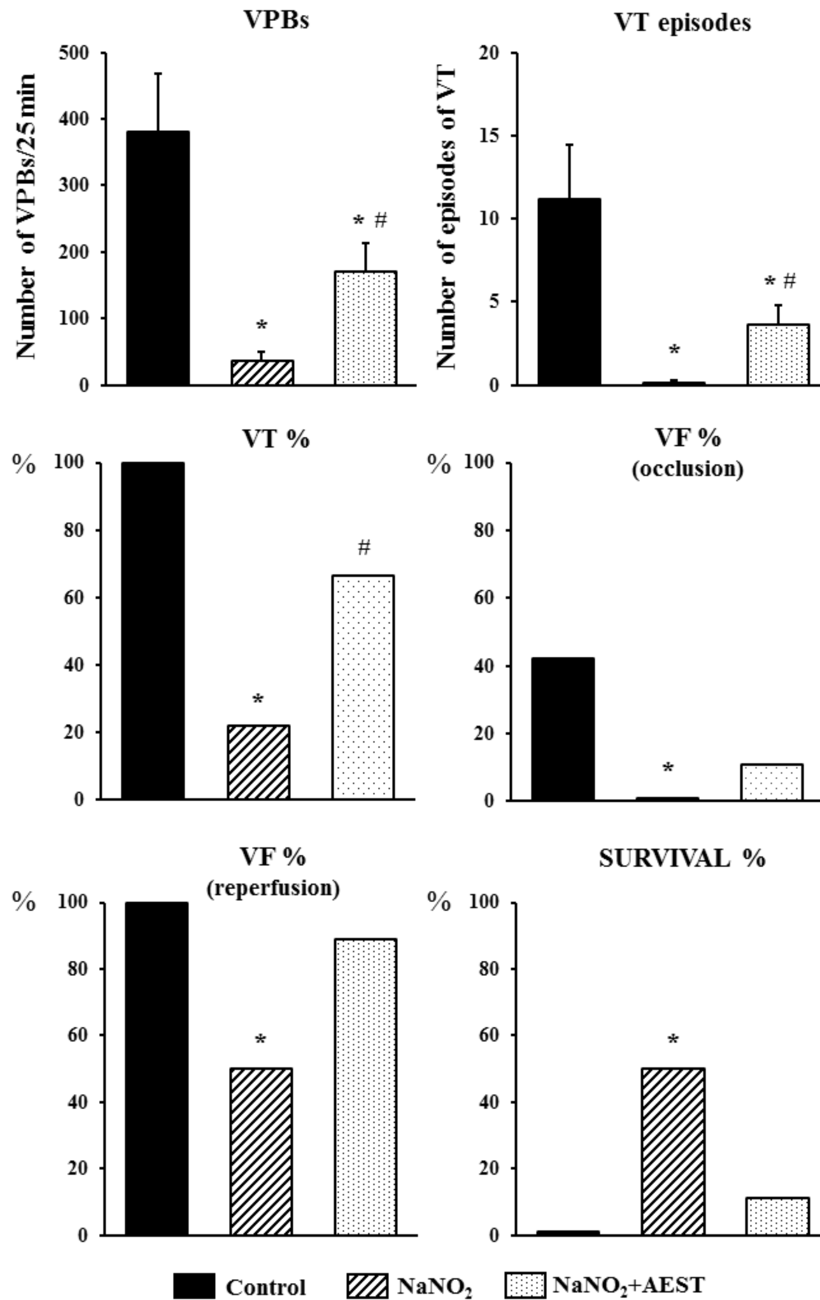

Figure 3. *The number and incidence of ventricular arrhythmias assessed during a 25 min occlusion and reperfusion of the LAD. Values are means ± SEM.* $^{*}P < 0.05$ *compared to the controls;* $^{\#}P < 0.05$ *compared to the* $NaNO_2$ *treated dogs.*

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

This was assessed by the measurement of two parameters; i.e. changes in the epicardial ST-segment and in the degree of inhomogeneity of electrical activation (Figure 4). 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 $NaNO_2$.

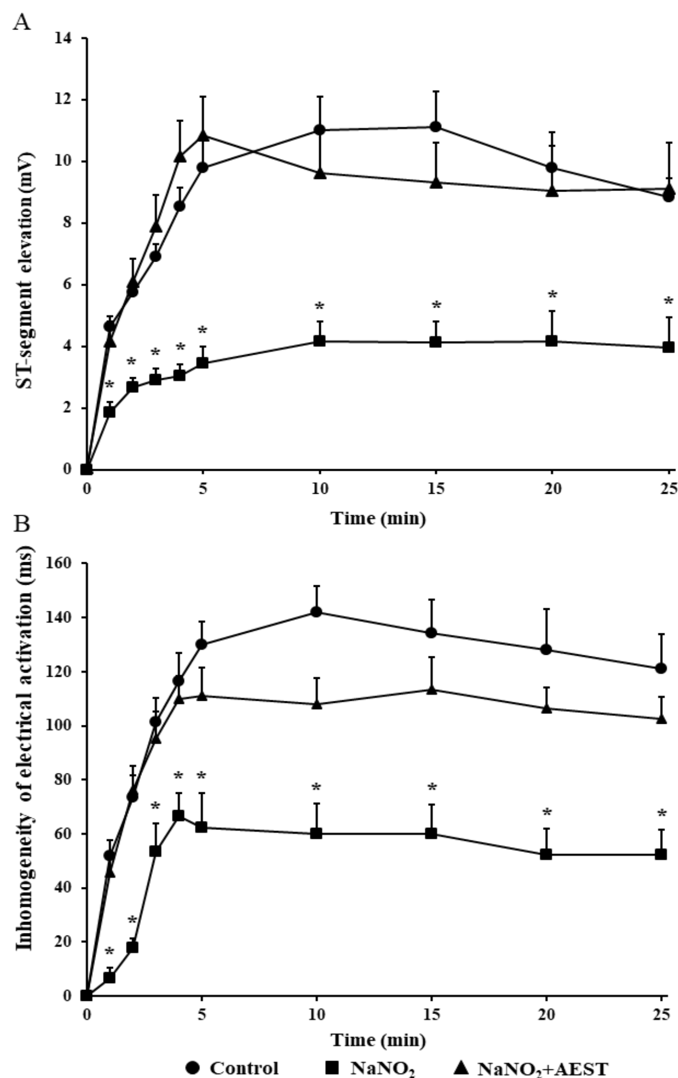

Figure 4. *Changes in the epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 25 min occlusion of the LAD. Values are mean ± SEM.* $^{*}P < 0.05$ *compared to the controls.*

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; i.e. before and after the infusion of $NaNO_2$ infusion, and 24 hours later before and 25 min after the coronary artery occlusion. There were no significant changes between the baseline NO values among the groups. 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; Figure 5). 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.

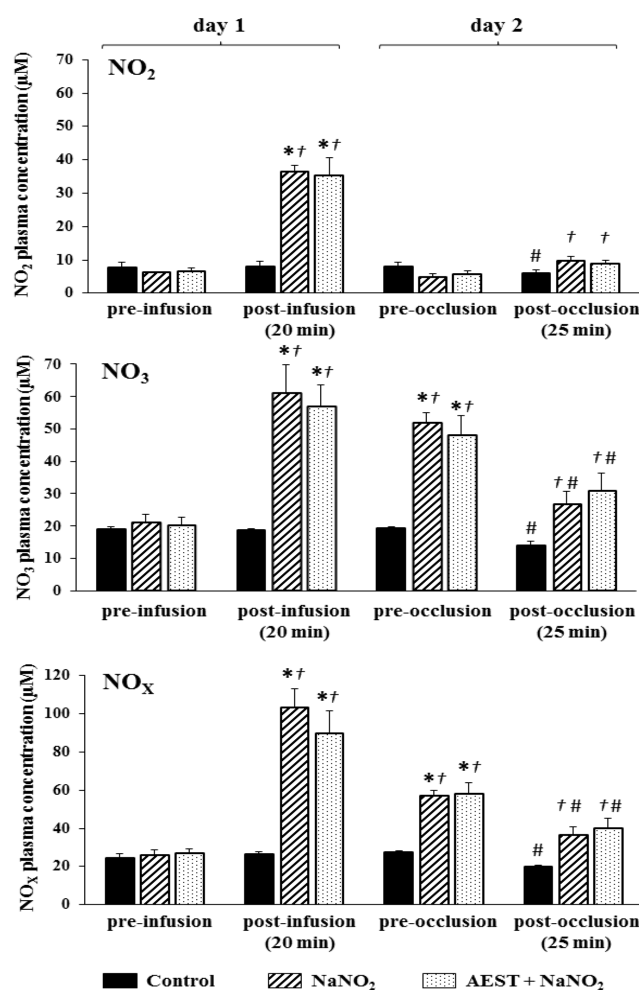

Figure 5. *Changes in plasma nitrate/nitrite (NO_x) concentrations. Values are means ± S.E.M. *P < 0.05 compared to the baseline (pre-infusion) value of the corresponding group, $^{\#}P$ < 0.05 compared to the pre-occlusion value of the corresponding group, and $^{\dagger}P$ < 0.05 compared to the control group.*

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 (Figure 6).

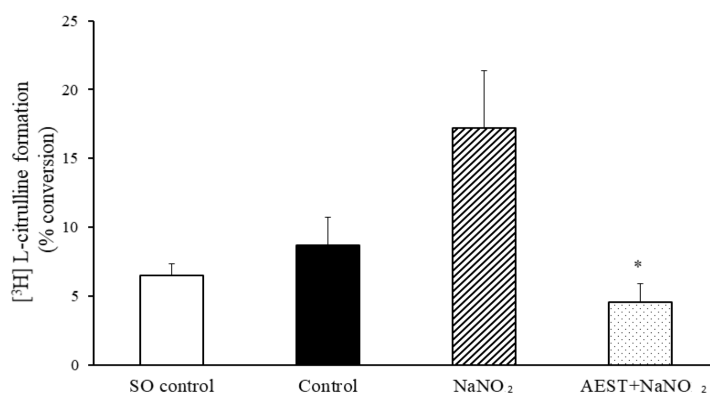

Figure 6. *Changes in iNOS enzyme activation, assessed by radioimmunoassay. Values are means ± SEM. *P<0.05 compared to the* $NaNO_2$ *group.*

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

These experiments planned to examine the involvement of changes in mitochondrial morphology and function in the delayed antiarrhythmic effect of sodium nitrite. Therefore, in four groups of dogs, each containing five animals, similar I/R protocols were performed as in the previous study. Since the haemodynamic alterations and the occurrence of arrhythmias, following ischaemia and nitrite administration were not significantly different from that we have previously observed, the results are not repeated here.

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

The representative images, taken by TEM, are shown in Figure 7, whereas the results obtained from the quantitative analysis of the pictures are illustrated in Figure 8 and summarized in Table 3.

The images show 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 could be observed (indicated by arrows, Figure 7), irrespective of their localization (SSM, PN and IMF). These alterations were less marked in dogs infused $NaNO_2$.

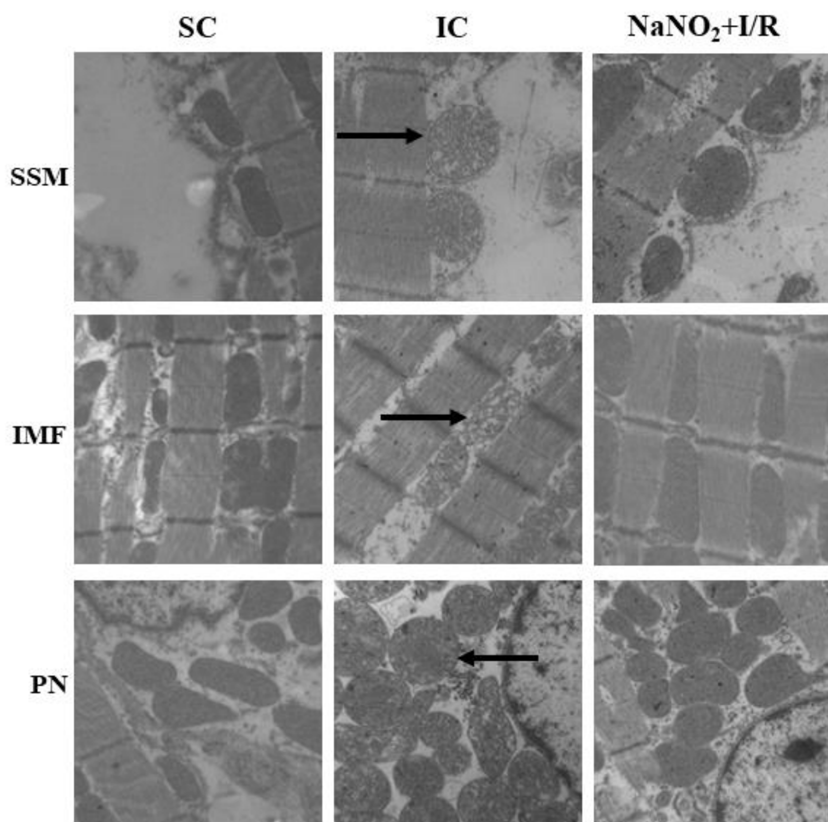

Figure 7. *Representative TEM pictures of the changes in the mitochondrial morphology. The images show that compared to the SC group, in dogs subjected to a 25 min I/R, swelling and disorganization of cristae of the mitochondrial matrix appeared in all the three examined regions (indicated by arrows).*

Data obtained following the quantitative analysis of mitochondria localized in the sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and perinuclear (PN) areas, are summarized in Table 3, whereas changes in the measured parameters in the IMF region are also shown in Figure 8. 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 (Table 3 and Figure 8). Sodium nitrite itself did not significantly changed the morphological parameters (Figure 8).

Table 3. Changes in morphology of three mitochondrial subsets

	Area (μm^2)	Perimeter (μm)	Feret diameter (μm)	Roundness
SC				
SSM	0.48±0.02	2.69±0.03	1.04±0.01	0.57±0.05
IMF	0.68±0.04	3.38±0.06	1.37±0.03	0.46±0.03
PN	0.49±0.02	2.77±0.09	1.09±0.04	0.58±0.02
IR				
SSM	$0.35±0.02^*$	$2.12±0.05^*$	$0.77±0.02^*$	$0.75±0.01^*$
IMF	$0.39±0.04^*$	$2.30±0.08^*$	$0.88±0.03^*$	$0.67±0.03^*$
PN	$0.39±0.02^*$	$2.26±0.05^*$	$0.82±0.02^*$	$0.75±0.02^*$
$NaNO_2$-IR				
SSM	$0.65±0.05^\#$	$3.15±0.12^\#$	$1.22±0.07^\#$	$0.58±0.07^\#$
IMF	$0.54±0.04^\#$	$2.88±0.06^\#$	$1.13±0.01^\#$	$0.53±0.04^\#$
PN	$0.51±0.02^\#$	$2.76±0.05^\#$	$1.04±0.02^\#$	$0.65±0.02^\#$
$NaNO_2$				
SSM	$0.58±0.06^\#$	$2.96±0.15^\#$	$1.13±0.05^\#$	$0.58±0.02^\#$
IMF	$0.67±0.08^\#$	$3.18±0.19^\#$	$1.22±0.03^\#$	$0.54±0.01^\#$
PN	$0.47±0.02^\#$	$2.64±0.08^\#$	$1.02±0.03^\#$	$0.60±0.02^\#$

Values are means ± S.E.M. $^*P < 0.05$ compared with SC; $^\#P < 0.05$ compared with IC. SSM: sub-sarcolemmal, IMF: inter-myofibrillar, PN: perinuclear.

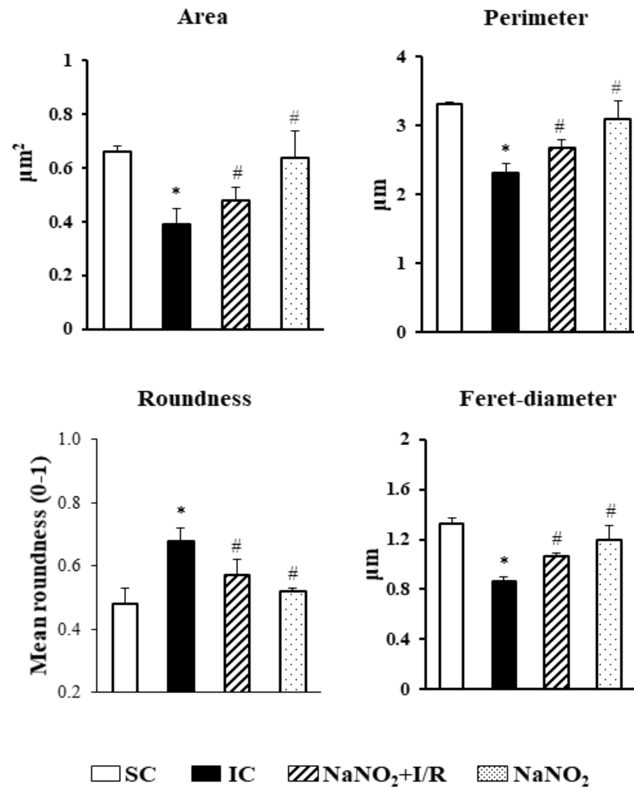

Figure 8. *Changes in mitochondrial morphology within the IMF region. Values are means ± S.E.M. $^*P < 0.05$ compared with SC; $^\#P < 0.05$ compared with IC.*

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. The results are shown in Figure 9 and Figure 10, respectively. 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 (Figure 9). These changes were less marked in case of the CII-dependent respiration (Figure 10). 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.

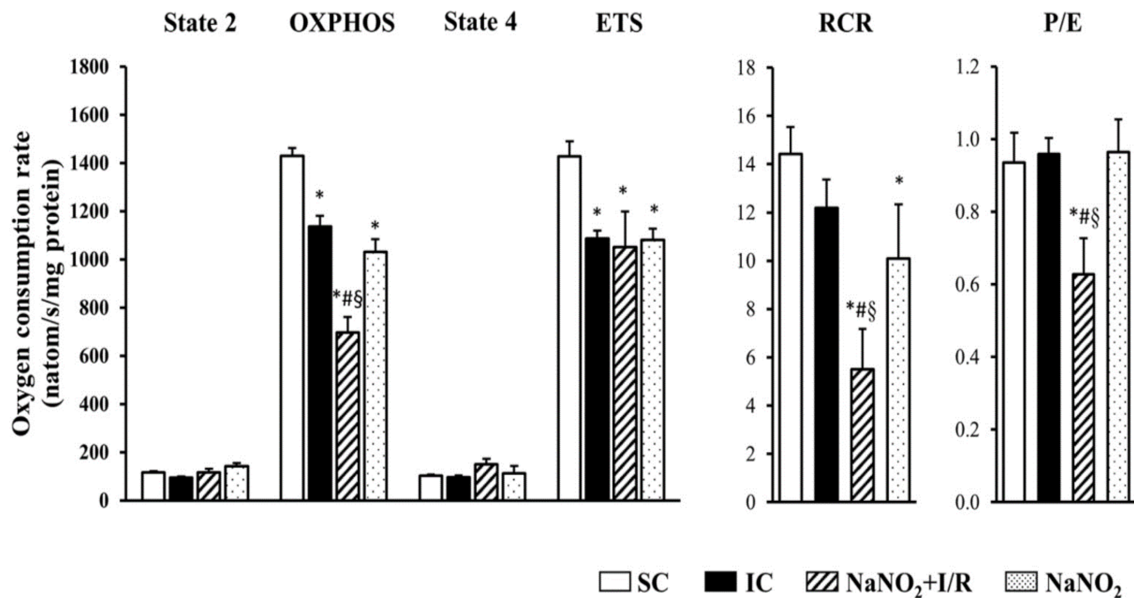

Figure 9. *The effect of $NaNO_2$ on CI-dependent mitochondrial respiration. Values are means ± S.E.M. $^{*}P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC, $^{\S}P < 0.0.5$ compared with nitrite alone.*

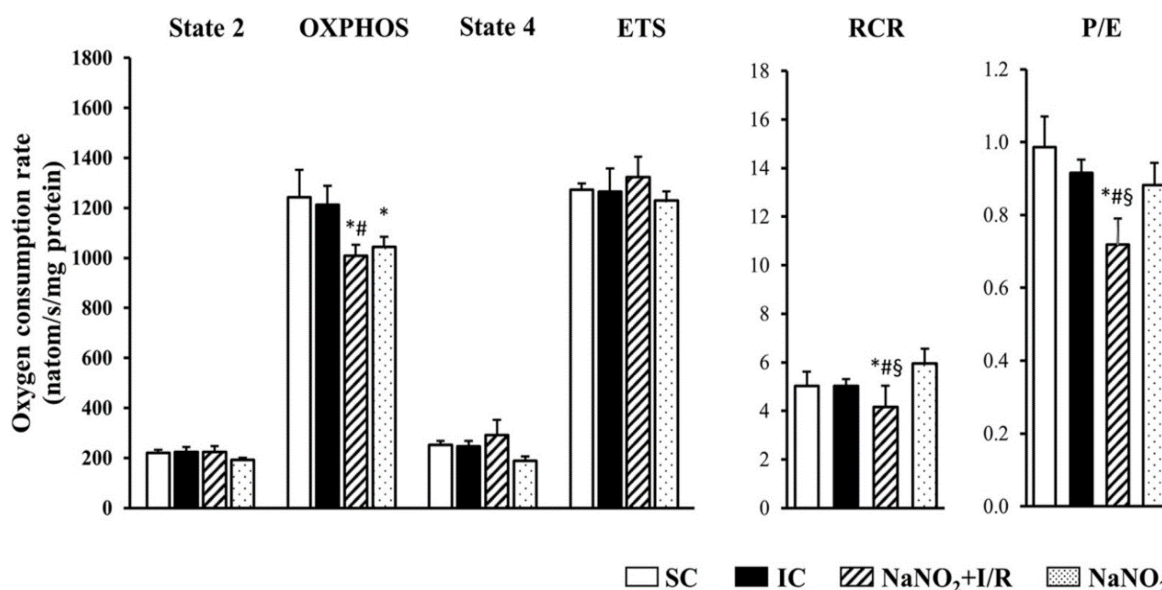

Figure 10. *The effect of $NaNO_2$ on CII-dependent mitochondrial respiration. Values are means ± S.E.M. $^{*}P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC, $^{\S}P < 0.0.5$ compared with nitrite alone.*

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

This is illustrated in Figure 11 and Figure 12. 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 (Figure 11).

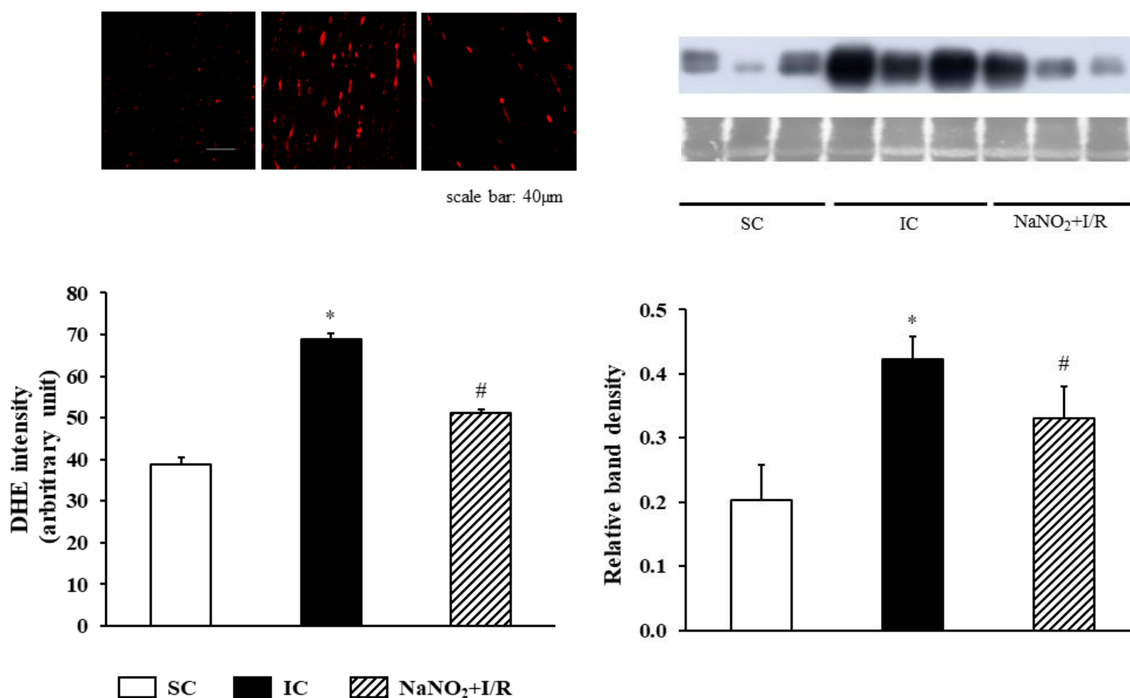

Figure 11. Changes in the tissue superoxide production. Values are means ± S.E.M. $^{*}P < 0.05$ *compared with SC;* $^{\#}P < 0.05$ *compared with IC.*

Figure 12. Changes in the tissue peroxynitrite production. Values are means ± S.E.M. $^{*}P < 0.05$ *compared with SC;* $^{\#}P < 0.05$ *compared with IC.*

Similarly, compared to the SC dogs, a 25 min occlusion and reperfusion insult significantly increased peroxynitrite production, as assed by changes in 3-nitrotyrosine (3-NT) formation (Figure 12). This increase in 3-NT formation was markedly reduced in the $NaNO_2$ treated dogs.

4. 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. Thus, first we have examined, whether $NaNO_2$, infused 24 hours before a coronary artery occlusion and reperfusion in anaesthetized dogs, provides protection against the severe ventricular arrhythmias. Furthermore, if so, we wanted to explore, whether the activation of iNOS enzyme would play a role in the antiarrhythmic effect of nitrite. There was previous evidence that in the delayed antiarrhythmic effect of preconditioning the NO-induced iNOS activation, and the subsequent increased NO formation plays a central role (Végh and Parratt 1996, Bolli et al., 1997, Kis et al., 1999a,b). Therefore, in the present study, the role of iNOS activation in the nitrite-induced protection was also examined by the administration of the iNOS enzyme inhibitor, AEST.

We have found that the infusion of $NaNO_2$, 24h prior to I/R, markedly reduced the severe ventricular arrhythmias during occlusion and increased survival following reperfusion. Since, the iNOS inhibitor AEST, although completely blocked the nitrite-induced iNOS activation, it only partially abolished the nitrite evoked antiarrhythmic effect, we concluded that in the delayed cardioprotective effect of nitrite other mechanisms than activation of iNOS, may play a role.

Therefore, we designed further studies, in which we have examined the role of the mitochondria in the delayed antiarrhythmic effect of nitrite. There were a few previous studies, which had already indicated that the modification of the mitochondrial respiration by S-nitrosylation of complexes of the respiratory chain would play an important role in the cardioprotective effects of nitrite (Shiva *et al.*, 2007; Shiva *et al.*, 2009). This mechanism by attenuating mitochondrial ROS production, similar to preconditioning (Shiva *et al.*, 2007), would be responsible for the protection. Since we had ample of evidence for the role of NO in preconditioning (Végh *et al.*, 1992b, Végh and Parratt, 1996, Kis *et al.*, 1999b), further there was a lack of evidence for the delayed effect of nitrite in large animal experiments, in a second study we have examined the role of the functional and structural changes of the mitochondria, following ischaemia and nitrite administration. We have found that $NaNO_2$ significantly reduced the I/R-induced morphological changes of the mitochondria. The administration of nitrite, however, caused a further depression in mitochondrial respiration resulting from I/R, without significantly modifying the rate of ATP production. The effect of nitrite on the mitochondrial respiratory complexes were examined previously, although this is

the first study which suggests that nitrite has an effect on the phosphorylation system. The influence of nitrite on the various components of the mitochondrial respiratory chain resulted in a marked reduction in the superoxide, and consequently on peroxynitrite productions under conditions of I/R, which mechanism certainly plays a role in the protection against arrhythmias.

According to the results of the two studies, the following conclusions can be drawn.

First, 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 been given 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.

Second, 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. This was given twice in nitrite treated dogs; i.e. prior to the infusion of sodium nitrite, and 24h later before the coronary artery occlusion. This protocol was similar to that we had used previously with ischaemic preconditioning (Kis *et al.*, 1999b; Hajnal *et al.*, 2005). 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 (Figure 3). 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 also reversed the anti-ischaemic effects of nitrite (Figure 4).

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; moreover, in the AEST treated dogs the iNOS activity was somewhat lower than in the sham-operated controls, 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 (Figure 5). 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 (Figure 5). 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 (Figure 5). 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 (Jansson *et al.*, 2008). 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 (Figure 5).

We conclude that following nitrite administration, the majority of nitrite is converted to nitrate, and it remains and circulates in this form over the next 24h period. We may speculate that even under oxygenated conditions, a part of the infused nitrite may convert to NO, which reductive process largely depends on the nitrite reductase activity of deoxy-haemoglobin (Hb) and xanthine oxidoreductase (Dejam et al., 2004). We also know that under ischaemic conditions the activity of the nitrite reductive mechanisms increases with decreasing pO_2, and pH, and with increasing NAD^+ concentration (Dejam et al., 2004). Although, we did not measure directly the NO formation and/or of nitrite reductase activity we have found an increase in iNOS activity 24h after nitrite administration, which almost certainly resulted from the enzyme stimulation by NO (Figure 5). This enzyme activation was completely abolished in the presence of AEST (Figure 6).

Our results show that under ischaemic conditions the heart of the nitrite treated dogs uses nitrate as a primary source for NO production via its reduction to nitrite and then to NO. This non-enzymatic NO formation provides adequate amount of NO during occlusion and the subsequent reperfusion to elicit protection against the ischaemic changes and arrhythmias. The fact that a part of the protection has still remained in the presence of AEST, further that the complete inhibition of the activity of iNOS did not modify the nitrite-induced effects on the concentration of NO metabolites suggest that iNOS, and the iNOS derived NO has only a minor role in the nitrite-evoked delayed antiarrhythmic protection during ischaemia. On the other hand, the fact that AEST markedly attenuated the nitrite-induced protection against the ischaemic changes and the reperfusion-induced severe ventricular arrhythmias suggests a role for iNOS-derived NO in the protective effect of nitrite. We suppose that this enzymatic NO formation becomes particularly important during reoxygenation, when the rapid change in the milieu stops the nitrate-nitrite-NO conversion that has provided NO during ischaemia.

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 (Figure 9 and Figure 10). 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 (Figure 11, Figure 12).

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 (Figure 11, Figure 12). 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 is substantial evidence that NO regulates ROS formation, and that this mechanism is largely involved in the protective effect of NO, for example, against those severe ventricular arrhythmias (Kiss et al., 2010), which occur during the first minutes of the reperfusion, when the burst of ROS is apparent (Xia and Zweier, 1997; Iwase et al., 2007; Burwell and Brookes, 2008). 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 (Clancy et al., 1992; 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 (St-Pierre et al., 2002; 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).

In summary, we propose that sodium nitrite provides a marked delayed antiarrhythmic and anti-ischaemic effect. This protection is manifested in a significant reduction in the number and severity of the I/R-induced serious ventricular arrhythmias, as well as in the ischaemic changes, such as the epicardial ST-segment and the degree of inhomogeneity of electrical activation 24h after the administration of nitrite. As concerns the mechanisms involved in the late cardioprotective effect of nitrite, the results suggest that in contrast to preconditioning the NO-induced iNOS activation plays only a minor role, whereas changes in mitochondrial morphology and respiration would be more important in the protection. We hypothesize that the preservation of the mitochondrial structure, the suppression of the mitochondrial

respiration with the subsequent reduction in ROS production during ischaemia would be protective and explain the antiarrhythmic effect of nitrite.

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.

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ANNEX

Reprints of full papers

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

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

I.

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ARTICLE

Is there a role of inducible nitric oxide synthase activation in the delayed antiarrhythmic effect of sodium nitrite?

Vivien Demeter-Haludka, László Juhász, Mária Kovác, János Gardi, and Ágnes Végh

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Abstract: This study aimed to examine whether inducible nitric oxide synthase (iNOS) plays a role in the delayed antiarrhythmic effect of sodium nitrite. Twenty-one dogs were infused intravenously with sodium nitrite (0.2 μmol·kg^{-1}·min^{-1}) for 20 min, either in the absence (n = 12) or in the presence of the iNOS inhibitor *S*-(2-aminoethyl)-isothiourea (AEST) (total dose 2.0 mg·kg^{-1} i.v., n = 9). Control dogs (n = 12) were given saline. Twenty-four hours later, all of the dogs were subjected to a 25 min period occlusion of the left anterior descending coronary artery followed by rapid reperfusion. Dogs treated with AEST and nitrite received again AEST prior to the occlusion. Compared with the controls, sodium nitrite markedly reduced the number of ectopic beats, the number and incidence of ventricular tachycardia, and the incidence of ventricular fibrillation during occlusion and increased survival (0% versus 50%) from the combined ischaemia and reperfusion insult. Although AEST completely inhibited iNOS activity, the nitrite-induced increase in NO bioavailability during occlusion was not substantially modified. Furthermore, AEST attenuated but did not completely abolish the antiarrhythmic effect of nitrite. The marked delayed antiarrhythmic effect of sodium nitrite is not entirely due to the activation of iNOS; other mechanisms may certainly play a role.

Key words: arrhythmias, sodium nitrite, delayed protection, ischaemia–reperfusion, nitric oxide.

Résumé : Cette étude portait sur le rôle éventuel de l'oxyde nitrique synthase inductible (iNOS) dans les effets antiarythmiques retards du nitrite de sodium. Nous avons perfusé par voie intraveineuse du nitrite de sodium (à 0,2 μmol·kg^{-1}·min^{-1}) chez 21 chiens pendant 20 min, en absence (n = 12) ou en présence de *S*-(2-aminoéthyl)-isothio-urée (AEST), un inhibiteur de l'iNOS (dose totale 2,0 mg·kg^{-1} i.v., n = 9). Nous avons administré une solution saline aux chiens témoins (n = 12). Vingt-quatre heures plus tard, nous avons procédé chez tous les chiens à une ligature de 25 min de l'artère interventriculaire antérieure, suivie d'une reperfusion rapide. Les chiens exposés à l'AEST et au nitrite ont de nouveau reçu de l'AEST avant l'occlusion. Par rapport au groupe témoin, le nitrite de sodium a entraîné une réduction importante du nombre de battements ectopiques, du nombre et du taux d'apparition de tachycardies ventriculaires ainsi que du taux d'apparition de fibrillations ventriculaires pendant l'occlusion. Le produit a aussi permis d'augmenter le taux de survie (0 % versus 50 %) après les interventions d'ischémie et de reperfusion combinées. Bien que l'AEST n'ait pas entraîné d'inhibition complète de l'activité de l'iNOS, l'augmentation de la biodisponibilité du NO provoquée par le nitrite pendant l'occlusion n'était pas modifiée de façon notable. De plus, l'AEST a entraîné une atténuation sans abolition complète des effets antiarythmiques du nitrite. L'effet antiarythmique retard marqué du nitrite de sodium n'est pas entièrement causé par l'activation de l'iNOS; d'autres modes d'action pourraient certainement avoir un rôle à jouer. [Traduit par la Rédaction]

Mots-clés : arythmies, nitrite de sodium, protection retard, ischémie–reperfusion, oxyde nitrique.

Introduction

There is experimental and clinical evidence that inorganic nitrite and nitrate, the natural products of nitric oxide (NO) metabolism, may serve as reservoirs of NO (Kevil and Lefer 2011). This would be particularly important under ischaemic conditions, when in the absence of oxygen, the generation of NO from nitric oxide synthase (NOS) enzyme activation is limited and when the drop in pH and oxygen tension favours the reduction of nitrite to NO (Zweier et al. 1995), thus providing better cardiac function during ischaemia (Lefer 2006; Lundberg et al. 2011).

Recently, we have shown that the intravenous infusion of sodium nitrite, in a concentration that does not significantly modify arterial blood pressure and coronary blood flow, profoundly reduced the severity of ventricular arrhythmias that resulted from a 25 min coronary artery occlusion and reperfusion in anaesthetized dogs (Kovács et al. 2015). This marked antiarrhythmic protection was associated with increased NO-mediated reduction in oxidative stress, perhaps through protein S-nitrosylation and (or) S-glutathionylation (Kovács et al. 2015).

We have evidence that in this dog model, preconditioning induced by various mechanical (coronary artery occlusion, cardiac pacing, exercise) (Végh et al. 1992*a*, 1994; Kis et al. 1999*a*, 1999*b*; Babai et al. 2002) and pharmacological stimuli (NO donors, statins) (György et al. 2000; Kisvári et al. 2014), results in a marked early and delayed antiarrhythmic effect (Végh et al. 1992*a*; György et al. 2000) and that this protection is associated with the maintenance of NO availability during ischaemia (Kiss et al. 2010). The importance of NO, both in the early and the delayed cardioprotection, is well established (Bolli et al. 1997; Végh et al. 1992*b*; Végh and Parratt 1996). For example, we know that in the delayed effect, NO, generated by the preconditioning stimulus via the rapid acti-

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vation of the endothelial NOS (eNOS), stimulates further NO synthesis by activating the inducible form of NOS (iNOS) (Bolli et al. 1997) and perhaps eNOS as well (Kovács et al. 2013). This mechanism is certainly involved in the late antiarrhythmic effect, since the inhibition of iNOS activation attenuates or even abolishes the protection (Végh et al. 1994; Kis et al. 1999*a*, 1999*b*; Hajnal et al. 2005).

There is also some evidence that sodium nitrite induces delayed protective effects in various experimental models (Shiva et al. 2007*b*). However, it is unknown whether it can evoke delayed protection against arrhythmias, and if so, what mechanisms would play a role. It has been proposed that nitrite causes early mitochondrial S-nitrosylation, which is stable for 24 h, and protects mitochondrial function, when the heart is subjected to ischaemia (Shiva et al. 2007*a*, 2007*b*). Although this mechanism seems to be very likely, we have now examined whether iNOS would also be involved in the delayed effect of nitrite. Therefore, we used *S*-(2-aminoethyl)-isothiourea (AEST), a relatively selective inhibitor of iNOS. The changes in the severity of ischaemia and arrhythmias in iNOS activity and in the plasma nitrate/nitrite levels were examined during the experiments.

Materials and methods

Ethics

The upkeep of the dogs was in accordance with Hungarian law (XVIII/VI/31) regarding large experimental animals, which conforms to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH publication No. 85-23, revised in 1996) and conformed to the European Parliament Directive 2010/63/EU. All animal experiments were supervised and approved by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (No. XIII/1211/2012) and the Ethical Committee for the Protection of Animals in Research of University of Szeged, Szeged, Hungary (No. I-74-5-2012).

Surgical procedures

Thirty-three adult mongrel dogs of either sex, with a mean body mass of 21 ± 4 kg, were used. The dogs were housed in a separated animal room (temperature 10–20 °C, humidity 40%–70%, lightening 12 h per day, two animals per pen) for 2 weeks and fed a standard diet and ad libitum access to water. Food was withdrawn 24 h before anesthesia.

On the first day, the dogs were lightly anaesthetized with sodium pentobarbitone (30 $mg \cdot kg^{-1}$, Euthasol 40% i.v. A.U.V.; Produlab Pharma B.V., Raamsdonksveer, The Netherlands). A polyethylene catheter was introduced into the jugular vein through which the drugs (sodium nitrite, AEST, saline) were administered intravenously. A Millar tip catheter (5F; Millar Instruments Inc., Houston, Texas) was positioned into the left carotid artery for monitoring changes in arterial blood pressure.

On the second day, the dogs were re-anaesthetized with a bolus injection of sodium pentobarbitone (30 $mg \cdot kg^{-1}$ i.v.) and the anesthesia was maintained with intravenous injections of a mixture of chloralose and urethane (60 and 200 $mg \cdot kg^{-1}$, respectively) (Sigma, St. Louis, Missouri). The depth of anaesthesia was monitored by the examination of the cornea and pain reflexes as well as by the measurement of blood pressure, and when it was necessary, a further bolus injection of the anaesthetic was given. The dogs were ventilated with room air using a Harvard respirator (Harvard Apparatus, Natick, Massachusetts) at a rate and volume sufficient to maintain arterial blood gases within normal limits (Végh et al. 1992). Body temperature was measured from the mid-oesophagus and maintained at 37 ± 0.5 °C.

A catheter (Cordis F4) was introduced into the right femoral artery to measure arterial blood pressure (systolic and diastolic). The Millar tip catheter (5F; Millar Instruments Inc., Houston, Texas), which had been introduced into the left carotid artery on day 1, was now pushed into the left ventricle (LV) for measuring LV systolic and end-diastolic pressure as well as LV positive and negative dP/dt_{max}. Through the right jugular vein, another catheter was positioned into the coronary sinus to obtain blood samples for the measurement of plasma nitrate/nitrite (NO_x) levels.

A thoracotomy was performed at the fifth intercostal space, the pericardium was transected, and the heart was explored. The anterior descending branch of the left coronary artery (LAD) was prepared for occlusion proximal to the first main diagonal branch.

The severity of myocardial ischaemia was assessed by the measurement of changes in the degree of electrical activation and in the epicardial ST segment using a composite electrode positioned within the potential ischaemic region as described in detail previously (Végh et al. 1992*a*). The composite electrode collects R waves from 28 epicardial points with a bipolar lead, and the degree of inhomogeneity of electrical activation is assessed as a time delay between the first and a last point activated under the electrode and expressed in ms. The electrode also contains four unipolar electrodes by which changes in the epicardial ST segment (in mV) are assessed. A chest lead II standard electrocardiogram (Plugsys Hemodynamic Apparatus; Hugo Sachs Electronik, March-Hugstetten, Germany) was also recorded to measure heart rate and assess the number and severity of arrhythmias.

Ventricular arrhythmias were evaluated according to the Lambeth Conventions (Walker et al. 1998) with a modification as previously outlined (Végh et al. 1992*a*). Thus, the total number of ventricular premature beats, the incidence and the number of episodes of ventricular tachycardia (VT), and the incidence of ventricular fibrillation (VF) were assessed during the occlusion period. During reperfusion, only the incidence of VF (which is a final event in this species) was determined. Dogs that were still alive 2 min after reperfusion (the end of the study) were considered to be survivors. Those dogs that survived reperfusion were euthanized by an excess dose of the anaesthetic. All recordings were assembled and evaluated by LabChart 7 software (AD Instruments Pty Ltd., Bella Vista, Australia).

Determination of iNOS activity

This was performed using the radioimmunoassay method. The preparation of the myocardial tissue samples (200 mg) was identical to that described previously (Kisvári et al. 2014). Total protein concentrations were determined by the method of Bradford.

The measurements were carried out in the absence of calcium and calmodulin using a NOS activity assay kit (Cayman Chemical, Ann Arbor, Michigan) based on the conversion of $[^3H]$L-arginine to $[^3H]$L-citrulline by NOS. To eliminate background NOS activity in control samples, 10 mmol of L-N^G-nitro-arginine, a nonspecific inhibitor of the NOS enzyme, was given to the reaction mixture before the addition of the tissue extract. A liquid scintillation counter (Wizard™; PerkinElmer, Waltham, Massachusetts) was used to detect the amount of radiolabeled L-citrulline formed during the reaction by iNOS and expressed as the percentage of the total counts corrected with the background counts per minute.

Assessment of plasma NO_x levels

This was determined by the Griess reaction as described previously (Kiss et al. 2010; Kisvári et al. 2014). Plasma samples were collected from the jugular vein (day 1) and after thoracotomy (day 2) from the coronary sinus at different time points as indicated in Fig. 1). The absorbance of the azo-compound was measured spectrophotometrically at 540 nm using a microplate reader (FLUOstar OPTIMA; BMG LABTECH GmbH, Ortenberg, Germany). The total NO_x concentration ($\mu mol \cdot L^{-1}$) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma, St. Louis, Missouri). Data were analyzed by OPTIMA software (Control and Data Analyses; BMG LABTECH GmbH, Ortenberg, Germany).

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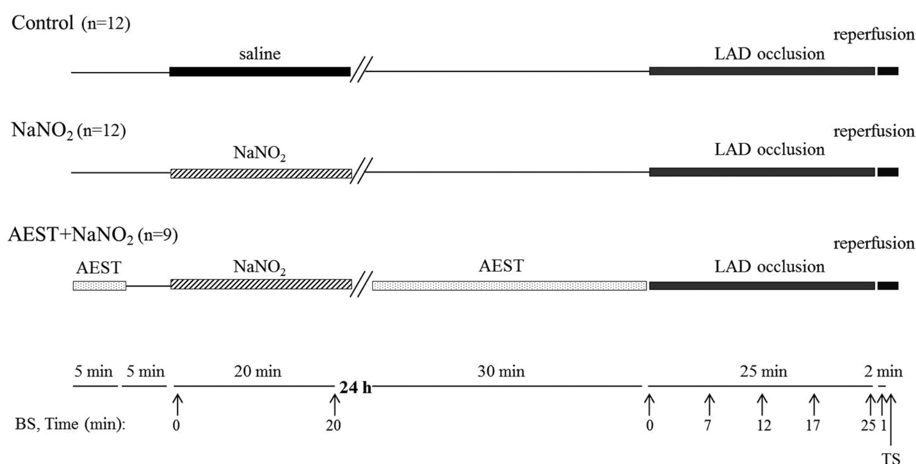
Fig. 1. Experimental protocol. Three groups of dogs were used. On day 1, control dogs (n = 12) received saline in intravenous infusion over a 20 min period. In 21 dogs, sodium nitrite was infused in a dose of 0.2 μmol·kg⁻¹·min⁻¹) for 20 min either in the absence ($NaNO_2$ group, n = 12) or in the presence of the iNOS inhibitor *S*-(2-aminoethyl)-isothiourea (AEST+$NaNO_2$ group, n = 9). AEST was slowly injected (over 5 min) intravenously in a total dose of 2 mg·kg⁻¹ 5 min before the commencement of the sodium nitrite infusion. Twenty-four hours later (day 2), all of the dogs were subjected to a 25 min occlusion of the anterior descending branch of the left coronary artery (LAD) followed by rapid reperfusion. In the AEST+$NaNO_2$ group, AEST was given again in intravenous infusion for 30 min, just prior to the coronary artery occlusion. During the experiments, blood samples (BS) were taken from the jugular vein before (0 min) and after (20 min) nitrite administration as well as 24 h later from the coronary sinus to determine changes in nitrate/nitrite levels before (0 min) and during (7, 12, 17, and 25 min) coronary artery occlusion as well as immediately (1 min) following reperfusion. Myocardial tissue samples (TS) were collected either 2 min after reperfusion (these animals were considered as survivors) or at the time when the fibrillation was observed for further biochemical analyses.



Experimental protocol

A total number of 33 dogs were used and randomly divided into three groups. On day 1, the dogs were slightly anaesthetized and infused intravenously either with saline (control group, n = 12) or sodium nitrite ($NaNO_2$ group, n = 21) (0.2 μmol·kg⁻¹·min⁻¹) for 20 min (Fig. 1). Twenty-four hours later, all of the dogs were subjected to a 25 min occlusion of the LAD followed by rapid reperfusion. In nine dogs out of the sodium nitrite treated dogs, the AEST+$NaNO_2$ group was slowly injected (over 5 min) intravenously in a total dose of 2 mg·kg⁻¹ 5 min before the commencement of the sodium nitrite infusion. In these dogs, 24 h later, the same dose of AEST was given again in intravenous infusion for 30 min, just prior to the coronary artery occlusion. The dose of AEST applied in the present study was identical to that used previously to inhibit iNOS activity (Kis et al. 1999). Since in that study, we proved that AEST given to control dogs (subjected only to ischaemia and reperfusion) does not modify arrhythmia severity (Kis et al. 1999), we did not include a separate AEST control group. Three dogs served as sham-operated controls (not included in the protocol figure); from these animals, myocardial tissue samples were collected to determine iNOS activity in healthy myocardium.

At the end of the experiments, the dogs were euthanized by an excess of the anaesthetic and myocardial tissue samples were collected either 2 min after reperfusion (these animals were considered as survivors) or at the time when the fibrillation was observed for further analyses (Fig. 1). Blood samples were also collected at different time points as indicated in Fig. 1. In some dogs (at least five dogs in each group), the size of the area, affected by the occlusion, was assessed using the same method that has been described in detail previously (Végh et al. 1992*a*). In brief, at the end of the experiments, the heart was removed and Patent Blue V dye was infused into the re-occluded LAD artery, whereas saline was infused into the patent's left circumflex artery at a pressure equivalent to that of the mean arterial pressure. The dyed area was cut out and weighed and the area at risk was expressed as a percentage of the left ventricular wall together with the septum.

Statistical analysis

The data were expressed as mean ± SEM and differences between means were compared by ANOVA for repeated measures and by one-way ANOVA as appropriate using the Fisher post hoc and Bonferroni tests. The number of ventricular premature beats and the number of episodes of VT were compared using the Kruskal–Wallis test. The incidence of VT and VF as well as survival from the combined ischaemia and reperfusion insult were compared using the Fisher exact test. Differences between groups were considered significant at $P < 0.05$.

Results

Haemodynamic effects of intravenously administered saline, sodium nitrite, and AEST

These are illustrated in Table 1. Compared with the saline-treated controls, the intravenous infusion of sodium nitrite resulted in significant reductions in arterial blood pressure and a slight increase in heart rate. AEST itself had no significant effect on any haemodynamic parameters, measured either on day 1, i.e., prior to the infusion of sodium nitrite, or on day 2, i.e., just prior to the occlusion, but significantly attenuated the haemodynamic effects of the intravenously administered sodium nitrite.

Haemodynamic changes following coronary artery occlusion

These are shown in Table 2. In all groups, occlusion of the LAD resulted in significant reductions in arterial blood pressure, LVSP, positive and negative dP/dt_{max}, and an increase in LVEDP, whereas the heart rate remained substantially unchanged. These haemodynamic alterations were somewhat less pronounced in dogs given sodium nitrite 24 h previously. The administration of AEST in the nitrite-treated dogs did not substantially modify the occlusion-induced haemodynamic changes compared with either the controls or the nitrite-treated dogs.

The severity of arrhythmias during coronary artery occlusion and reperfusion

The number and the incidence of various types of arrhythmias occurring during a 25 min occlusion are illustrated in Fig. 2. In

Table 1. Haemodynamic effects of saline, sodium nitrite, *S*-(2-aminoethyl)-isothiourea (AEST), and sodium nitrite plus AEST.

	Saline		$NaNO_2$		AEST[a] Day 1		AEST[a] Day 2		AEST+$NaNO_2$	
	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change
SABP (mmHg)	148±6	3±2	155±6	−11±4*	161±5	−1±4	142±4	5±6*	153±3	−3±3†
DABP (mmHg)	100±6	3±2	122±4	−7±2*#	129±4	−4±2*#	101±5	5±6*	117±3	−2±2#†
MABP (mmHg)	116±6	3±2	133±5	−8±3*	140±4	−3±2*#	115±4	4±6*	136±3	−3±2#†
HR (beats·min^{-1})	159±5	3±1	166±6	6±2*#	187±7	−13±3*#	166±2	−2±2*	173±4	−3±3#†

Note: Values are mean ± SEM calculated from n = 8 experiments. *P < 0.05 versus baseline value, #P < 0.05 versus control group, †P < 0.05 versus $NaNO_2$ group. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; HR, heart rate.

[a]These data represent the acute haemodynamic effects of AEST when it was given before the administration of sodium nitrite (day 1) and 24 h later (day 2) when it was infused for 30 min prior to occlusion in the nitrite-treated dogs.

Table 2. Haemodynamic changes during a 25 min occlusion of the anterior descending branch of the left coronary artery.

	Saline		$NaNO_2$		AEST+$NaNO_2$	
	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change
SABP (mmHg)	140±13	−17±3*	146±5	−11±4*	146±8	−14±3*
DABP (mmHg)	99±9	−18±3*	98±4	−11±5*	105±6	−12±3*
MABP (mmHg)	113±10	−17±2*	114±4	−11±3*	119±7	−13±4*
LVSP (mmHg)	141±9	−19±5*	148±12	−10±5*	146±9	−16±2*
LVEDP (mmHg)	6.0±1.1	7.3±1.3*	3.0±0.4	5.3±0.6*#	2.7±0.3	6.4±0.6*
$+dP/dt_{max}$ (mmHg·s^{-1})	2792±210	−720±84*	2906±136	−535±130*	3431±114	−710±130*
$-dP/dt_{max}$ (mmHg·s^{-1})	2526±164	−583±167*	2347±75	−166±112*#	2523±149	−535±62*
HR (beats·min^{-1})	167±6	4±4	161±8	−4±2	161±4	4±2

Note: Values are mean ± SEM calculated from n = 8 experiments. *P < 0.05 versus baseline value, #P < 0.05 versus saline-treated control group. AEST, *S*-(2-aminoethyl)-isothiourea; SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate.

control dogs, there were high numbers of ventricular premature beats and episodes of VT that were apparent in all the examined dogs. Furthermore, in five dogs out of the 12 (42%) control animals, VF occurred during the occlusion period and the rest of the dogs fibrillated on reperfusion; thus, no dog in this group survived the combined ischaemia and reperfusion insult. In contrast, dogs infused with sodium nitrite 24 h previously exhibited only a few ectopic beats and episodes of VT, which occurred in two of the nine (22%) nitrite-treated dogs. Furthermore, in this group, no dog fibrillated during occlusion and 50% of the animals survived reperfusion (Fig. 2). When AEST was given both prior to the infusion of nitrite (on day 1) and 24 h later, just before the occlusion, the number of ectopic beats and the episodes of VT as well as the incidence of VT during occlusion were again increased but still remained significantly less than in the untreated controls. Inhibition of iNOS with AEST did not significantly modify the protective effect of nitrite on the incidence of ischaemia-induced VF, i.e., in the presence of AEST, only one nitrite-treated dog out of the nine dogs (11%) fibrillated during occlusion. AEST, however, abolished the protective effect of nitrite on the reperfusion-induced VF. In the AEST+$NaNO_2$ group, only one dog (11%) survived the combined ischaemia and reperfusion insult.

The severity of ischaemia during coronary artery occlusion

This was assessed by measuring changes in the epicardial ST segment and in the degree of inhomogeneity of electrical activation (Fig. 3). In control dogs, both the epicardial ST segment (Fig. 3A) and the degree of inhomogeneity of electrical activation (Fig. 3B) were rapidly elevated during the first 5 min of the occlusion and reached a maximum value of around 11 mV and 130 ms, respectively, at the 5 min of ischaemia. The administration of sodium nitrite 24 h previously significantly suppressed these ischaemic changes; both the development and the absolute values of the elevation of the epicardial ST segment and of the degree of inhomogeneity were much slower and less than in the controls. The administration of AEST almost completely abolished the anti-ischaemic effects of sodium nitrite.

Changes in plasma NO_x levels

These were determined both before and after the intravenous administration of sodium nitrite from the venous blood and also 24 h later before and after the occlusion of the LAD in blood samples taken from the coronary sinus, as shown in the protocol in Fig. 1. In control dogs infused with saline intravenously, there were no significant differences between the baseline (pre-infusion) and the 20 min values of nitrate and nitrite levels. In contrast, the infusion of sodium nitrite significantly increased the plasma nitrate and nitrite as well as NO_x concentrations irrespective of whether AEST was present or not (Fig. 4).

Whereas on day 1, there were no significant differences between the baseline values of NO metabolites among the groups, on day 2, a marked difference occurred between the control and nitrite-treated dogs in the NO_x levels measured just prior to the occlusion in the blood of the coronary sinus. Thus, in both the nitrite and the AEST+$NaNO_2$ groups, there was a significant increase in the NO_x levels that resulted primarily from the marked elevation in the nitrate levels, since the nitrite concentrations were almost the same in all groups as the day 1 baseline values. This result indicates that sodium nitrite, under oxygenated conditions, occurred as nitrate in the blood 24 h later.

When the LAD coronary artery was occluded, in the control dogs, both the nitrate and the nitrite levels were significantly reduced, resulting in a marked decrease in NO_x by the end of the occlusion period. Compared with these changes, in the nitrite-

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Fig. 2. Number and incidence of ventricular arrhythmias (ventricular premature beats (VPBs), ventricular tachycardia (VT), and ventricular fibrillation (VF)) during a 25 min occlusion and reperfusion of the anterior descending branch of the left coronary artery in control, in sodium nitrite treated dogs ($NaNO_2$), and in dogs that were infused with nitrite in the presence of *S*-(2-aminoethyl)-isothiourea (AEST+$NaNO_2$). Compared with the controls, nitrite significantly reduced the number and incidence of ventricular arrhythmias during occlusion and increased survival on reperfusion. AEST partially reversed the antiarrhythmic effect of nitrite during occlusion, but it abolished the protection against the reperfusion-induced arrhythmias. Values are means ± SEM. *P < 0.05 compared with the controls, #P < 0.05 compared with the nitrite-treated dogs.

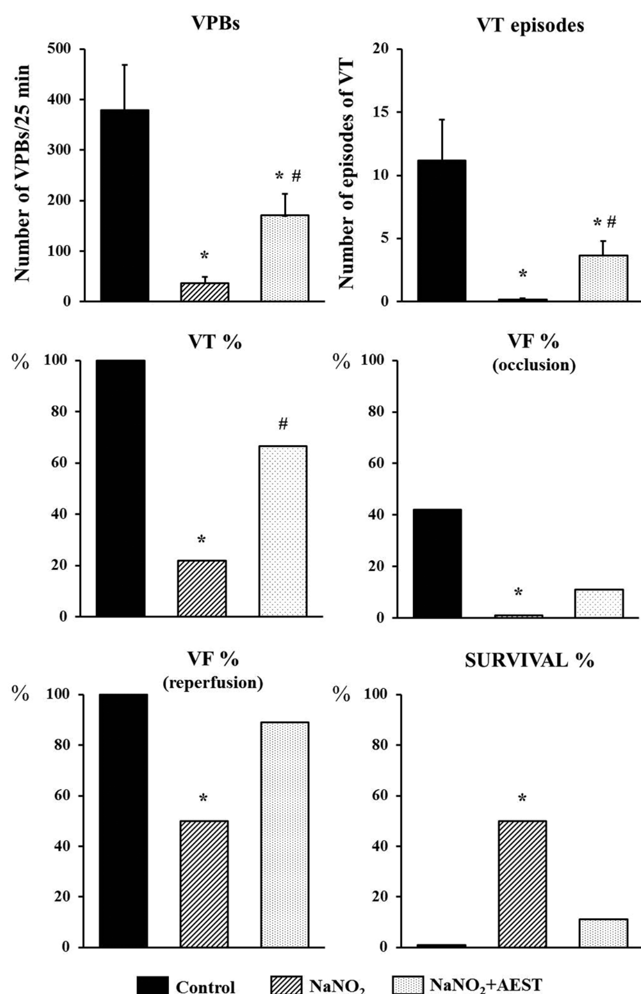
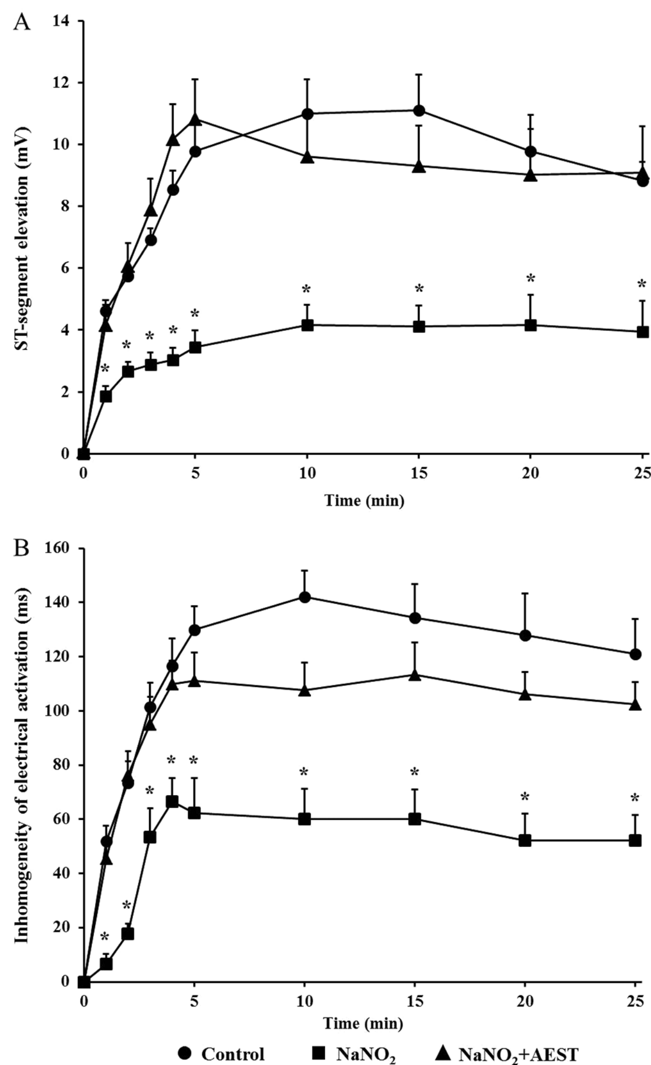


Fig. 3. Changes in the (A) epicardial ST segment and (B) degree of inhomogeneity of electrical activation during a 25 min occlusion of the anterior descending branch of the left coronary artery. Compared with controls, sodium nitrite significantly attenuated both indices of ischaemia severity. These effects of nitrite were markedly attenuated in the presence of AEST. Values are means ± SEM. *P < 0.05 compared with the controls.



treated dogs, the nitrate concentrations were considerable decreased and the nitrite concentrations increased during coronary artery occlusion, suggesting a possibility for nonenzymatic NO formation under reductive conditions. Since in these dogs, the reduction in the nitrate concentrations was more marked than the increase in nitrite concentrations, the NO_x levels also showed a decrease during the occlusion. Nevertheless, the NO bioavailability in the nitrite-infused dogs was significantly higher during ischaemia than in the saline-infused controls. The administration of AEST did not significantly modify the nitrite-induced changes in the level of NO metabolites.

The effect of nitrite on iNOS activity

This is illustrated in Fig. 5. Compared with the sham-operated controls, in samples taken from dogs subjected only to a 25 min occlusion and reperfusion insult, no significant changes could be observed in the activation of iNOS. The administration of nitrite resulted in a slight increase in iNOS activation, which was completely abolished by the administration of AEST.

Area at risk

There were no significant differences in the area at risk among the groups. Thus, the risk area was 36% ± 3% in the controls, 37% ± 3% in the nitrite-treated group, and 35% ± 4% in the AEST+$NaNO_2$-treated group.

Discussion

The aim of the present study was to test the hypothesis in our canine model of ischaemia and reperfusion whether the administration of sodium nitrite can produce a delayed antiarrhythmic effect, and if so, whether this effect, similar to preconditioning, involves the activation of iNOS. This question was raised because there is evidence, albeit in different models, that sodium nitrite evokes delayed cardioprotection (Shiva et al. 2007*b*; Shiva and

Fig. 4. Changes in nitrite (NO_2), nitrate (NO_3), and NO_x plasma levels determined in the venous blood before and after the administration of sodium nitrite and also 24 h later in the blood of the coronary sinus before and after the occlusion of the anterior descending branch of the left coronary artery (LAD). Compared with the pre-infusion values, the infusion of nitrite elevated the nitrite and nitrate levels, irrespective of whether *S*-(2-aminoethyl)-isothiourea (AEST) was present or not. Twenty-four hours later, the nitrite concentrations in all groups were similar to the normal, initial values, whereas the nitrate levels were markedly elevated in the nitrite-treated dogs. Occlusion of the LAD significantly reduced NO_x in all groups, but the levels of NO metabolites in the nitrite-treated dogs were significantly higher than in the controls. These changes were also independent from the presence of AEST. Values are means ± SEM. $^*P < 0.05$ compared with the pre-infusion (baseline) value of the corresponding group, $^{\#}P < 0.05$ compared with the pre-occlusion value of the corresponding group, and $^{\dagger}P < 0.05$ compared with the control group.

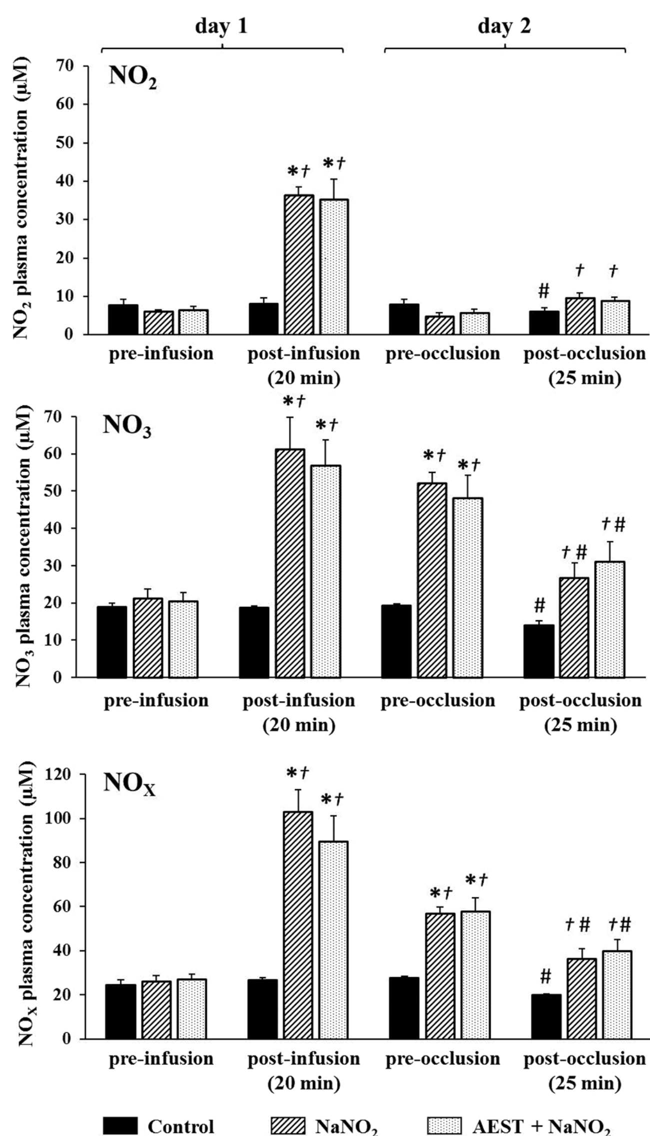
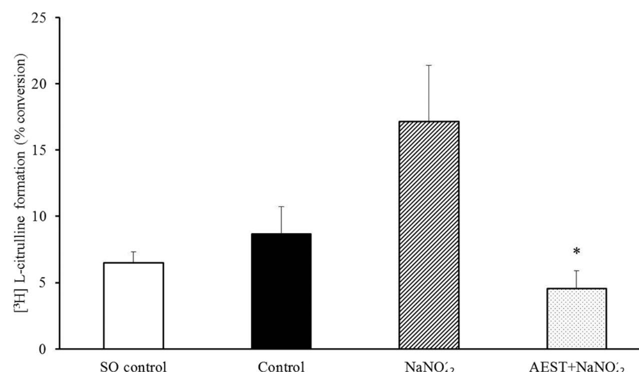


Fig. 5. Changes in iNOS enzyme activity determined by radioimmunoassay in the sham-operated (SO) control ($n = 3$), in the ischaemic control ($n = 6$), and in the sodium nitrite infused dogs without ($NaNO_2$, $n = 7$) and with the administration of *S*-(2-aminoethyl)-isothiourea ($NaNO_2$+AEST, $n = 6$). Compared with the SO group, there were no significant changes in iNOS activity in the ischaemic controls, but in the nitrite-treated dogs, a detectable, albeit statistically not significant, increase in enzyme activation could be observed. AEST completely inhibited the activation of iNOS resulting from nitrite administration. Values are mean ± SEM. $^*P < 0.05$ compared with the $NaNO_2$ group.



Gladwin 2009), but the mechanism by which this protection is attained is not well understood. We do not know whether nitrite itself or after converting to NO would elicit the protection. It is also not clear how this conversion will take place under physiological conditions. To give answers to at least a part of these questions, we first designed studies to examine the possibility of whether the activation of iNOS plays a role in the nitrite-induced delayed antiarrhythmic protection.

This hypothesis was based on those previous findings that clearly showed that iNOS has a crucial role in the delayed cardioprotective effects of preconditioning (Végh and Parratt 1996; Bolli et al. 1997). It was proposed that the preconditioning stimulus, via the activation of eNOS, enhances the formation of NO, which then stimulates iNOS (and most probably eNOS as well; Kovács et al. 2013), resulting in further NO generation (Bolli et al. 1997). This iNOS-derived enhanced NO production certainly contributes to the protection 24 h later when the hearts are subjected to an ischaemia and reperfusion challenge (Végh et al. 1994; Bolli et al. 1997). The evidence for this NO-induced NO formation via the activation of NOS enzyme isoforms comes from the direct measurement of enzyme activity (Bolli et al. 1997; Kovács et al. 2013) and from the use of the relatively selective inhibitors of iNOS, such as aminoguanidine (Kis et al. 1999*a*) or *S*-(2-aminoethyl)-isothiourea (Kis et al. 1999*b*; Hajnal et al. 2005). However, as to whether sodium nitrite infused 24 h prior to an ischaemia–reperfusion insult uses a similar pathway to induce protection is not known, and to the best of our knowledge, it has not yet been investigated.

We have found that sodium nitrite administered in dogs 24 h before coronary artery occlusion and reperfusion results in a significant antiarrhythmic effect. Thus, the number and incidence of the various types of arrhythmias, resulting from a 25 min occlusion and then reperfusion of the LAD, were markedly reduced compared with the untreated controls. Similarly, the infusion of nitrite significantly attenuated the ischaemic changes, assessed by measuring the epicardial ST segment and the degree of inhomogeneity of electrical activation. We have also found that AEST, given twice in dogs infused with sodium nitrite, only partially, but not completely, abolished the protective effects of nitrite against arrhythmias. 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 (Fig. 2). 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 also reversed the anti-ischaemic effects of nitrite (Fig. 3).

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Thus, we may conclude that the activation of iNOS might have a role in the delayed antiarrhythmic effect of sodium nitrite.

This conclusion is supported by the results obtained from the measurement of iNOS activity. These show that sodium nitrite increased (although it was statistically not significant) the activity of iNOS. This increase was completely abolished in the presence of AEST; moreover, in the AEST-treated dogs, the iNOS activity was somewhat lower than in the sham-operated controls. This result confirms the findings of our previous studies 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. 1999*b*). However, whereas AEST completely blocked the delayed antiarrhythmic effect of the preconditioning stimuli, such as cardiac pacing (Kis et al. 1999*b*) and treadmill exercise (Hajnal et al. 2005), it only attenuated the nitrite-induced late antiarrhythmic effect. This finding raises the possibility that in some aspects, there might be a difference between the preconditioning and the nitrite-induced protection, as regards 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. 1999*a*, 1999*b*); in the 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.

To examine the source of NO 24 h after the administration of sodium nitrite, we measured plasma nitrate and nitrite levels twice, i.e., on day 1 before and after the 20 min infusion of nitrite in the systemic blood and also on day 2 before and after a 25 min coronary artery occlusion in the blood of the coronary sinus. We 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 concentrations of NO metabolites were markedly increased by the end of the infusion period compared with the saline-infused controls (Fig. 4). Twenty-four hours later, when the level of these NO metabolites had been assessed again, now in the blood of the coronary sinus, we observed that in all groups, the nitrite levels were almost the same as the basal plasma nitrite concentrations 24 h previously (Fig. 4). In contrast, the nitrate concentrations were highly elevated in the nitrite-treated dogs compared with the controls. We think that by this time, nitrite had converted to nitrate and in the absence of food intake, the nitrate levels were only affected by the renal function over the 24 h observation period (Lauer et al. 2001). At this time, if the dogs had been subjected to coronary artery occlusion, the total NO_x concentrations were reduced in all groups compared with their corresponding pre-occlusion values, but in the nitrite-treated dogs, NO_x was significantly higher than in the untreated controls (Fig. 4). Furthermore, the reduction in NO_x of the nitrite-infused animals resulted mainly from the marked decrease in nitrate concentrations, whereas the nitrite levels were rather increased, indicating that nitrate reduced to nitrite and then possibly to NO during occlusion. 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 the nitrite concentrations (Fig. 4).

What conclusions we may draw from these results? First, it seems that following nitrite administration, the majority of nitrite is converted to nitrate and it remains and circulates in this form over the next 24 h period. We may speculate that even under normal, oxygenated conditions, a part of the infused nitrite may convert to NO, which reductive process largely depends on the nitrite reductase activity of deoxyhaemoglobin and xanthine oxidoreductase (Dejam et al. 2004). We also know that the activity of these nitrite reductive mechanisms increases with decreasing pO_2, and pH and with increasing NAD^+ concentration, which milieu is rather unusual under physiologic conditions (Dejam et al. 2004). Therefore, in the absence of direct measurement of NO formation and (or) of nitrite reductase activity, we are not able to provide evidence for the existence of such a nitrite to NO conversion in our experiments, but of course, the possibility of this mechanism cannot be excluded. The possible operation of these abovementioned nitrite reductase mechanisms (Lauer et al. 2001) in our experiments is supported by the fact that we have found an increase in iNOS activity 24 h after nitrite administration, which almost certainly resulted from the enzyme stimulation by NO (Fig. 5). This enzyme activation was completely abolished in the presence of AEST.

Second, our results show that under ischaemic conditions, the heart of the nitrite-treated dogs uses nitrate as a primary source for NO production via its reduction to nitrite and then to NO. This nonenzymatic NO formation provides an adequate amount of NO during occlusion and the subsequent reperfusion to elicit protection against the ischaemic changes and arrhythmias. The fact that a part of the protection, such as against the ischaemia-induced ventricular arrhythmias, has still remained in the presence of AEST and further that the complete inhibition of the activity of iNOS did not modify the nitrite-induced effects on the concentration of NO metabolites suggests that iNOS and the iNOS-derived NO have only a minor role in the nitrite-evoked delayed antiarrhythmic protection during ischaemia. On the other hand, the fact that AEST markedly attenuated the nitrite-induced protection against the ischaemic changes and the reperfusion-induced severe ventricular arrhythmias suggests a role for iNOS-derived NO in the protective effect of nitrite. We suppose that this enzymatic NO formation becomes particularly important during reoxygenation, when the rapid change in the milieu stops the nitrate–nitrite–NO conversion that has provided NO during ischaemia. Thus, under these conditions, the blockade of the additional NO source (iNOS) would result in the abolition of the nitrite-induced protection.

The first evidence that inorganic nitrites may provide delayed protection resulted from studies of Shiva and his colleagues (Shiva et al. 2007*a*, 2007*b*; Shiva and Gladwin 2009). They showed in various in vitro and in vivo models that nitrite administered 24 h prior to ischaemia produces similar protection by the same mechanism as nitrite given acutely (Shiva and Gladwin 2009). They proposed that nitrite through S-nitrosylation of mitochondrial proteins, particularly complex I, mediates both the early and delayed protection (Shiva et al. 2007*b*). It was suggested that mitochondrial S-nitrosylation occurs rapidly following the administration of nitrite and it remains stable for 24 h (Shiva and Gladwin 2009). The role of S-nitrosylation as a potential mechanism in the acute effect of nitrite has been confirmed in our own studies as well. We have shown that when sodium nitrite was administered either prior to and during the occlusion or just prior to reperfusion, it provided a marked antiarrhythmic protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias and this effect was associated with protein S-nitrosylation and glutathionylation (Kovács et al. 2015). Although the role of stable protein S-nitrosylation cannot be ruled out as one of the potential mechanisms for the explanation of the delayed cardioprotection resulting from nitrite administration (Shiva et al. 2007*b*), there is a lack of sufficient information on how this prolonged protein S-nitrosylation would take place. The results of studies in vitro from the same group (Shiva et al. 2007*a*) suggest that deoxymyoglobin, which nitrite reductase activity is much higher than deoxyhemoglobin to reduce nitrite, would be the candidate for conserving and generating NO via nitrite reduction under physiological conditions (Shiva et al. 2007*a*). Although in the present study, we did not assess protein S-nitrosylation, we are not fully convinced that the same mechanism operates under in

vivo conditions and fully explains the marked delayed antiarrhythmic effect of sodium nitrite. What we can propose as an alternative and (or) additional hypothesis for the delayed protective effect of nitrite that results from the NO_x measurements in our large animal experiments is that under physiological conditions, the majority of the infused nitrite converts to nitrate and in this form is stored over the next 24 h. This is supported by the fact that the nitrate, but not the nitrite, levels were markedly increased 24 h after the infusion of nitrite. As to whether mitochondrial protein S-nitrosylation would take place before the conversion of nitrite to nitrate we do not know yet, but the possibility of such a mechanism cannot be excluded. This warrants further examination in our model. 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. In contrast, the fact that AEST, even though it completely blocked the activity of iNOS, it did not modify the concentration of NO metabolites, and the occlusion-induced arrhythmias suggest 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 are attained, such as during coronary artery occlusion, when we could observed 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 is only attenuated but not completely abolished the protection. Exploration of the mechanisms that may contribute to the marked delayed antiarrhythmic effect of sodium nitrite warrants further investigations, which are in progress.

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II.

frontiers in Pharmacology

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Examination of the Role of Mitochondrial Morphology and Function in the Cardioprotective Effect of Sodium Nitrite Administered 24 h Before Ischemia/Reperfusion Injury

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Background: We have previous evidence that in anesthetized dogs the inorganic sodium nitrite protects against the severe ventricular arrhythmias, resulting from coronary artery occlusion and reperfusion, when administered 24 h before. The present study aimed to examine, whether in this effect changes in mitochondrial morphology and function would play a role.

Methods: Thirty dogs were infused intravenously either with saline ($n = 15$) or sodium nitrite (0.2 μmol/kg/min; $n = 15$) for 20 min, and 24 h later, 10 dogs from each group were subjected to a 25 min period of occlusion and then reperfusion of the left anterior descending coronary artery. The severity of ischaemia and ventricular arrhythmias were examined *in situ*. Left ventricular tissue samples were collected either before the occlusion (5 saline and 5 nitrite treated dogs) or, in dogs subjected to occlusion, 2 min after reperfusion. Changes in mitochondrial morphology, in complex I and complex II-dependent oxidative phosphorylation (OXPHOS), in ATP, superoxide, and peroxynitrite productions were determined.

Results: The administration of sodium nitrite 24 h before ischemia/reperfusion significantly attenuated the severity of ischaemia, and markedly reduced the number and incidence of ventricular arrhythmias. Nitrite also attenuated the ischaemia and reperfusion (I/R)-induced structural alterations, such as reductions in mitochondrial area, perimeter, and Feret diameter, as well as the increase in mitochondrial roundness. The administration of nitrite, however, enhanced the I/R-induced reduction in the mitochondrial respiratory parameters; compared to the controls, 24 h after the infusion of nitrite, there were further significant decreases, e.g., in the complex I-dependent OXPHOS (by −20 vs. −53%), respiratory control ratio (by −14 vs. −61%) and in the P/E control coupling ratio (by 2 vs. −36%). Nitrite also significantly reduced the I/R-induced generation of superoxide, without substantially influencing the ATP production.

Conclusions: The results suggest that sodium nitrite may have an effect on the mitochondria; it preserves the mitochondrial structure and modifies the mitochondrial function, when administered 24 h prior to I/R. We propose that nitrite affects primary the phosphorylation system (indicated by the decreased P/E ratio), and the reduction in superoxide production would result from the subsequent suppression of the ROS producing complexes; an effect which may certainly contribute to the antiarrhythmic effect of nitrite.

Keywords: ischaemia/reperfusion, arrhythmia, sodium nitrite, cardioprotection, mitochondrial structure, mitochondrial respiration

INTRODUCTION

We have previous evidence that the acute administration of sodium nitrite (0.2 μmol/kg/min; i.v.), protects against the ischaemia and reperfusion (I/R)-induced severe ventricular arrhythmias, in anesthetized dogs (Kovács et al., 2015). This protection was associated with protein S-nitrosylation (SNO) and glutathionylation by nitric oxide (NO) derived from nitrite (Kovács et al., 2015). More recently, we have reported that sodium nitrite, administered 24 h prior to a similar period of I/R, evokes also an antiarrhythmic effect (Demeter-Haludka et al., 2017). This particular study has also examined whether this post-poned effect of nitrite against arrhythmias involves the mechanism of the nitric oxide (NO)-induced iNOS activation, which is known to play a significant role in the preconditioning-induced delayed cardioprotection (Végh and Parratt, 1996; Bolli et al., 1997). We have found that, in contrast to preconditioning, where the pharmacological inhibition of iNOS by S-(2-aminoethyl)-isothiourea completely abolished the delayed antiarrhythmic protection (Kis et al., 1999a,b; Babai et al., 2002), the nitrite-induced effect was only partially diminished following iNOS inhibition (Demeter-Haludka et al., 2017). This finding suggested that the nitrite-induced cardioprotective effect that occur 24 h after nitrite administration may involve additional mechanisms, which are most probably independent from the activation of iNOS (Demeter-Haludka et al., 2017).

There is some previous evidence for the late occurring cardioprotective effect of sodium nitrite in various *in vivo* and *in vitro* models of ischaemia and reperfusion (Shiva et al., 2007a,b; Shiva and Gladwin, 2009). For example, it has been found that sodium nitrite administered in rats, 24 h prior to I/R, reduced myocardial infarct size and hepatic reperfusion injury (Shiva et al., 2007a). This protection was attributed to a stable post-translational modification of the mitochondrial complexes (particularly complex I) via S-nitrosylation (Shiva et al., 2007b). Since there were no changes in mitochondrial respiration and ATP generation of the hepatic mitochondria, isolated from the nitrite treated rats until subjected them to anoxia and re-oxygenation (Shiva et al., 2007a), it was concluded that the rapid and prolonged S-nitrosylation of mitochondrial proteins, plays an important role in the delayed protective effect of nitrite (Shiva and Gladwin, 2009).

Starting from the assumption that the target of the cardioprotective effect of nitrite might be a mitochondria-mediated process, we designed studies, in which changes in mitochondrial morphology and in respiratory function were examined in dogs undergoing a 25 min period of coronary artery occlusion and reperfusion, 24 h after the administration of sodium nitrite.

Abbreviations: 3-NT, 3-nitrotyrosine; ADP, Adenosine 5′-diphosphate; ATP, Adenosine 5′-triphosphate; CI, Mitochondrial respiratory chain complex I; CII, Mitochondrial respiratory chain complex II; CIII, Mitochondrial respiratory chain complex III; CytC, Cytochrome c; DABP, Diastolic arterial blood pressure; DHE, Dihydroethidium; ETS, Electron transport system; FCCP, Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone; HR, Heart rate; IC, Ischaemic control group; I/R, Ischaemia and reperfusion; IMF, Inter-myofibrillar; LAD, Left anterior descending coronary artery; LV, Left ventricle; LVEDP, Left ventricular end-diastolic pressure; LVSP, Left ventricular systolic pressure; MABP, Mean arterial blood pressure; MPTP, Mitochondrial permeability transition pore; NO, Nitric oxide; OXPHOS, Oxidative phosphorylation; PN, Perinuclear; RCR, Respiratory control ratio; RLU, Relative luminescence unit; ROS, Reactive oxygen species; SABP, Systolic arterial blood pressure; SC, Sham-operated control group; SNO, S-nitrosylation; SSM, Sub-sarcolemmal; TEM, Transmission electronmicroscopy; VF, Ventricular fibrillation; VPBs, Ventricular premature beats; VT, Ventricular tachycardia

MATERIALS AND METHODS

Ethics

The upkeep of the dogs was in accordance with Hungarian law (XVIII/VI/31) regarding large experimental animals, which conforms to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH publication No.85-23, revised in 1996), and conformed to the European Parliament Directive 2010/63/EU. All animal experiments were supervised and approved by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (No.XIII/1211/2012) and the Ethical Committee for the Protection of Animals in Research of University of Szeged, Szeged, Hungary (No.XIII./4657/2016).

Surgical Procedures

Thirty adult mongrel dogs of either sexwith a mean body weight of 22 ± 4 kg were used. The animals were housed in a separated animal room (temperature: 10–20°C, humidity: 40–70%, lightening: 12 h per day, 2 animals per pen) for 2 weeks and fed a standard diet and *ad libitum* access to water. Food was withdrawn 24 h before anesthesia. The surgical interventions were as the same as described previously (Végh et al., 1992;

Demeter-Haludka et al., 2017). In brief, on day one, the dogs were lightly anesthetized with intravenous sodium pentobarbitone (30 mg/kg; Euthasol 40%, Produlab Pharma B.V., Netherlands), and a polyethylene catheter was introduced into the jugular vein for the administration of saline and sodium nitrite. A Millar tip catheter (5F, Millar Instruments Inc., USA) was also positioned into the left carotid artery to measure changes in arterial blood pressure. Twenty-four hours later (on day 2), the dogs were re-anesthetized with a bolus injection of sodium pentobarbitone (30 mg/kg, i.v.), and the anesthesia was maintained with intravenous injections of a mixture of chloralose and urethane (60 and 200 mg/kg respectively; Sigma, USA). The depth of anesthesia was monitored, and when it was necessary, a further bolus injection of the anesthetic was given. The dogs were ventilated with room air using a Harvard respirator (Harvard Apparatus, USA) at a rate and volume sufficient to maintain arterial blood gases within normal limits (Végh et al., 1992). Body temperature was measured from the mid-esophagus and maintained at $37 \pm 0.5°C$.

A Cordis F4 catheter was introduced into the right femoral artery to measure arterial blood pressure, whereas the Millar tip catheter, introduced previously into the left carotid artery, was pushed into the left ventricle (LV) to measure LV systolic and end-diastolic (LVEDP) pressure, as well as the LV positive and negative dP/dt_{max}. After thoracotomy, the left anterior descending (LAD) coronary artery was prepared for occlusion proximal to the first main diagonal branch. Myocardial ischaemia was induced by a 25 min period of LAD occlusion, followed by 2 min reperfusion (Végh et al., 1992). The severity of ischaemia was assessed by measuring changes in the degree of inhomogeneity of electrical activation (expressed in milliseconds) and in the epicardial ST-segment (expressed in mV), using a composite electrode positioned within the ischaemic area (Végh et al., 1992; Demeter-Haludka et al., 2017). A chest lead II standard electrocardiogram was recorded to measure heart rate (HR) and to assess the severity of arrhythmias, such as the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT), the incidence of ventricular fibrillation (VF) during occlusion, and the incidence of VF following reperfusion (Végh et al., 1992). Dogs that were still alive 2 min after reperfusion were considered to be survivors. These dogs were euthanized by an excess dose of the anesthetic 2 min after reperfusion. All parameters were recorded (Plugsys Hemodynamic Apparatus; Hugo Sachs Electronik, Germany), stored and evaluated by LabChart 7 (AD Instruments, Australia) software.

In Vitro Measurements

Assessment of Mitochondrial Morphology

This was performed by transmission electron-microscopy (TEM). Blocks of fresh tissue samples ($1\ mm^3$), excised from the ischaemic region, were fixed in Karnovsky solution (Karnovsky, 1965) for 240 min at room temperature, rinsed and post-fixed in 2% OsO_4 (Millonig, 1961). After dehydration with ethanol, the samples were embedded in epoxy resin (Durcupan ACM, Sigma, USA) and polymerized at 56°C for 2 days. Ultrathin sections (50 nm) were prepared and contrasted with uranyl acetate (Hayat, 1970) and lead citrate (Reynolds, 1963). Transmission electron-microscope (Zeiss CEM 902, Germany) was used in conventional transmission mode (80 keV) to capture sub-sarcolemmal (SSM), perinuclear (PN) and inter-myofibrillar (IMF) mitochondria, using a Spot RT 14.0 CCD camera (Diagnostic Instruments, USA) at 12,000 x magnifications. Five images were taken from each area per samples, and the mitochondria were segmented with ImageJ 2 (FIJI; NIH, Bethesda, USA). Changes in mitochondrial morphology were evaluated using the built-in applications of ImageJ 2; such as we determined the area (μm^2) and perimeter (μm), the measures of the size of the mitochondria, as well as the roundness ($4x[Area]/(\pi x[Major\ axis]^2)$) and the Feret diameter (μm), which describe the level of circularity and the shape of the mitochondria. Data obtained from the five images in each animal were averaged, and the results obtained from the individual dogs within a certain group were also averaged. These values served for comparison among the groups.

Assessment of Mitochondrial Respiration and ATP Production

Mitochondrial respiration was measured by Clarke-type oxygen electrode (Strathkelvin 782 oxygen system, Strathkelvin, Germany). Tissue samples collected from the ischaemic area was homogenized in isolation medium (Grainer, Strathkelvin, Germany), containing trypsin and sucrose, and the mitochondria were separated by centrifugation. The concentrations of the mitochondrial proteins were determined by the method of Bradford.

The respiratory parameters for CI and CII were determined as described previously (Duicu et al., 2013a,b). We measured the basal respiration (State 2), the active respiration (OXPHOS; State 3), the capacity of the inhibition of OXPHOS (State 4; oligomycin, $2\ \mu M$, Sigma, USA) and the electron transport system (ETS). The intactness of the outer mitochondrial membrane (Pc) was evaluated by the administration of $10\ \mu M$ cytochrome C (Sigma, USA). The uncoupling was determined using carbonyl-cyanide-p-(trifluoro-methoxy) phenyl-hydrazone (FCCP; $0.5\ \mu M$, Sigma USA). Antimycin A (Sigma, USA) was administered to assess the residual oxygen consumption. From the measured parameters the respiratory control ratio (RCR = OXPHOS/State4) and the P/E coupling control ratio (OXPHOS/ETS) were calculated. The measurements were repeated three times in each sample per dog, and the results were averaged. Data obtained from the individual dogs within a group were also averaged, and these means served for the comparison among the groups.

Mitochondrial ATP production was assessed by bioluminescence assay, using an ATP Determination Kit (Invitrogen, USA) according to the manufacturer's protocol. Malate and pyruvate (Sigma, USA) were used as substrates. The emitted light was measured with luminescent optic using a micro plate reader (FLUOstar OPTIMA, Germany). Data were expressed as relative luminescence units (RLU). Three samples in each dog were evaluated and then averaged within a certain group. These means were compared among the groups.

Assessment of Tissue Superoxide Production

Superoxide production was determined as described previously (Kiss et al., 2010). The preparation of tissue samples, collected from the ischaemic and non-ischaemic areas within 2 min of the reperfusion. Longitudinal cryosections (20 μm) were cut, stained with dihydroethidium (DHE, 10 μM, Sigma, USA). N-acetyl-L-cysteine (100 mM, Sigma, USA) was used as a negative control. Both from the stained and negative control samples, ten images were captured by a confocal laser scanning microscope (Olympus FV 1000, Japan). The intensity of the fluorescent signals was analyzed by ImageJ, and expressed in arbitrary units. The intensity values, evaluated from four images in each dogs, were averaged, and data obtained from dogs within a certain group were also averaged. These values served for comparison among the groups.

Assessment of Peroxynitrite Production

This was assessed by measuring 3-nitrotyrosine (3-NT) formation using Western blot. Tissue samples (70 mg), taken from the ischaemic myocardium within 2 min of reperfusion, were prepared as described previously (Kiss et al., 2010). The formation of 3-NT was assessed from 25 μg of total protein loaded onto SDS-PAGE gel (10%) and transferred to PVDF membrane. Mouse monoclonal anti-nitrotyrosine was used as primary antibody (diluted to 1:3000; Chemicon, Millipore, USA), and horseradish peroxidase-conjugated rabbit anti-mouse IgG (diluted to 1:1000, Dakocytomation, Denmark) was used as a secondary antibody. The blot was developed with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare, UK), exposed to X-ray film and scanned. The intensity of the 3-NT bands was determined using Image J software, and expressed in percentage of the sham-operated animals. Equal loading of the samples was controlled by Coomassie Brilliant Blue staining, and normalized for total protein. Protein samples, isolated from four dogs in each experimental group, were used for western blot. The measurements were repeated three times in each dog, and the results were averaged. Data obtained from the individual dogs within a group were also averaged, and these means served for the comparison among the groups.

Experimental Protocol

Thirty dogs of both sexes were randomly divided into four groups. On day one, 15 dogs (7 female and 8 male) were infused intravenously with saline, and another 15 dogs (6 female and 9 male) with sodium nitrite (0.2 μmol/kg/min) for 20 min. Twenty-four hours later, 10 control (IC) and 10 nitrite ($NaNO_2$+I/R) treated dogs underwent a 25 min period of LAD occlusion followed by rapid reperfusion. In 5 nitrite ($NaNO_2$) and in 5 saline (SC) treated dogs (both groups contained 2 female and 3 male, undergoing the same surgical interventions, without subjecting them to I/R), the hearts were removed 24 h after nitrite and saline administration, respectively.

At the end of the experiments, the hearts were stopped by an excess of anesthetic, removed and myocardial tissue samples were taken for *in vitro* analyses. In dogs that were fibrillated on reperfusion, the samples were collected at the time of the fibrillation observed. The samples were either immediately used (for the mitochondrial measurements) or frozen in liquid nitrogen and stored on −80°C. In 4 or 5 dogs from the IC and $NaNO_2$+I/R groups, the "risk area" was assessed using Patent Blue V dye, as described previously (Végh et al., 1992; Demeter-Haludka et al., 2017).

Statistical Analysis

The data were expressed as mean ± SEM, and differences between means were compared by Welch-ANOVA for repeated measures the Bonferroni-Holm *post-hoc* test. The number of VPBs and the number of episodes of VT were compared using the Kruskal-Wallis test. The incidence of VT and VF, as well as survival from the combined I/R insult was compared by the Fisher Exact test. Differences between groups were considered significant at $P < 0.05$.

RESULTS

Haemodynamic Changes Following Nitrite Administration and Coronary Artery Occlusion

The intravenous infusion of sodium nitrite significantly reduced the mean arterial blood pressure from 132 ± 5 to 122 ± 6 mmHg ($P < 0.05$), without a substantial increase in the heart rate (from 167 ± 7 to 168 ± 11 beats/min). Twenty-four hours later, when the dogs had been subjected to a 25 min period of occlusion, there were similar changes in most of the haemodynamic parameters, except that the increase in LVEDP and the decrease in negative dP/dt_{max} were significantly less in the nitrite than in the saline infused dogs (**Table 1**).

The Administration of Sodium Nitrite Reduces the Number and Incidence of Ventricular Arrhythmias During Coronary Artery Occlusion and Reperfusion

This is illustrated in **Figure 1**. Control dogs, showed a great number of VPBs and episodes of VT that occurred in all dogs

TABLE 1 | Haemodynamic changes during a 25 min occlusion of the LAD.

	Saline		$NaNO_2$	
	Baseline	Max. change	Baseline	Max. change
SABP (mmHg)	145 ± 14	−17 ± 3*	141 ± 4	−10 ± 5*
DABP (mmHg)	101 ± 10	−18 ± 3*	97 ± 4	−11 ± 6*
MABP (mmHg)	116 ± 11	−17 ± 2*	111 ± 3	−11 ± 5*
LVSP (mmHg)	139 ± 11	−25 ± 5*	143 ± 13	−9 ± 6*
LVEDP (mmHg)	6.6 ± 1.0	7.1 ± 1.4*	4.4 ± 1.6	5.4 ± 0.7*#
$+dP/dt_{max}$(mmHg/s)	2869 ± 226	−769 ± 78*	2839 ± 138	−557 ± 148*
$-dP/dt_{max}$ (mmHg/s)	2609 ± 163	−574 ± 193*	2295 ± 63	−147 ± 118*#
HR (beats/min)	168 ± 7	5 ± 5	165 ± 8	−5 ± 2

*Mean ± SEM, calculated from n = 10 experiments.*P < 0.05 vs. baseline value, #P < 0.05 vs. saline treated control group. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate.*

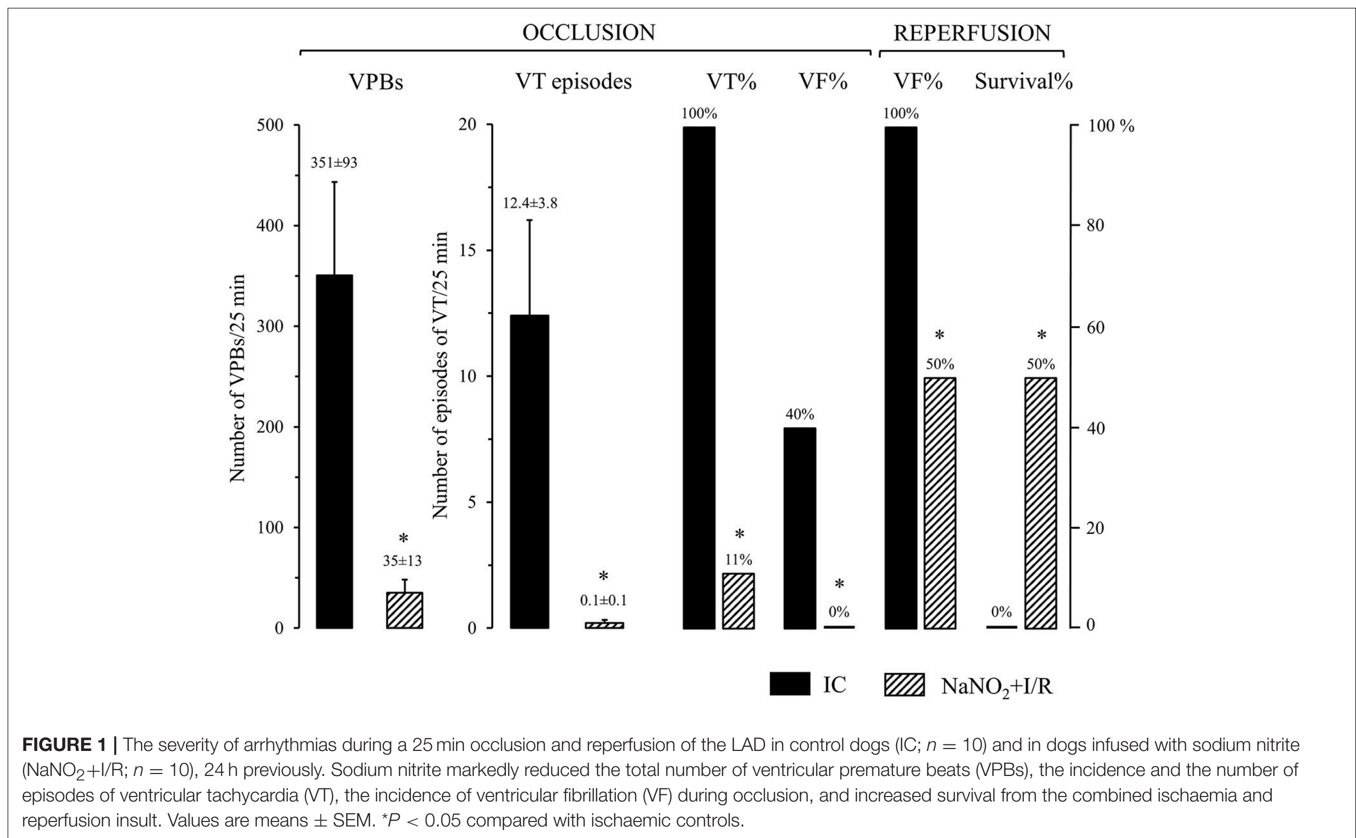

FIGURE 1 | The severity of arrhythmias during a 25 min occlusion and reperfusion of the LAD in control dogs (IC; $n = 10$) and in dogs infused with sodium nitrite ($NaNO_2$+I/R; $n = 10$), 24 h previously. Sodium nitrite markedly reduced the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT), the incidence of ventricular fibrillation (VF) during occlusion, and increased survival from the combined ischaemia and reperfusion insult. Values are means ± SEM. *$P < 0.05$ compared with ischaemic controls.

(100%) during the 25 min LAD occlusion. Further, four animals out of the 10 (40%) fibrillated during the occlusion and all the remaining dogs fibrillated on reperfusion; thus no control dog survived the combined I/R insult. In contrast, dogs infused with sodium nitrite 24 h previously, exhibited significantly less number of VPBs and episodes of VT that occurred in only 1 dog (10%) during the occlusion period. Moreover, no dog in the nitrite treated group fibrillated during the occlusion and 50% of the dogs survived reperfusion.

The Administration of Sodium Nitrite Attenuates the Severity of Ischaemia During Coronary Artery Occlusion

This was assessed by measuring changes in the epicardial ST-segment and the degree of inhomogeneity of electrical activation during a 25 min occlusion of the LAD as described previously (Végh et al., 1992). In control dogs both indices of ischaemia severity were steeply increased, reaching the maximum value (epicardial ST segment: 9.3 ± 0.9 mV, degree of inhomogeneity: 125 ± 12 mV) by the 5 min of the occlusion, and these were maintained over the rest of the occlusion. The administration of nitrite significantly attenuated these ischaemia-induced changes in the epicardial segment (3.7 ± 0.6 mV) and inhomogeneity (63 ± 13 ms) during the entire occlusion period, although there were no significant differences between the groups, regarding the risk area (39.2 ± 1.2 vs. 40.3 ± 1.2 in the control and in the nitrite group, respectively).

The Administration of Sodium Nitrite Reduces the Ischaemia and Reperfusion-Induced Morphological Changes of the Mitochondria

The representative images acquired by TEM are illustrated in **Figure 2A**, whereas data of the quantitative analysis obtained from mitochondria localized in the sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and perinuclear (PN) areas, are summarized in **Table 2**, and the results of mitochondria, assessed in the IMF region, are also illustrated in **Figure 2B**. The images show that compared to the SC dogs, in dogs of the IC group a substantial swelling and disorganization of cristae of the mitochondrial matrix could be observed, irrespective of their localization (SSM, PN, and IMF). These I/R-induced alterations were less marked in dogs infused with sodium nitrite, 24 h previously (**Figure 2A**). Furthermore, there were slight, but statistically not significant structural differences between the mitochondria, assessed in the three subsets in the sham control dogs (**Table 2**). A 25 min I/R resulted in similar tendency of changes in all mitochondria; thus, compared to the SC dogs, in dogs subjected to I/R there were significant reductions in the mitochondrial area, perimeter, and Feret diameter, and a significant increase in mitochondrial roundness (**Table 2**, **Figure 2B**). These alterations were significantly less marked in the nitrite treated animals (**Table 2**, **Figure 2B**). Sodium nitrite itself without ischaemia did not cause significant alterations in the assessed morphological parameters (**Table 2**, **Figure 2B**).

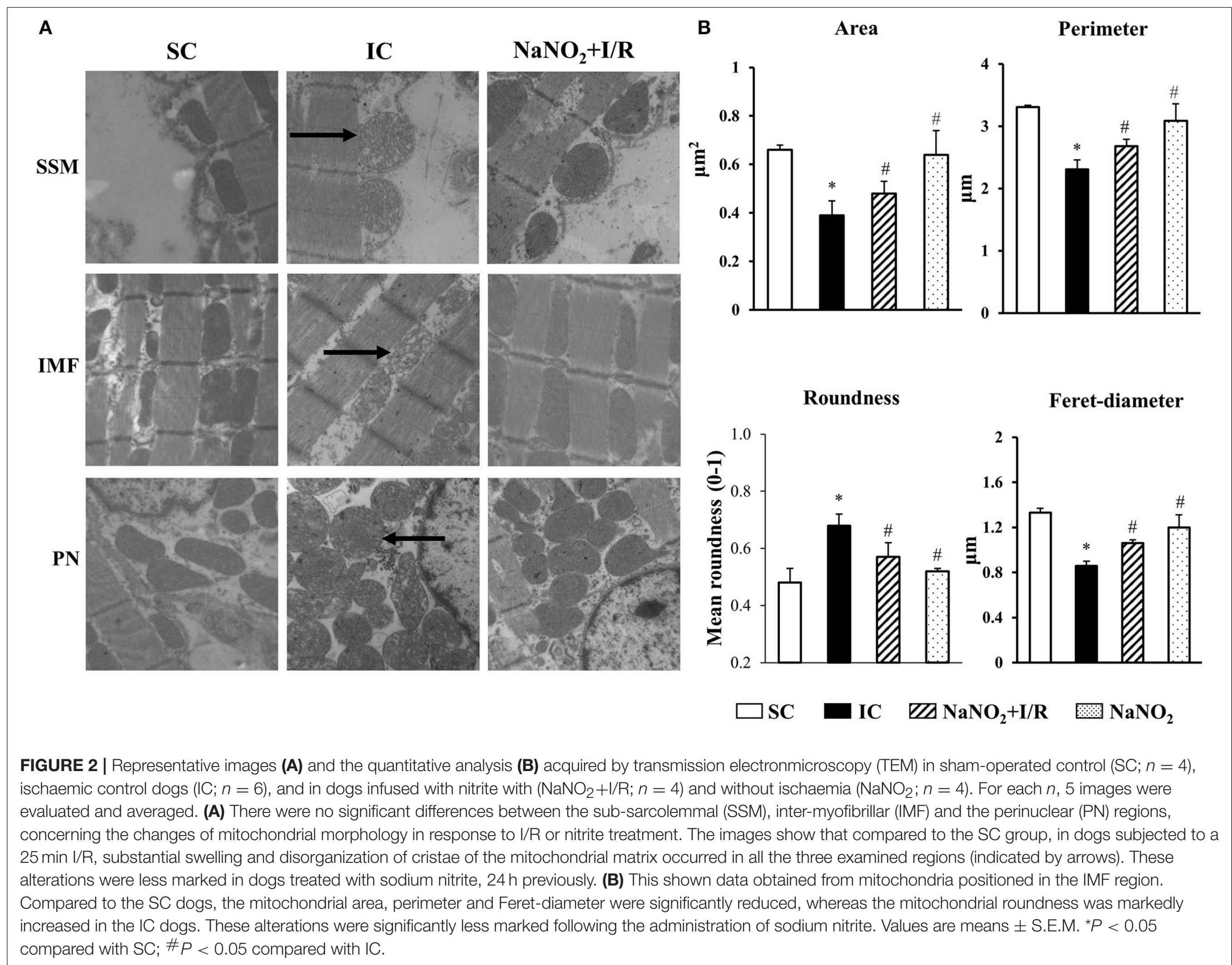

FIGURE 2 | Representative images **(A)** and the quantitative analysis **(B)** acquired by transmission electronmicroscopy (TEM) in sham-operated control (SC; $n = 4$), ischaemic control dogs (IC; $n = 6$), and in dogs infused with nitrite with ($NaNO_2$+I/R; $n = 4$) and without ischaemia ($NaNO_2$; $n = 4$). For each n, 5 images were evaluated and averaged. **(A)** There were no significant differences between the sub-sarcolemmal (SSM), inter-myofibrillar (IMF) and the perinuclear (PN) regions, concerning the changes of mitochondrial morphology in response to I/R or nitrite treatment. The images show that compared to the SC group, in dogs subjected to a 25 min I/R, substantial swelling and disorganization of cristae of the mitochondrial matrix occurred in all the three examined regions (indicated by arrows). These alterations were less marked in dogs treated with sodium nitrite, 24 h previously. **(B)** This shown data obtained from mitochondria positioned in the IMF region. Compared to the SC dogs, the mitochondrial area, perimeter and Feret-diameter were significantly reduced, whereas the mitochondrial roundness was markedly increased in the IC dogs. These alterations were significantly less marked following the administration of sodium nitrite. Values are means ± S.E.M. $^*P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC.

The Administration of Sodium Nitrite Reduces Mitochondrial Respiration Following Coronary Artery Occlusion and Reperfusion

The changes in the CI and CII-dependent respiratory parameters are illustrated in **Figures 3**, **4**, respectively. Whereas, there was no significant difference in the basal respiration between the examined groups, the CI-dependent OXPHOS and the ETS were markedly reduced in dogs subjected to a 25 min period of occlusion and then reperfusion. The respiratory control ratio (RCR), a classical parameter for the mitochondrial qualitative control, indicating the coupling between oxygen consumption and oxidative phosphorylation (Montaigne et al., 2010) was only slightly, but not significantly reduced following such aperiod of I/R insult (**Figure 3**). Furthermore, the P/E control coupling ratio, a measure of the limitation of OXPHOS capacity by the phosphorylation system, was almost the same in the ischaemic (IC group) as in the non-ischaemic (SC group) dogs, regarding both the CI and the CII-dependent respiration (**Figures 3**, **4**, respectively). Interestingly, compared to the SC dogs, nitrite alone (without I/R) significantly reduced the CI-dependent OXPHOS, ETS, and RCR, without substantially modifying State 4 and the P/E coupling ratio (**Figure 3**). Furthermore, in dogs infused with nitrite and 24 h later subjected to a 25 min period of ischaemia and reperfusion, significant decreases occurred both in CI and CII-dependent OXPHOS and RCR, and an increase in State 4, compared with the ischaemic controls (**Figure 3**, **4**). Since, in these dogs the ETS was slightly but not significantly increased compared with the untreated ischaemic (IC) dogs, the P/E coupling ratio was markedly reduced (**Figures 3**, **4**), indicating that under conditions of ischaemia and reperfusion, nitrite limits OXPHOS capacity by influencing the phosphorylation system.

Changes in the Mitochondrial ATP Production 24 h After Sodium Nitrite Administration

Changes in total ATP production were determined in three samples of each animal, collected from the sham control (SC; $n = 4$), ischaemic control (IC; $n = 5$) dogs, as well as from dogs

TABLE 2 | Morphological changes in the different mitochondria subsets following ischaemia and reperfusion, and sodium nitrite administration.

	Area (µm^2)	Perimeter (µm)	Feret diameter (µm)	Roundness
SC ($n = 4$)				
SSM	0.48 ± 0.02	2.69 ± 0.03	1.04 ± 0.01	0.57 ± 0.05
IMF	0.68 ± 0.04	3.38 ± 0.06	1.37 ± 0.03	0.46 ± 0.03
PN	0.49 ± 0.02	2.77 ± 0.09	1.09 ± 0.04	0.58 ± 0.02
IC ($n = 6$)				
SSM	0.35 ± 0.02*	2.12 ± 0.05*	0.77 ± 0.02*	0.75 ± 0.01*
IMF	0.39 ± 0.04*	2.30 ± 0.08*	0.88 ± 0.03*	0.67 ± 0.03*
PN	0.39 ± 0.02*	2.26 ± 0.05*	0.82 ± 0.02*	0.75 ± 0.02*
$NaNO_2$-IC ($n = 4$)				
SSM	0.65 ± 0.05#	3.15 ± 0.12#	1.22 ± 0.07#	0.58 ± 0.07#
IMF	0.54 ± 0.04#	2.88 ± 0.06#	1.13 ± 0.01#	0.53 ± 0.04#
PN	0.51 ± 0.02#	2.76 ± 0.05#	1.04 ± 0.02#	0.65 ± 0.02#
$NaNO_2$ ($n = 4$)				
SSM	0.58 ± 0.06#	2.96 ± 0.15#	1.13 ± 0.05#	0.58 ± 0.02#
IMF	0.67 ± 0.08#	3.18 ± 0.19#	1.22 ± 0.03#	0.54 ± 0.01#
PN	0.47 ± 0.02#	2.64 ± 0.08#	1.02 ± 0.03#	0.60 ± 0.02#

*For each n, 5 images were evaluated and averaged. Values are means ± S.E.M. *$P < 0.05$ compared with SC; #$P < 0.05$ compared with IC. SSM, sub-sarcolemmal; IMF, inter-myofibrillar; PN, perinuclear.*

that had been infused with nitrite with ($NaNO_2$+I/R; $n = 5$) and without ($NaNO_2$; $n = 5$) ischaemia. The production of ATP was expressed in RLU (over 30 s/mg protein). Compared with the SC group, a 25 min period of I/R almost halved the ATP production (12232 ± 1291 cp. 7213 ± 1117 RLU/30 s/mg protein; $P < 0.05$). The administration of nitrite alone (13001 ± 3109 RLU/30 s/mg protein cp. SC group), and under ischaemic conditions (7130 ± 1560 RLU/30 s/mg protein cp. IC group) did not significantly modify the rate of ATP production.

Changes in the Ischaemia and Reperfusion-Induced Superoxide Production 24 h After Sodium Nitrite Infusion

This is illustrated in **Figure 5A**. Compared to the SC dogs, the generation of superoxide was markedly increased in the IC dogs. This I/R-induced increase in superoxide production was attenuated by the prior administration of nitrite.

Changes in the Ischaemia and Reperfusion-Induced Peroxynitrite Production 24 h After the Infusion of Sodium Nitrite

The changes in 3-NT production are shown in **Figure 5B**. Compared to the SC dogs, a 25 min I/R resulted in a significant increase in 3-NT production. This increase in 3-NT formation was markedly reduced in the nitrite treated dogs.

DISCUSSION

We have previous evidence that the infusion of sodium nitrite provides a marked immediate (Kovács et al., 2015), and also a later appearing (24 h later; Demeter-Haludka et al., 2017) protective effect against those severe ventricular arrhythmias that result from a 25 min period of coronary artery occlusion and reperfusion in anesthetized dogs. We have now examined, whether the cardioprotective effect of sodium nitrite, occurring 24 h later, involves changes in mitochondrial morphology and function. This question was raised because our previous studies, examining the role of NO-induced iNOS activation in this protection against arrhythmias showed that the NO/iNOS/NO pathway (Végh and Parratt, 1996; Bolli et al., 1997) may have some role in the protection, but it does not fully explain the marked antiarrhythmic effect of nitrite (Demeter-Haludka et al., 2017). Since, there has been some previous evidence, albeit from different experimental models, which suggests that the mitochondria might be important target organelles in the delayed protective effect of nitrite (Shiva et al., 2007a,b; Shiva and Gladwin, 2009), we designed studies to examine the effects of nitrite on mitochondrial structure and function in our established *in vivo* canine model of ischaemia and reperfusion (e.g., Végh et al., 1992; Kiss et al., 2010). Using various *in vitro* methods, we have determined the changes in mitochondrial morphology, the alterations in the CI and CII-dependent mitochondrial respiration, as well as in ATP, superoxide and peroxynitrite productions in myocardial tissue samples, collected from the heart of dogs during the early period (2 min) of reperfusion, following a 25 min ischaemic insult.

There is emerging evidence that changes in mitochondrial morphology play an important role both in the normal and the diseased myocardium; by the dynamic nature of the mitochondria their morphological changes may occur during cardiac development, and also in response to injurious conditions, such as ischaemia and reperfusion, heart failure, diabetes, apoptotic, and autophagy cell death (Ong and Hausenloy, 2010). In our study the qualitative and quantitative analyses of the TEM images showed that a 25 min period of ischaemia and 2 min reperfusion resulted in substantial structural alterations in the mitochondria, irrespective whether they were inter-myofibrillar, sub-sarcolemmal, or perinuclear mitochondria (**Figure 2A**).We have found that the electron density of the mitochondrial matrix was markedly reduced and the normally tightly packed cristae became disconnected and disorganized. There were also signs of mitochondrial swelling. In many of these severely damaged mitochondria, large and empty blebs could be observed that led to membrane disruption. In other mitochondria, a rearrangement of the cristae was apparent (**Figure 2A**). Furthermore, the reduction in the mitochondrial area, perimeter, and Feret-diameter, as well as the increase in roundness indicated that the mitochondria become smaller and more spherical following a 25 min period of ischaemia and reperfusion insult (**Figure 2B**). A recent finding also shows that in mouse subjected to a 20 min global ischaemia without reperfusion, the sphericity of the mitochondria, in all the three subsets, was significantly increased (Kalkhoran et al., 2017). We

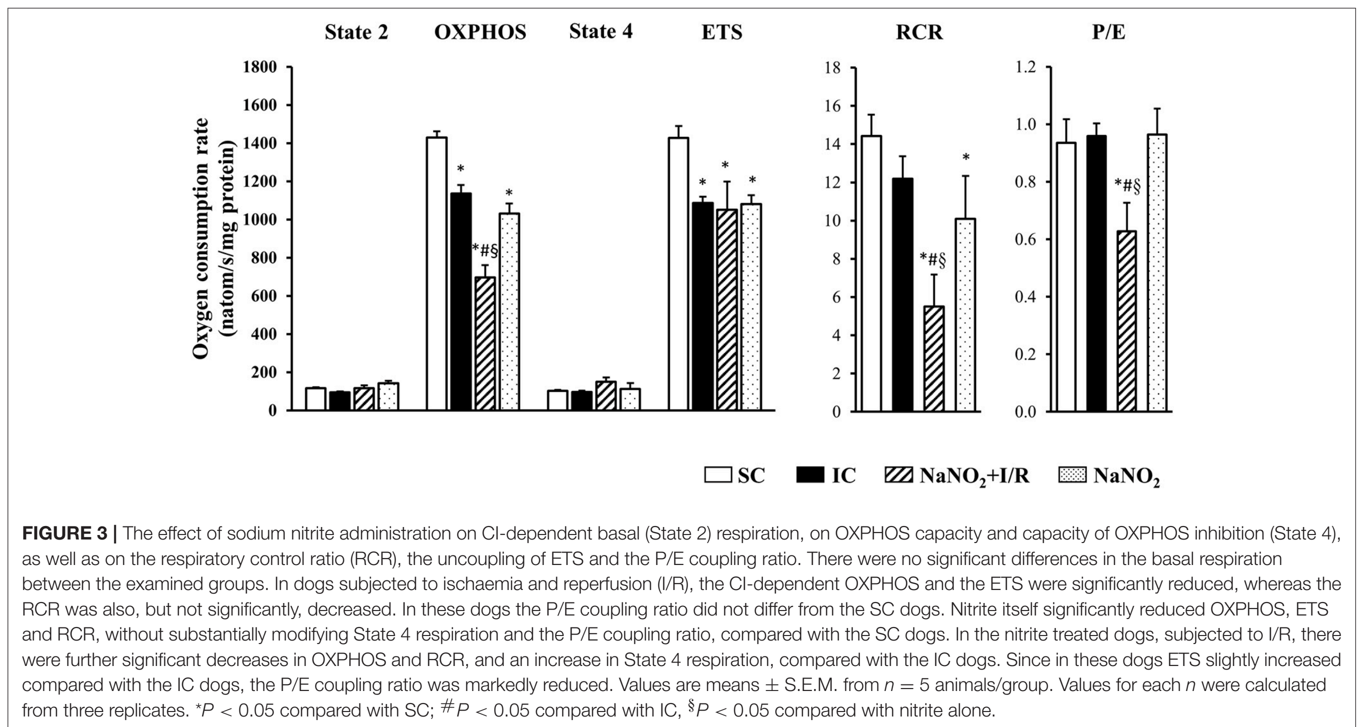

FIGURE 3 | The effect of sodium nitrite administration on CI-dependent basal (State 2) respiration, on OXPHOS capacity and capacity of OXPHOS inhibition (State 4), as well as on the respiratory control ratio (RCR), the uncoupling of ETS and the P/E coupling ratio. There were no significant differences in the basal respiration between the examined groups. In dogs subjected to ischaemia and reperfusion (I/R), the CI-dependent OXPHOS and the ETS were significantly reduced, whereas the RCR was also, but not significantly, decreased. In these dogs the P/E coupling ratio did not differ from the SC dogs. Nitrite itself significantly reduced OXPHOS, ETS and RCR, without substantially modifying State 4 respiration and the P/E coupling ratio, compared with the SC dogs. In the nitrite treated dogs, subjected to I/R, there were further significant decreases in OXPHOS and RCR, and an increase in State 4 respiration, compared with the IC dogs. Since in these dogs ETS slightly increased compared with the IC dogs, the P/E coupling ratio was markedly reduced. Values are means ± S.E.M. from $n = 5$ animals/group. Values for each n were calculated from three replicates. $^{*}P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC, $^{\S}P < 0.05$ compared with nitrite alone.

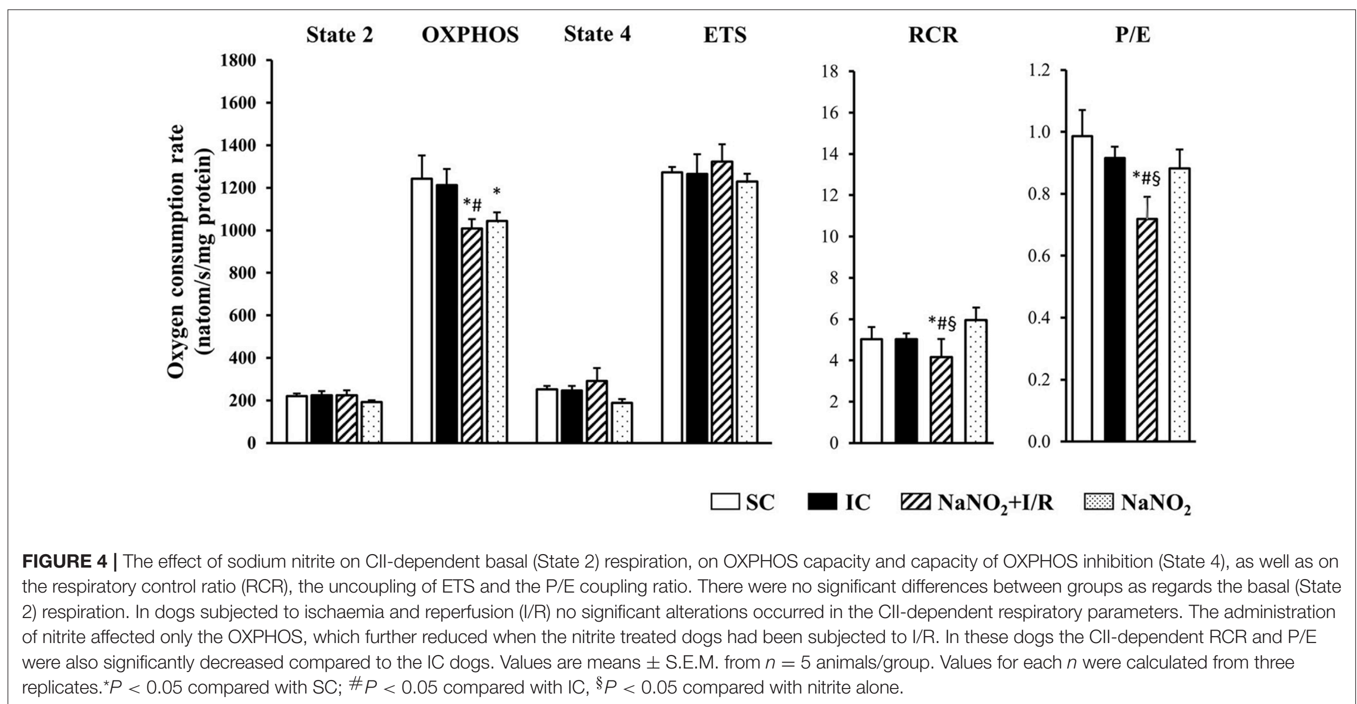

FIGURE 4 | The effect of sodium nitrite on CII-dependent basal (State 2) respiration, on OXPHOS capacity and capacity of OXPHOS inhibition (State 4), as well as on the respiratory control ratio (RCR), the uncoupling of ETS and the P/E coupling ratio. There were no significant differences between groups as regards the basal (State 2) respiration. In dogs subjected to ischaemia and reperfusion (I/R) no significant alterations occurred in the CII-dependent respiratory parameters. The administration of nitrite affected only the OXPHOS, which further reduced when the nitrite treated dogs had been subjected to I/R. In these dogs the CII-dependent RCR and P/E were also significantly decreased compared to the IC dogs. Values are means ± S.E.M. from $n = 5$ animals/group. Values for each n were calculated from three replicates.$^{*}P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC, $^{\S}P < 0.05$ compared with nitrite alone.

have also found that the I/R-induced structural changes of the mitochondria were significantly less marked, if the dogs had been infused with sodium nitrite, 24 h previously (**Figure 2B**). To the best of our knowledge, this is the first study, which has examined the effect of nitrite on mitochondrial morphology in a large animal model, and showed that nitrite may modify the ischaemia and reperfusion-induced structural changes of the mitochondria; an effect which might have a role in the cardioprotective effect of nitrite. However, as to whether nitrite directly acts on the mitochondria, or whether the preservation of mitochondrial morphology results from other effects of nitrite, we do not know; this warrants further examinations.

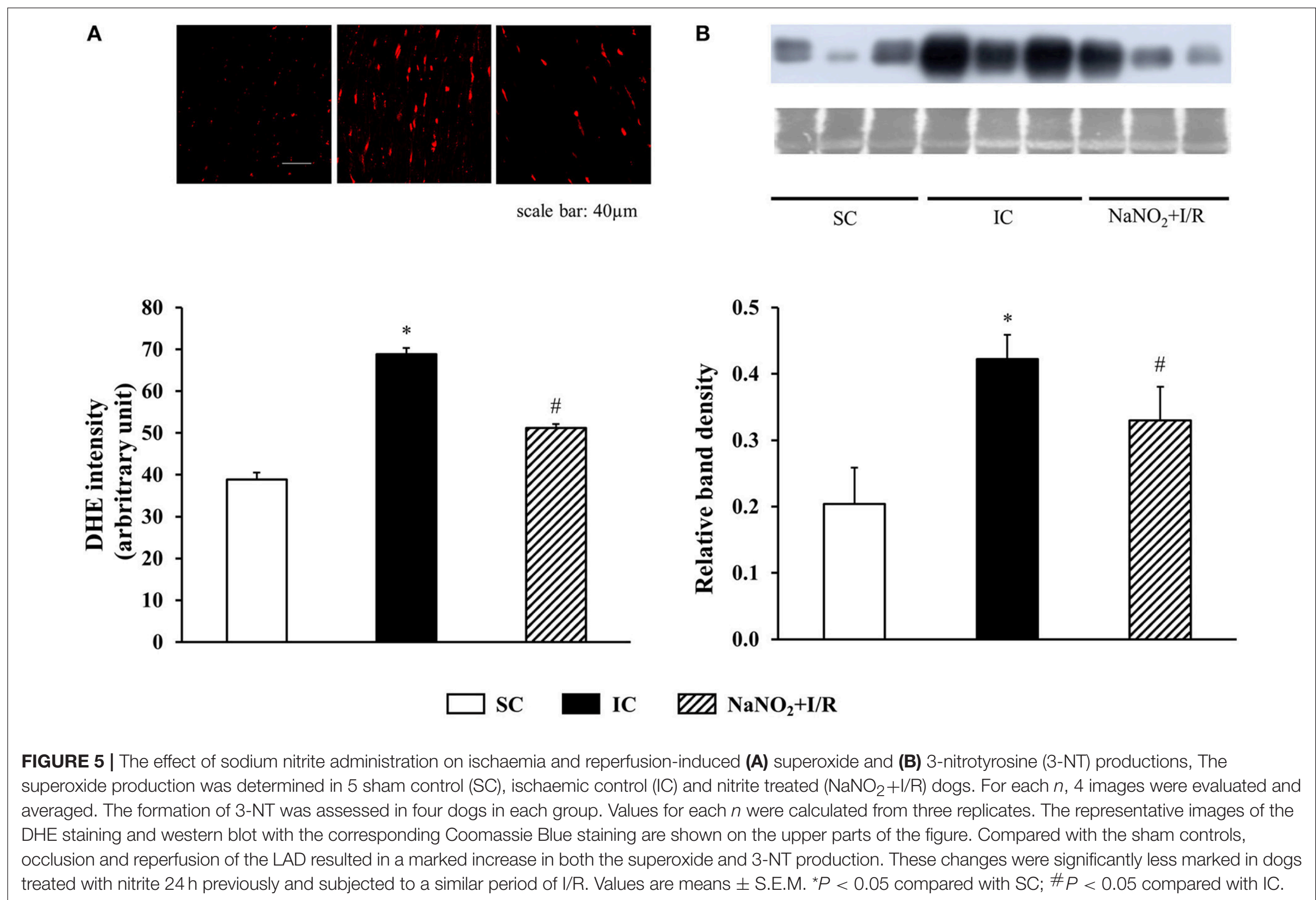

FIGURE 5 | The effect of sodium nitrite administration on ischaemia and reperfusion-induced **(A)** superoxide and **(B)** 3-nitrotyrosine (3-NT) productions, The superoxide production was determined in 5 sham control (SC), ischaemic control (IC) and nitrite treated ($NaNO_2$+I/R) dogs. For each *n*, 4 images were evaluated and averaged. The formation of 3-NT was assessed in four dogs in each group. Values for each *n* were calculated from three replicates. The representative images of the DHE staining and western blot with the corresponding Coomassie Blue staining are shown on the upper parts of the figure. Compared with the sham controls, occlusion and reperfusion of the LAD resulted in a marked increase in both the superoxide and 3-NT production. These changes were significantly less marked in dogs treated with nitrite 24 h previously and subjected to a similar period of I/R. Values are means ± S.E.M. $^{*}P < 0.05$ compared with SC; $^{\#}P < 0.05$ compared with IC.

Also, we do not have direct evidence whether the preservation of mitochondrial structure by nitrite contributes to better mitochondrial function, but the results of the functional measurements show that nitrite modifies mitochondrial respiration and ROS production as well. Although there are many possibilities to assess mitochondrial function and dysfunction, in our experiments we measured mitochondrial respiration, as the generally accepted indicator of mitochondrial function (Brand and Nicholls, 2011) in isolated mitochondria, obtained from the control and the nitrite treated dog hearts. 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 (**Figures 3**, **4**). 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, compared with the ischaemic controls (IC group), in the nitrite treated dogs both the CI and CII-dependent OXPHOS, the RCR, and the P/E coupling control ratio were significantly reduced. Furthermore, nitrite significantly reduced the superoxide and the 3-NT productions, resulted from a 25 min period of occlusion and reperfusion insult (**Figure 5**).

There is substantial evidence that NO regulates ROS formation, and that this mechanism is largely involved in the protective effect of NO, for example, against those severe ventricular arrhythmias (Kiss et al., 2010), which occur during the first minutes of the reperfusion, when the burst of ROS is apparent (Xia and Zweier, 1997; Iwase et al., 2007; Burwell and Brookes, 2008). 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 (Clancy et al., 1992; 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 late 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 (St-Pierre et al., 2002; 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).

In our dog model a 25 min ischaemia and 2 min reperfusion (this reperfusion interval was selected because the severe reperfusion-induced arrhythmias occur almost immediately after the reopening of the coronary artery; **Figure 1**) resulted in a mild, but significant reduction in the CI (24%; $P < 0.05$ compared to the SC group; **Figure 3**), and also in the CII-supported OXPHOS (**Figure 4**), a decrease in ATP and an increase in superoxide (**Figure 5**) productions. Furthermore, in these ischaemic dogs, the P/E coupling ratio was similar to that observed in the sham controls (SC), suggesting that such a period of I/R limits the capacity of the respiratory complexes of the ETS, and consequently, increases the generation of ROS. In contrast, the administration of nitrite itself (without I/R), and also following an occlusion and reperfusion insult, substantially reduced mitochondrial respiration; i.e., there was a marked decrease in the CI-dependent OXPHOS (48% compared with 24% in the IC group), in RCR and, in particular, in the P/E coupling ratio. The decrease in P/E following nitrite 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 (**Figure 5**). This latter might be associated with the observation that the State 4 respiration was significantly increased in the $NaNO_2$+I/R dogs, indicating an increase in proton leakage in the inner membrane, which results in a reduction in ROS production (Brand et al., 1999; Divakaruni and Brand, 2011).

Although we do not have direct evidence that in the protective effect of nitrite the modification of the phosphorylation system plays a major role, the fact that following the administration of the uncoupler FCCP, the decrease in ETS was similar both in the control and in the nitrite treated dogs, supports this idea. We assume that nitrite (or NO) acts on one of the components of the phosphorylation system, such as, for example, the ATP synthase, the phosphate transporter or the ADP/ATP translocator ANT. It might well be that nitrite interferes with the interaction of ATP synthase and cyclophilin D, which interaction plays a role in the formation and opening of mitochondrial permeability transition pores (MPTP), resulting in decreased ATP synthesis and increased ROS formation under conditions of I/R (Halestrap and Richardson, 2015). Moreover, the inhibition of the pore forming and opening interactions between the inner mitochondrial membrane proteins and cyclophilin D results in protection by reducing ATP loss and ROS formation (Javadov and Kuznetsov, 2013; Halestrap and Richardson, 2015). Recent evidence suggests that the cysteine 203 residue of cyclophilin D is necessary for cyclophilin D activation and subsequent MPTP opening (Nguyen et al., 2011), and that this residue undergoes protein SNO (Kohr et al., 2011). It has been suggested that in a NO-enriched environment, the formation of SNO is protective by preventing the crucial proteins from the irreversible modification of oxidation, occurring during I/R (Sun et al., 2006). It is tempting to speculate that in the delayed cardioprotective effect of nitrite, the S nitrosylation of mitochondrial proteins involved in the regulation of MPTP, plays an important role.

In summary, the results of this study confirm that the administration of sodium nitrite provides protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias, 24 h later. We have now shown that this protective effect may involve, among a number of other NO-dependent effects, changes in mitochondrial morphology and function. Nitrite prevents the I/R-induced structural alterations of the mitochondria, and most probably by interfering with the phosphorylation system, inhibits the ROS producing components of the ETS and reduces the ROS formation during the early phase of reperfusion. As to whether the nitrite-induced protection attains through S-nitrosylation of proteins or one of the crucial proteins involved in the regulation of MPTP, warrants further investigations.

AUTHOR CONTRIBUTIONS

ÁV, VD-H, and MK contributed to the conception and design of this study. The *in vivo* and *in vitro* experiments, as well as the data acquisition and analysis were performed by VD-H, MK, AP (mitochondrial respiratory measurements), and RP (TEM analysis). The drafting and revising the work was made by ÁV with the contribution of DM and LS to data interpretation.

All authors participated in the manuscript revision, read and approved the submitted version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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