Further evidence for the role of nitric oxide in the antiarrhythmic effect of ischaemic preconditioning: The effect of peroxynitrite and changes in NOS-dependent NO production

PhD Thesis

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LIST OF PUBLICATIONS

Full papers

I. **Juhász L,** Kiss A, Nyeső E, Kovács M, Seprényi G, Kaszaki J, Végh Á. Is there a trigger role of peroxynitrite in the anti-arrhythmic effect of ischaemic preconditioning and peroxynitrite infusion? Eur J Pharmacol (2011). 667:306-313. **IF: 2.737**.

II. **Juhász L,** Déri S, Kisvári G, Kiss A, Seprényi G, Gardi J, Végh Á. The effect of ischaemic preconditioning on nitric oxide synthase activity during myocardial ischaemia and reperfusion in anaesthetized dogs. Curr Res Cardiol (2014). 2:73-78.

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Other publications

IV. Kiss A, **Juhász L,** Huliák I, Végh Á. Peroxynitrite decreases arrhythmias induced by ischaemia reperfusion in anaesthetized dogs, without involving mitochondrial KATP channels. Br J Pharmacol (2008). 155:1015-24. **IF: 4.902**.

V. Kiss A, **Juhász L,** Seprényi G, Kupai K, Kaszaki J, Végh Á. The role of nitric oxide, superoxide and peroxynitrite in the anti-arrhythmic effects of preconditioning and peroxynitrite infusion in anaesthetized dogs. Br J Pharmacol (2010). 160:1263-72. **IF: 4.925**.

ABSTRACTS

Abstracts for oral presentations

I. Kiss A, **Juhász L,** Huliák I, Végh Á. Az exogén peroxinitrit hatása az okklúziós és reperfúziós arrhythmiákra altatott kutyákban (2006). Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged. Abstract book:125: E67.

II. **Juhász L,** Kiss A. Az exogén peroxinitrit hatása az okklúziós és reperfúziós arrhythmiákra altatott kutyában (2007). SZTE Szent-Györgyi Albert Orvos- és Gyógyszerésztudományi

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III. **Juhász L,** Kiss A. A peroxinitrit szerepének vizsgálata a prekondicionálás korai antiarrhythmiás hatásában (2007). XXVIII. Országos Tudományos Diákköri Konferencia, Állatélettan és -szervezettan szekció, Debrecen. Abstract book 52.

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V. Kiss A, **Juhász L,** Végh Á. Az exogén peroxinitrit hatása az okklúziós és reperfúziós arrhythmiákra altatott kutyában. Magyar Kardiológus Társaság Kongresszusa, Balatonfüred. Cardiologia Hungarica (2007). 37: 19.

VI. **Juhász L,** Kiss A, Végh Á. A mitokondriális ATP-függő kálium csatornák (mitoK_{ATP}) nem játszanak szerepet a peroxynitrit antiarrhythmiás hatásában altatott kutyában. XV. TDK konferencia, Targu Mures. Orvosi és Gyógyszerészeti Szemle (2008). S54:12.

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IX. **Juhász L,** Kiss A, Nyeső E, Seprényi Gy, Kaszaki J, Végh Á. Az ischaemiás prekondicionálás és az exogén peroxinitrit antiarrhythmiás hatásának vizsgálata húgysav jelenlétében (2009). Magyar Élettani Társaság Vándorgyűlése, Budapest. Abstract book (un-numbered page).

X. **Juhász L,** Kiss A, Nyeső E, Seprényi Gy, Kaszaki J, Végh Á. A prekondicionálás antiaritmiás hatásának vizsgálata peroxinitrit scavanger jelenlétében. Magyar Kardiológus Társaság Kongresszusa, Balatonfüred. Cardiologia Hungarica (2010). 40: 41.

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XI. **Juhász L,** Kiss A, Huliák I, Ferdinándy P, Végh Á. Mito-K_{ATP} channels are not involved in the antiarrhytmic effect of peroxynitrite. ISHR XIX. World Congress, Bologna. J Mol Cell Cardiol (2007). 42: S18.

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XIV. Kiss A, **Juhász L,** Seprényi Gy, Kaszaki J, Végh Á. The effect of preconditioning and exogenous peroxynitrite on the generation of nitric oxide, superoxide and peroxynitrite in anaesthetised dogs. Joint Meeting of the European Heart Failure Association and the ISHR-European Section, Nice. Eur Heart J (2009). S8-2.

XV. **Juhász L,** Kiss A, Nyeső E, Kovács M, Seprényi G, Kaszaki J, Végh Á. The role of peroxynitrite in the induction of antiarrhythmic effect of ischaemic preconditioning in anaesthetised dogs. ISHR XX. World Congress, Kyoto. J Mol Cell Cardiol (2010). 48: S41.

XVI. **Juhász L,** Haludka V, Seprényi Gy, Kaszaki J, Gardi J, Végh Á. Examination of the role of monoamine oxidase inhibition on ischaemia and reperfusion-induced ventricular arrhythmias in anaesthetised dogs (2010). Magyar Élettani Társaság és Magyar Kísérletes és Klinikai és Farmakológiai Társaság konferencia, Szeged. Abstract book: 148-149: P74.

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XXI. **Juhász L,** Déri Sz, Kisvári G, Kiss A, Gardi J, Seprényi Gy, Kaszaki J, Végh Á. The effect of ischaemic preconditioning on nitric oxide synthase activity during myocardial ischaemia and reperfusion in anaesthetized dogs. Frontiers in Cardiovascular Biology Congress, Barcelona. Cardiovasc Res (2014). 103 (suppl 1): S75-S76.

SUMMARY

It is well established that peroxynitrite (PN), formed endogenously by the reaction of nitric oxide (NO) and superoxide, largely contributes to the development of myocardial injury, resulting from ischaemia and reperfusion (I/R). However, more recent evidence suggests that PN in a lower (nanomolar/low micromolar) concentration range, may exert preconditioning (PC)-like protective effects. For example, we have shown in a previous study that PN administered in 100 nM concentration, markedly reduced the severity of ventricular arrhythmias that resulted from acute coronary artery occlusion and reperfusion in anaesthetized dogs (Kiss et al., 2008). This study, however, did not examine whether PN, generated during the brief periods of preconditioning I/R insults, plays also a trigger role in the PC-induced antiarrhythmic protection. Therefore, in the first series of experiments (Study I) we examined this by the use of uric acid (UA; 0.2 mg/kg/min, over 30 min), a relatively selective scavenger of PN, and the effects obtained in PC dogs were compared to those dogs that had been received PN exogenously, 25 min before the occlusion of the left anterior descending coronary artery (LAD). In these experiments the severity of ischaemia and of ventricular arrhythmias, changes in plasma nitrate/nitrite (NOx) levels, as well as myocardial superoxide and nitrotyrosine (NT) production (a marker of PN generation) were assessed.

We have found that both the PC procedure (2x5 min occlusion/reperfusion) and the administration of PN resulted in a significant increase in NT formation, which was abolished or markedly attenuated in the presence of UA. This attenuation of PN formation in PC dogs, however, did not influence the PC-induced protection; i.e. the number and the incidence of ventricular arrhythmias during the prolonged occlusion remained to be suppressed, whereas the increase in NO bioavailability and the decrease in superoxide production were as the same as in the PC animals. In contrast, UA completely abrogated the protection that resulted from the administration of PN. Interestingly, UA itself also reduced the arrhythmias; an effect which might be associated with the antioxidant property of the compound. Our conclusion was that PN administration results in a PC-like protection against arrhythmias, but PN, generated during the PC procedure, is not necessary for triggering the PC-induced antiarrhythmic protection.

The second series of experiments (Study II) aimed to examine whether the increased NO bioavailability that occurs in PC dogs, is the direct consequence of an enhanced nitric oxide

synthase (NOS) activity or other NO producing mechanisms, such as the non-enzymatic NO formation, may also play a role. Therefore, we designed studies in which the time-course changes in NOS activity, NO bioavailability, as well as superoxide and NT productions were simultaneously examined in control dogs and in dogs subjected to PC. We have found that in control dogs subjected to a 25 min LAD occlusion, there was an initial increase in NOS activation that occurred around 5 min of the occlusion. Afterwards the enzyme activity continuously decreased up to the end of the occlusion period. These changes in NOS activity were almost parallel with the alterations in NO levels. In control dogs, there were also marked increases in tissue superoxide and NT concentrations, determined at the end of the 25 min of the occlusion. In contrast, in dogs subjected to PC the activation of NOS and the production of NO were significantly increased during the PC procedure, and these were maintained over the entire period of the subsequent prolonged ischaemic insult. Although the PC procedure increased the superoxide and NT levels, the generation of these oxidative stress products was markedly suppressed during the prolonged occlusion. We concluded from these results that PC preserves the NOS enzyme-dependent NO formation, and perhaps through this mechanism, it reduces the harmful consequences of the reperfusion-induced oxidative stress. We propose that this NOS-dependent increase in NO bioavailability during ischaemia plays a mandatory role in the antiarrhythmic effect of PC.

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LIST OF ABBREVIATIONS

5-HD: 5-hydroxydecanoate
AU: arbitrary units
BH4: tetrahydrobiopterin
BK: bradykinin
cGC: soluble guanylyl cyclase
DABP: diastolic arterial blood pressure
DHE: dihydroethidine
EDTA: Ethylenediaminetetraacetic acid
ERK: extracellular signal-regulated kinase
eNOS: endothelial nitric oxide synthase
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR: heart rate
iNOS: inducible nitric oxide synthase
I/R: ischaemia and reperfusion
LAD: left anterior descending coronary artery
L-NAME: L-NG-nitroarginine methyl ester
LVEDP: left ventricular end-diastolic pressure
LVSP: left ventricular systolic pressure
MABP: mean arterial blood pressure
mitoKATP: mitochondrial ATP-sensitive potassium channels
NAC: N-acetyl-L-cysteine
NADPH: nicotinamide adenine dinucleotide phosphate
$NaNO_2$: sodium nitrite
$NaNO_3$: sodium nitrate
NO: nitric oxide
NOS: nitric oxide synthase
nNOS: neuronal nitric oxide synthase
NOx: nitrate/nitrite
$O_2^{\cdot-}$: superoxide
p38MAPK: p38 mitogen activated protein kinase
PBS: phosphate buffer solution
PKC: protein kinase C
PN: peroxynitrite
ROS: Reactive oxygen species
SABP: systolic arterial blood pressure
UA: uric acid, urate
VF: ventricular fibrillation
VPBs: ventricular premature beats
VT: ventricular tachycardia
WHO: World Health Organization

1. INTRODUCTION

The reduced blood supply to the myocardium, resulting from coronary artery occlusion causes ischaemic changes that are often accompanied by the occurrence of lethal ventricular arrhythmias. According to the statistic of the World Health Organization (WHO), both in men and women the ischaemia-induced sudden cardiac death results in more than 7 million deaths per year in the world (Finegold et al., 2013). Consequently, the search for novel strategies, which aim to prevent or reduce the acute ischaemia and reperfusion (I/R)-induced life-threatening arrhythmias is still a major challenge in both the experimental and clinical cardiology. Such a novel strategy might be the phenomenon of ischaemic preconditioning (Murry et al., 1986).

1.1. The phenomenon of ischaemic preconditioning: from experimental studies to clinical exploitation

It is now 28 years that PC, as one of the endogenous adaptive phenomena, was first described by Murry and colleagues (1986) in anaesthetized dogs. They showed that four 5 min periods of occlusion of the left circumflex coronary artery 5 min prior to a 40 min occlusion of the same artery markedly reduced the size of the infarct, the myocardial ATP consumption and the drop in pH (Murry et al., 1986). Since then the protective effects of PC have been described and confirmed in various experimental settings. It was shown that PC markedly reduces the severity of ischaemia and reperfusion-induced ventricular arrhythmias (Shiki and Hearse, 1987; Végh et al. 1990, 1992a) and improves the restoration of contractile dysfunction following reperfusion (Cave and Hearse, 1992; Hendrikx et al., 1993). Somewhat later it was also turned out that not only the brief periods of I/R insults, but other stimuli, such as cardiac pacing (Végh et al., 1991a), heavy physical exercise (Babai et al., 2002), heat stress (Cumming et al., 1996), an increase in myocardial stretch (Ovize et al., 1994), as well as various pharmacological agents (Cohen et al., 2000) can induce a PC-like protection.

It was also an early recognition that PC is a general phenomenon; the protection can be initiated in all species thus far investigated, including humans (Tomai et al., 1994; Arstall et al., 1998), suggesting a possibility for clinical exploitation of the phenomenon. Another important aspect of PC is that the protection is time dependent; it occurs in two distinct phases. There is an ‘early phase’ which appears immediately after the PC stimulus, but it

fades within 1 or 2 hours (Végh et al., 1992a). However, the protection reappears 20-24h later (Marber et al., 1993; Kuzuya et al., 1993; Végh et al., 1994a), and this phase is termed as late or delayed or "second window of protection" (Szekeres et al., 1989; Yellon and Baxter, 1995).

Of particular importance, the PC phenomenon can occur in humans as well (Schwarz et al., 1999). The first evidence for this was provided by Deutsch et al. (1990). They showed that in patients with coronary artery disease, and undergoing percutaneous transluminal coronary angioplasty, the repeated balloon inflations resulted in less marked ST-segment changes and increases in lactate levels (Deutsch et al., 1990). Similarly, the TIMI studies indicated that the clinical outcome of myocardial infarction is better in those patients, who suffered from preinfarct angina within 24 hours of the onset of the infarction (Kloner et al., 1995, 1998). Similarly, in patients undergoing coronary artery bypass grafting, the intermittent aortic cross-clamping procedure (2-times for 3 min with 2 min reperfusion intervals) prior to a 10 min global ischaemia, preserved ATP levels and reduced myocardial injury, as assessed by a lower troponin T release (Yellon et al., 1993; Jenkins et al., 1997). Also, human studies have revealed that PC reduced the infarct size (Ottani et al., 1995), lowered creatine kinase values (Kloner et al., 1998), ameliorated the ejection fraction, preserved contractile function (Nakagawa et al., 1995; Napoli et al., 1998), enhanced the development of new collaterals and suppressed the occurrence of tachyarrhythmias (Tamura et al., 1997). A novel promising clinical exploitation of PC is proposed in relation to heart transplantation by the preservation of the donor heart (Karck et al., 1996).

1.2. Initiation of ischaemic preconditioning: characteristics and possible mechanism

Despite of the intensive research, several key questions have still remained to be unanswered as regards the molecular and cellular mechanisms of PC. As mentioned above, the phenomenon was first described in anaesthetized dogs (Murry et al., 1986), but it has turned out soon that a similar protection can also be induced in other species, such as in swine (Schott et al., 1990), rabbit (Cohen et al., 1991), rat (Li and Kloner, 1992), sheep (Burns et al., 1995) and in mouse (Sumeray and Yellon, 1998). It is a common feature of the PC-induced adaptation that the stimulus should be reached a threshold to elicit protection, although this threshold might be different in the various species. The development of protection is dependent on the strength of the PC stimulus, which can be modified by the duration and/or the number of the PC cycles. The strength of the stimulus may also be different for the

experimental end-points examined (Li et al., 1990; Van Winkle et al., 1991; Miura et al., 1992; Végh et al., 1992a; Seyfarth et al., 1994; Barbosa et al., 1996; Schulz et al., 1998). For example, in dogs one or two cycles of 5 min PC occlusion resulted in almost similar antiarrhythmic protection, whereas in rats the optimal stimulus to achieve protection against arrhythmias was 3 min (Végh et al., 1992a). In case of the examinations of infarct size, there were no differences between a single 5 min ischaemia and 6 or 12 cycles in the canine (Li et al., 1990). In rabbits, one single 2 min period of occlusion was ineffective to induce protection, but two 2 min occlusions resulted in similar reduction in infarct size than a single 5 min occlusion (Miura and Iimura, 1993).

It is now also well established that PC induces the formation and release of several endogenous substances from the heart, which may have either beneficial or deleterious effects on the cardiovascular system (Curtis et al., 1993; Parratt, 1993; Cohen et al., 2000; Downey et al., 2007). It is proposed that these substances, via receptor-dependent and receptor-independent mechanisms, may activate various signalling pathways and modify end-effectors (one or more), leading ultimately to cardioprotection (Downey et al., 2007). The first endogenous substances that were associated with the PC-induced cardioprotection, were prostacyclin (Végh et al., 1990), bradykinin (Végh et al., 1991b), and adenosine (Liu et al., 1991). For example, Downey and colleagues showed that adenosine is released during the short periods of I/R insults (Liu et al., 1991), and by acting on the Gi-coupled A1 receptor, stimulates the translocation of protein kinase C (PKC) from the cytosol to the membrane (Cohen et al., 2000). Furthermore, the blockade of A1 receptors abolished, whereas the administration of A1 agonist mimicked the cardioprotective effects of PC (Liu et al., 1991; Thornton et al., 1992). Parallel with this adenosine hypothesis, our research group proposed that bradykinin (BK), formed rapidly after the onset of ischaemia, plays also an important trigger role in the antiarrhythmic effect of PC (Végh et al., 1991b, 1993; 1994b). The administration of BK (Végh et al., 1991b) and the combined ACE/NEP inhibitor Z13752A, which increases endogenous BK levels by inhibiting the degradation of BK (Rastegar et al., 2000) mimicked, whereas the blockade of BK2 receptors with icatibant markedly attenuated the antiarrhythmic effect of PC (Végh et al., 1994b). Furthermore, it was suggested that BK, by activating B2 receptors, stimulates the generation and the release of nitric oxide (NO) from the endothelium, and possibly also from cardiac myocytes, since the protective effect of BK

was abolished by the NOS inhibitor L-NAME (Végh et al., 1993).

There is now substantial evidence that NO plays an essential role in the cardioprotective effect of PC (Végh et al., 1992b; Bilinska et al., 1996; Lochner et al., 2000). The first evidence for this came from studies in anaesthetized dogs which showed that the antiarrhythmic effect of PC was abolished by the administration of the nitric oxide synthase (NOS) enzyme inhibitor, L-NAME (Végh et al., 1992b). It was proposed that in antiarrhythmic effect of PC, the activation of the BK-NO-soluble guanylyl cyclase (sGC) pathway and the resulting increase in cGMP, plays a mandatory role (Végh et al., 1994a; Végh and Parratt, 1996), since the administration of methylene blue, a non-selective inhibitor of sGC, completely abolished the protection against arrhythmias, resulting from PC (Végh et al. 1992c). There are many ways by which cGMP can lead to cardioprotection; e.g. it activates the cGMP-dependent phosphodiesterase enzyme, which reduces cAMP and thereby the influx of calcium through the L-type calcium channels (Parratt and Végh, 1996), or it stimulates protein kinase G (PKG) and opens mitochondrial ATP-sensitive potassium channels (Oldenburg et al., 2004; Costa et al., 2005), etc. Indeed, closing of mitoK_{ATP}-channels with 5-hydroxydecanoate (5-HD), attenuated the PC-induced antiarrhythmic protection (Végh and Parratt, 2002). Both mechanisms (reduction in calcium influx and opening of mitoK_{ATP} channels) during ischaemia may have a protective role against the generation of arrhythmias.

There are, of course, several other substances, such as angiotensin II (Liu et al., 1995), endothelin-1 (Wang et al., 1996) and opioids (acting mainly on κ and/or δ opioid receptors; Miki et al., 1998), which have also been proposed to participate in the initiation of PC. Their role, however, in the PC-induced cardioprotection is not well established and, indeed, seems rather controversial (Miki et al., 1998; Dickson et al., 2001; Weinbrenner et al., 2002). For example, the AT2 and ET1 receptor blockers failed to inhibit the angiotensin II and endothelin-induced protection (Sharma and Sing, 1999; Erikson et al., 1996), indicating that these substances may have a negligible role in the PC-induced cardioprotection.

Substances that are generated mainly during reperfusion, and are thought to play an important role in I/R injury, such as, for example, superoxide and peroxynitrite, have also been proposed to play a trigger role in PC (Baines et al., 1997; Altug et al., 2000, 2001). Their importance, as triggers of the PC-induced protection is discussed in sections 1.3 and 1.4.

It might well be that several substances (protective and deleterious) are released from the myocardium during the brief transient ischaemia and reperfusion insults, and contribute to the protection afforded by PC (Parratt, 1993). For example, according to the concept of Downey and colleagues, the release of these endogenous substances depends on the strength of the PC stimulus, i.e. the stimulus should reach a threshold to induce the release of a certain substance (Goto et al., 1995). Thus, if a stimulus is adequately strong (i.e. reaches the threshold of the release of many substances), then the inhibition of the release of one of the substances would not result in the abolition of the protection, since the other substances that are also released by this stimulus, are still able to evoke the protection (Cohen et al., 2000).

In summary, it seems more than likely that the short periods of ischaemia and reperfusion insults initiate the formation and the release of several endogenous substances. Some of them might be protective, whereas the others might be potentially injurious. It might well be that these substances are acting in concert to trigger and mediate the protection. Our work, described in the first part of the present thesis, focused on the further examination of those endogenous substances, namely nitric oxide, superoxide and peroxynitrite, which have already known to be formed and released during preconditioning and are thought to play a role in cardioprotection associated with PC.

1.3. The role of reactive oxygen species (ROS) in ischaemia and reperfusion

There is no doubt that reactive oxygen species (ROS) play a substantial role in ischaemia and reperfusion injury (Simpson and Lucchesi 1987; Zweier et al., 1987; Ferrari et al., 1989, 1991; Richard et al., 1990). It has been shown that a burst of ROS that occurs mainly during reperfusion provokes serious ventricular arrhythmias and contributes to further injury of myocardial cells (Kloner et al., 1989; Giordano 2005). Several sources of ROS were identified in the heart, but it is still a matter of debate what is the major source of these oxygen products during myocardial ischaemia. Besides the mitochondria, which is one of the main sources of ROS formation, NAD(P)H oxidases, cytochrome P-450, xanthine oxidoreductases, auto-oxidation of catecholamines, etc. are candidates for ROS generation. Within the mitochondria, the elements of the electron transport chain (complex I and III), monoamine oxidases, phosphorylated $p66^{shc}$ protein, NADPH oxidase 4 might be the sources of intracellular ROS production (Kloner et al., 1989; Giordano 2005; DiLisa et al., 2009). Under aerobic conditions, ROS is eliminated by the endogenous antioxidants, such as

superoxide dismutase, urate, glutathione and catalase, but since under ischaemic conditions the antioxidant defence system is also injured, an imbalance develops between the capacity of the antioxidants and the generation of ROS. Interestingly, the increase of antioxidant capacity, for example by the combined administration of two antioxidants (i.e. superoxide dismutase and catalase) reduced the reperfusion-induced injury (Jolly et al., 1984).

As regards the deleterious effects of oxygen radicals in arrhythmia point of view, it is suggested that ROS is involved in the generation of early and delayed afterdepolarizations, and causes action potential abnormalities by influencing the function of various ion channels (e.g. late Na^+, L-type $Ca^{2+}$and K^+ channels), ion transporters (e.g. Na/Ca exchanger), as well as the activity of enzymes, such as Ca/calmodulin kinase (Xie et al., 2009). The oxidation of ion channel subunits by ROS (e.g. L-type Ca^{2+} channel α1 subunit) is suggested to contribute to the action potential abnormalities and S-nitrosylation, a posttranslational protein modification of sulfhydryl residues by NO prevented the ROS-induced oxidative modification of channel subunits (Sun et al., 2006a, 2006b).

There is also evidence that ROS by opening of mitochondrial permeability transition pore is involved in the apoptotic and necrotic cell death (Hausenloy et al., 2002).

On the other hand, ROS, generated after the short episodes of ischaemia (i.e. during PC), is thought to play a trigger role in the cardioprotective effect of PC (Baines et al., 1997). The evidence for this came from studies which showed that scavenging ROS during the PC procedure attenuated the protective effects of PC, whereas the enhancement of ROS formation induced a PC-like cardioprotective effects (Tritto et al., 1997). Although the precise mechanisms by which ROS induces protection is still not clear, a role for PKC-mediated pathway (Von Ruecker et al., 1989; Gopalakrishna et al., 1989) and for the modulation of mitoK_{ATP} channels (Zhang et al., 2001; Lebuffe et al., 2003) are suggested. It is proposed that ROS generated during PC, results from mitochondrial sources, i.e. the opening of mitoK_{ATP} channels enhances the mitochondrial ROS formation. This, through the activation various kinases and survival signalling pathways, such as ERK pathway would result in protection by reducing ROS generated during the prolonged ischaemia and reperfusion insult (Yue et al., 2000; Hausenloy et al., 2004, 2005; Yang et al., 2010). ERK can also phosphorylate connexin43 resulting in reduction in gap junction permeability (Naitoh et al., 2006). Opening these channels may also lead to the inhibition of mitochondrial calcium overload (O'Rourke

et al., 2000) and they are involved in the regulation of mitochondrial volume by ameliorating energy transfer between mitochondria and cellular ATP-ases (Kowaltowski et al., 2001).

1.4. The role of peroxynitrite generation during myocardial ischaemia and reperfusion

Wang and colleagues reported that during early reperfusion, there is a simultaneous elevation in NO and ROS production, and that these radicals react, by nearly a diffusion-limited rate, to produce another potent oxidant, peroxynitrite (PN; Wang et al., 1996; Wang and Zweier, 1996). This non-radical molecule PN may have both deleterious and beneficial effects, depending largely on its concentration (Yasmin et al., 1997; Ferdinandy and Schultz, 2001, Ferdinandy, 2006). For instance, high concentration (>20 μM) of PN, formed either endogenously or administered exogenously, results in endothelial dysfunction (Zou et al., 2004), contributes to cell necrosis (Virág et al., 1998), induces the breakage of DNA (Virág et al., 2003) and may provoke cardiac arrhythmias (Tecder-Unal and Kanzyk, 2004). The underlying mechanisms of the cytotoxic effects of PN could be associated with the oxidation of lipids and proteins, in which the modification of protein tyrosine residues by nitration may play an important role (Yasmin et al., 1997).

There is, however, increasing evidence that PN, in low (nanomolar) concentrations, can elicit protective effects in the heart. It has been shown that PN preserves endothelium function via the inhibition of leukocyte-endothelial cell interaction (Nossuli et al., 1997), reduces platelet aggregation (Lefer et al., 1997), produces coronary vasodilatation (Liu et al., 1994), and decreases infarct size following I/R (Nossuli et al., 1997). The proposed mechanisms include (*i*) the formation of S-nitrosothiols and the subsequent NOS-independent NO release (Wu et al., 1994), (*ii*) the activation of soluble guanylate cyclase and the subsequent increase in cGMP (Al-Sa'doni and Ferro, 2000), as well as (*iii*) the activation of various protein kinases, such as PKC, ERK and p38MAPK (Agbani et al., 2011).

There is also evidence that PN is generated during the PC procedure and may play a trigger role in the antiarrhythmic effect of PC (Altug et al., 2000, 2001). In rat isolated hearts the inhibition of PN formation by the administration of uric acid (UA) during PC markedly attenuated the antiarrhythmic effect of PC (Altug et al., 2000, 2001). Similarly, the protection resulted from the exogenous administration of PN was abolished in the presence of UA (Altug et al., 2000, 2001). In our own experiments using anaesthetized dogs we showed that PN

infused directly into the coronary circulation in a concentration of 100 nM, significantly reduced the severity of ventricular arrhythmias that resulted from a 25 min coronary artery occlusion and reperfusion insult (Kiss et al., 2008). This protection was similar to that seen with ischaemic PC (Kiss et al., 2008, 2010), or after the administration of NO donors (Végh et al., 1996; György et al., 2000; Gönczi et al., 2009). The fact that PN administration results in an antiarrhythmic effect raised the question whether in this large animal arrhythmia model the endogenously formed PN would contribute to the PC-induced antiarrhythmic protection.

1.5. The source and the role of NO during myocardial ischaemia and reperfusion

The first evidence that NO may play a role in the cardioprotective effect of PC came from those dog studies in which we showed that inhibition of the L-arginine-NO synthesis pathway, abolishes the protective effect of PC against arrhythmias (Végh et al., 1992b). Although it is still not quite clear how PC stimulates the generation and release of NO, it was proposed that the stress stimulus-induced rapid BK formation and the subsequent release of NO from the endothelium may play a role (Végh et al., 1993; Parratt and Végh, 1996). In the meantime it was reported that chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial nitric oxide synthase (eNOS) gene expression (Sessa et al., 1994), suggesting a BK-independent pathway for NO formation. More recently the PI3/Akt pathway is proposed to play an important role in the rapid activation of eNOS (Fulton et al., 1999).

According to our best knowledge, there are, at least, three isoforms of NOS enzyme, which synthesize NO: the endothelial (eNOS), neuronal (nNOS) and the inducible (iNOS) forms. These are different in their amino acid sequence, cellular localization, inhibitors and calcium dependency (Förstermann et al., 1994). More recently, it has also become evident that there are possibilities for enzyme-independent NO production as well (Kuppusamy et al., 2001), which occur mainly under ischaemic conditions (Zweier et al., 1995 a,b). In isolated heart preparations, using electron paramagnetic resonance for NO measurement, a considerable increase in NO production was detected (Zweier et al., 1995a), suggesting that during ischaemia NO is preferably generated from nitrite, rather than synthesized by NOS. This excess in NO formation would be detrimental, since NO, by reacting with superoxide, promotes the formation of toxic peroxynitrite. This can be especially detrimental during reperfusion, when a burst of superoxide occurs. According to this hypothesis, the reduction of NO generation would result in protection. Indeed, there is some evidence that in the rat

isolated hearts, PC reduced the generation of NO and that this reduced NO was associated with cardioprotection (Csonka et al., 1999). These *in vitro* studies were, however, not supported by the results of the *in vivo* experiments. In these studies, measuring tissue NO availability in the interstitial fluid by microdialysis (Mori et al., 1998; Stevens et al., 2002) or by electrochemical techniques (Engelman et al., 1995; Prasan et al., 2007), a reduced NO formation was found during myocardial ischaemia (Stevens et al., 2002; Prasan et al., 2007).

In our own experiments, where the plasma nitrate/nitrite (NOx) levels in the blood of the coronary sinus and the generation of superoxide and nitrotyrosine (a biomarker of PN) in myocardial tissue samples were simultaneously measured, we showed that in dogs subjected to a 25 min occlusion/reperfusion insult, the plasma NOx levels were significantly elevated at the beginning (around 7 min) of the occlusion, but after that the NOx levels started to markedly decline (Kiss et al., 2010). Reperfusion of the ischaemic myocardium in these dogs resulted in marked increases in superoxide and NT levels, which almost certainly largerly contributed to a marked increase in the reperfusion-induced fatal ventricular arrhythmias (Kiss et al., 2010). In contrast, in dogs subjected to PC the plasma NOx levels were elevated, and this increased NO bioavailability was present over the entire prolonged period of occlusion (Kiss et al., 2010). Moreover, PC reduced the increased superoxide and NT formation that resulted from the combined ischaemia and reperfusion insult. On the basis of these results we proposed that the preservation of NO bioavailability during ischaemia may have an influence on the generation of superoxide and peroxynitrite during reperfusion and this mechanism has a role in the marked antiarrhythmic effect of PC (Kiss et al., 2010). Indeed, there are studies which show that NO regulates mitochondrial superoxide production (Burwell and Brookes, 2008), and that protects the myocardium against reperfusion injury via protein S-nitrosylation (Kohr et al., 2011).

Although it seems more than likely that PC may influence NOS activity (Xuan et al., 2000) and expression (Sessa et al., 1994; Kovács et al., 2010), there is a lack of evidence whether the preservation of NO bioavailability following PC results from the influence of the PC stimulus on the enzyme-dependent or/and an enzyme-independent NO formation. It is also not well established, what is the time-course changes in the generation of other substances, namely superoxide and peroxynitrite, which can also be generated during the PC stimulus, and which formation is modified by NO.

AIMS

The aims of studies, involved in the present Thesis were as follows:

STUDY I.

On the basis of our own and others previous studies (Altug et al., 2000, 2001; Kiss et al., 2008, 2010), we hypothesized that peroxynitrite (PN) is produced during the PC stimulus, and it may act as a trigger for the PC-induced antiarrhythmic protection. To examine this we designed experiments in which uric acid (UA), a relatively selective scavenger of PN, was administered in dogs subjected to PC, and the results were compared to those that had been obtained in dogs treated with 100 nM concentration of PN. Urate was given in intravenous infusion in a dose of 0.2 mg/kg/min, both 10 min prior to and during PC procedure (total duration is 20 min), as well as together with the intracoronary administration of the 100 nM concentration of PN.

STUDY II.

The experiments involved in this part of the Thesis aimed to examine whether the PC-induced preservation in NO bioavailability during prolonged ischaemia is due to changes in NOS enzyme activity, or other enzyme-independent NO formation may also play a role. To achieve this aim, we assessed the time-course changes in NOS activation in parallel with alterations in plasma NOx levels, tissue superoxide and nitrotyrosine production in control dogs (without PC), and in dogs that were subjected to PC by two 5 min occlusion and reperfusion insults 5 min prior to a 25 min occlusion of the LAD.

2. MATERIALS AND METHODS

Since in Study I and Study II the experimental procedures, the measured parameters and methods for the *in vivo* and *in vitro* measurements were identical, these sections are valid for both studies, and only the 'Experimental protocols' are described separately.

2.1. Ethics statement

The origin and upkeep of these dogs were in accord with Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals, which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and conformed to the Directive 2010/63/EU of the European Parliament. The protocols have been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval number XIII/1211/2012).

2.2. Surgical procedures

Inbred adult mongrel dogs of either sex, and with a mean body weight of 19 ± 5 kg (Study I) and 18 ± 4 (Study II) were anaesthetized with an intravenous bolus injection of pentobarbitone (0.5 mg kg^{-1} i.v., Euthanyl® Bimeda-MTC Animal Health Inc.) and all efforts were made to minimize suffering. Prior to the studies the dogs were housed in an animal room (temperature: 10-20 ^{o}C, humidity: 40-70%, lightening: 12 h per day, 2 animals per pen), for at least two weeks and fed a standard diet with ad libitum access to water. Food was withdrawn 24 h before anaesthesia.

After catheterization of the right femoral vein, the anaesthesia was maintained with the intravenous administration of a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively; Sigma, St. Louis, MO, USA). 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 at a rate and volume sufficient to maintain arterial blood gases within normal limits (Végh et al., 1992a). Body temperature was measured from the mid-oesophagus and maintained at 37 ± 0.5 ^{o}C.

Polyethylene catheters (Cordis F4) were introduced into the right femoral artery for monitoring arterial blood pressure (systolic, diastolic and mean), and into the left ventricle (LV), via the left carotid artery, for the measurement of LV systolic and end-diastolic (LVEDP) pressures and LVdP/dt (isovolumic contraction and relaxation). All these parameters together with a chest lead electrocardiogram were measured with a Plugsys Hemodynamic Apparatus (Hugo Sachs Electronics, Germany) and recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany).

A thoracotomy was performed at the left fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) prepared for occlusion just proximal to the first main diagonal branch. Distal to the occlusion site, a small side branch of the same artery was also prepared and cannulated for the administration of peroxynitrite or saline. Through the right jugular vein a catheter was positioned into the coronary sinus to collect blood samples for the measurement of plasma nitrate/nitrite (NOx). The left jugular vein was also catheterized for the intravenous infusion of uric acid (UA).

2.3.1. Assessment of ventricular arrhythmias

The number and severity of ventricular arrhythmias were assessed from the chest lead II electrocardiogram, according the Lambeth conventions (Walker et al., 1988), modified by us as described elsewhere (Végh et al., 1992a). Thus, the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT; defined as a run of four or more VPBs at a rate faster than the resting sinus rate), 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.

2.3.2. Assessment of ischaemia severity

The severity of myocardial ischaemia was evaluated by two parameters; changes in the epicardial ST-segment and in the degree of inhomogeneity of electrical activation. These were measured by means of a composite electrode positioned within the potential ischaemic area of the left ventricular wall. This electrode gives a summarised recording of R waves from 24 epicardial measuring points as described previously (Végh et. al., 1992a). Under normal

myocardial perfusion and oxygenation, all sites are activated simultaneously, resulting in a single large spike. However, following occlusion, widening and fractionation of this summarized R-wave occurs, indicating that the adjacent fibres are not simultaneously activated because of the inhomogeneity of conduction. The greatest delay in activation occurring within the ischaemic area following coronary artery occlusion was expressed in ms. The composite electrode also contains four unipolar electrodes by which changes in epicardial ST-segment were evaluated (in mV) within the ischaemic zone.

2.4. In vitro measurements

2.4.1. Determination of plasma nitrate/nitrite levels

Plasma nitrate/nitrite (NO_x) levels were measured by Griess reaction as outlined by Moshage et al., (1995). Blood samples, collected from the coronary sinus at different time points (Figure 1 and 2.) were centrifuged at 10 000 *g* for 15 min at 4°C. The plasma was mixed with β-nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide, nitrate reductase (Sigma, MO) and incubated for 30 min at 37°C. After the enzymatic conversion of nitrate to nitrite, the mixture was incubated with Griess reagent for further 10 min at room temperature. The absorbance of the azo compound was measured spectrophotometrically at a wavelength of 540 nm and the total nitrate/nitrite (NOx) concentration (μmol/L) was assessed using a standard calibration curve of sodium nitrite ($NaNO_2$, MO) and sodium nitrate ($NaNO_3$, Sigma, MO).

2.4.2. Assessment of myocardial superoxide ($O_2^{-\cdot}$) generation

This was determined using dihydroethidine (DHE) fluorescent dye method. DHE enters freely into the cell and is oxidized by superoxide to yield fluorescent ethidium, which binds to DNA (Eth-DNA). Tissue blocks (0.5 x 0.5 x 2 cm) were excised from the ischaemic area and embedded in optical cutting temperature compounds. These samples were cut into 20 μm cryosections and stained with DHE (1μM) dissolved in phosphate buffer solution (PBS; pH 7.4), and incubated at 37 °C for 30 min in a dark humidified chamber. Samples, where the reaction was blocked with a thiol-containing antioxidant, N-acetyl-L-cysteine (100 mM, Sigma, St Louis, MO, U.S.A), served as a negative controls. Both from the stained and negative control samples 10 to 15 serial images were captured by a confocal laser scanning microscope (Olympus FV1000). The intensity of the fluorescent signals were analysed by

ImageJ software (NIH, Bethesda, MD) and expressed in arbitrary units (AU).

2.4.3. Determination of nitrotyrosine formation

Myocardial nitrotyrosine (NT), a biomarker of peroxynitrite (PN) formation was evaluated by Western blot. Tissue samples were taken from the ischaemic area within two minutes of the reperfusion as described previously (Kiss et al., 2008). To assess tyrosine nitrosylation, 20 μg of total protein was separated on 8% polyacrylamide gel and the protein bands were transferred to polyvinylidene membrane (100 V, 400 mA, for 120 min). After blocking in 5% non-fat milk, the membrane was incubated with mouse monoclonal anti-nitrotyrosine antibody (in a dilution of 1:1000; MAB5404, Chemicon, Millipore Corp., Billerica, MA, USA), and then with horseradish peroxidase-conjugated rabbit anti-mouse IgG (in a dilution of 1:1000, P0161, Dakocytomation A/S, Glostrup, Denmark) as a secondary antibody. For visualisation, the membrane was developed with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare, Little Chalfont, Buckinghamshire, UK), exposed to X-ray film and scanned. The intensity of bands was determined using ImageJ software (NIH Bethesda, MD), and expressed in percentage of the sham-operated dogs. Equal protein loading was confirmed by monitoring GAPDH.

2.4.4. Assessment of nitric oxide synthase activity (NOS)

In Study II this was performed using a NOS assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. This method is based on the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. From the tissue samples (100 mg) proteins were isolated, homogenized in ice-cold homogenization buffer (Cayman Chemical, Ann Arbor, MI, USA), and centrifuged at 10000 g for 15 min, at 4 °C. The supernatant was added to the reaction mixture and incubated for 60 min at room temperature. A liquid scintillation counter (Wizard^{TM}, USA) was used to determine total NOS activity by measuring the amount of the radio-labelled citrulline formed during the reaction, and expressed as the percentage of the total counts corrected with the background counts per minute.

2.4.5. Preparation of synthetic peroxynitrite

For the experiments in Study I. peroxynitrite was synthesized at the Department of Biochemistry (University of Szeged, Hungary) according to the method of Beckman and colleagues (Beckman et al., 1994), from acidified nitrite and hydrogen peroxide (Kiss et al.,

2008). The absorbance of synthesized PN was evaluated spectrophotometrically at 302 nm wavelength. Stock solutions (50-150 mM) were stored in Eppendorf tubes wrapped in aluminium foil at -80 °C. Before use in each experiment, the concentrations were evaluated and then diluted to 100 nM with appropriate volume of pH 8.4 saline (Nossuli et al., 1997).

2.5. Statistical analysis

Data analysis was performed using STATISTICA software (Statsoft, Oklahoma, USA). All values are expressed as means ± s.e. mean and differences between means compared by ANOVA for repeated measures or by one-way ANOVA as appropriate, using the Fisher post hoc test. Ventricular premature beats and episodes of ventricular tachycardia were compared using the Kruskal-Wallis test. The incidence of arrhythmias (such as VT and VF) and survival from the combined ischaemia and reperfusion insult was compared by the Fisher Exact test. Differences were considered significant at $P<0.05$.

2.6. Experimental protocols

2.6.1. Protocol for Study I. This is shown in Figure 1. The dogs were randomly divided into eleven groups. After completing the surgical preparations, a 20 min recovery period was allowed to stabilise the haemodynamic and blood gas parameters. In all groups, myocardial ischaemia was induced by a 25 min occlusion of LAD followed by rapid reperfusion. In the preconditioned dogs this was preceded by two 5 min periods of LAD occlusion either in the absence (PC; n=10) or in the presence of urate (UA+PC; n=8). Urate was infused intravenously into the jugular vein in a dose of 0.2 mg/kg/min, over 30 min before the sustained ischaemic period. In further two groups, synthetic PN, dissolved in pH 8.4 saline, was given in a concentration of 100 nM in intracoronary infusion (rate: 0.5 ml/min) for identical periods to the PC occlusions, either with (UA+PN; n=8) or without urate (PN, n=10). The PN containing syringes were covered with foil to protect the solution against photo-degradation. There were two control groups, in which pH 8.4 saline (C1; n=14) or urate (UAC; n=9) were given prior to the prolonged I/R insult.

In five additional groups the hearts were stopped after two periods of saline (pH 8.4, C2; n=4) or PN infusions (PN; n=3), or the PC occlusion/reperfusion insults (PC; n=4) in the absence of UA and also in the presence of urate (UA+PC; n=3; UA+PN; n=3). In these myocardial samples the formation of NT was assessed. In those animals that were underwent prolonged

ischaemia and were alive 2 min after reperfusion, the hearts were stopped with an excess of anaesthetic and tissue samples were taken from the ischaemic myocardial wall for the determination of superoxide and NT.

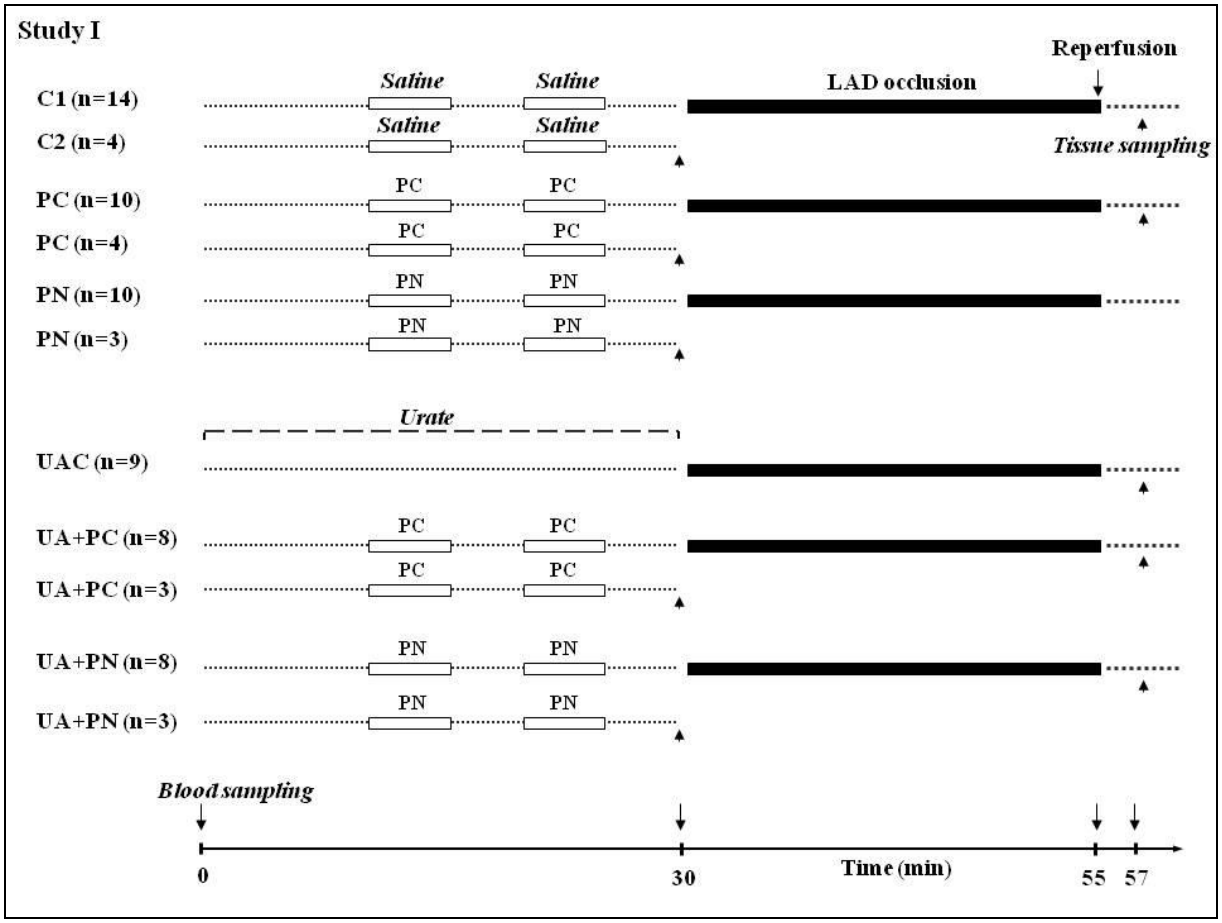

Figure 1. Experimental protocol to examine the trigger role of PN in cardioprotection induced by ischaemic PC and by the administration of PN.

2.6.2. Protocol for Study II. This is illustrated in Figure 2. In this study dogs were selected randomly to form seven groups, each containing from 3 to 5 animals. After surgery, the dogs were allowed to recover for 30 min. In all groups, except the sham-operated controls, myocardial ischaemia was induced by the occlusion of the LAD. Three groups served as controls. In these groups after euthanasia with an excess of the anaesthetic, myocardial tissue samples were collected either at the end of the 30 min recovery period (C0; n=5) or at 5 min (C1; n=4) and 25 min (C2; n=3) of the coronary artery occlusion. In four groups, the dogs were preconditioned by two 5 min occlusion of the LAD interspersed with a 5 min

reperfusion interval. In these groups after stopping the heart by the administration of an overdose of the anaesthetic, samples were taken either at the end (PC1; n=5) or 5 min after (PC2; n=4) of the second PC occlusion, as well as also at the 5 min (PC3; n=4) and 25 min (PC4; n=3) of the prolonged ischaemia. In each group tissue samples for the determination of NOS activity and superoxide production were excised from the ischaemic area supplied by the occluded LAD. Blood samples (BS) were also taken from the coronary sinus at various time points of the experiments to measure plasma NOx levels.

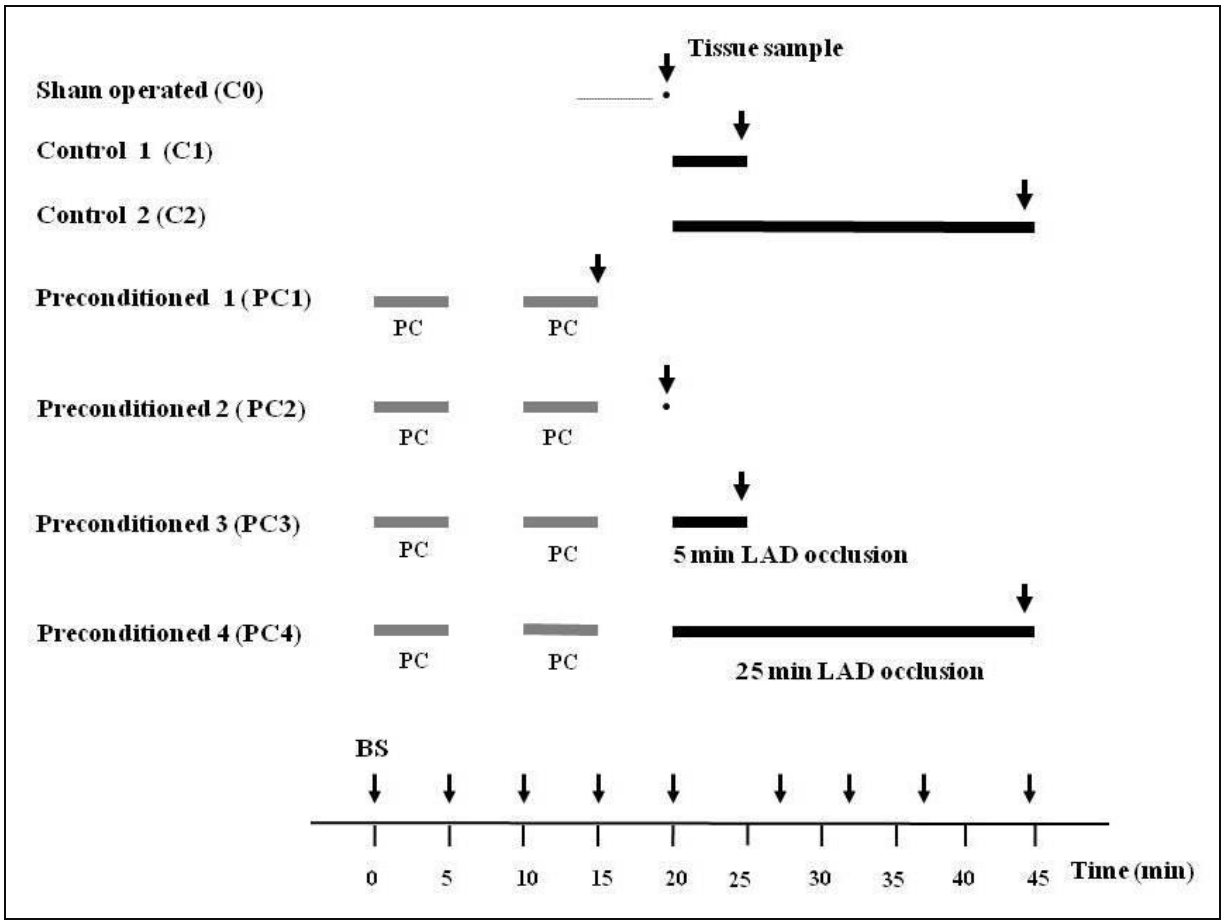

Figure 2. Experimental protocol for the examination of the time-dependent changes in NOS enzyme activity, NOx concentrations, as well as superoxide and nitrotyrosine productions in control and in preconditioned dogs.

3. RESULTS

3.1. Study I. Evaluation of the trigger role of peroxynitrite in the antiarrhythmic effect of ischaemic preconditioning

3.1.1. Haemodynamic effects of pH 8.4 saline, peroxynitrite and urate

These are summarised in Table 1. None of the infusion of pH 8.4 saline, peroxynitrite and uric acid caused significant alterations in the haemodynamic parameters.

Table 1. Haemodynamic effects of pH 8.4 saline, urate and peroxynitrite

Haemodynamic parameters	Saline pH 8.4		PN		UA	
	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change
SABP (mmHg)	127 ± 4	3 ± 3	124 ± 5	-3 ± 2	136 ± 6	1 ± 3
DABP (mmHg)	83 ± 7	3 ± 1	84 ± 3	-2 ± 3	93 ± 5	-1 ± 3
MABP (mmHg)	97 ± 6	3 ± 3	97 ± 4	-2 ± 2	108 ± 8	0 ± 3
LVSP (mmHg)	128 ± 11	1 ± 2	124 ± 6	-3 ± 2	137 ± 5	-1 ± 3
LVEDP (mmHg)	3.6 ± 0.5	-0.4 ± 0.2	3.7 ± 0.3	-0.4 ± 0.4	3.6 ± 0.6	-0.1 ± 3.2
+dP/dt$_{max}$ (mmHg·s^{-1})	2801 ± 167	45 ± 101	2900 ± 111	-48 ± 97	2849 ± 147	-73 ± 53
-dP/dt$_{max}$ (mmHg·s^{-1})	2058 ± 109	45 ± 29	1967 ± 106	-65 ± 85	2119 ± 88	-31 ± 57
HR (beats·min^{-1})	156 ± 2	2 ± 2	145 ± 6	-1 ± 2	153 ± 8	-5 ± 2

Data are presented as changes, measured 5 min after starting the infusions. 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. Data are means ± s.e.m.*

3.1.2. Haemodynamic changes following coronary artery occlusion

These are illustrated in Table 2. In all groups, occlusion of the LAD resulted in significant decreases in arterial blood pressure, LVSP, positive and negative dP/dt$_{max}$ and an increase in LVEDP. These haemodynamic changes were, however, significantly less both in the PC and the PN treated animals. Although UA itself did not influence either the ischaemia or the PC-induced haemodynamic changes, it abolished the effects of PN infusion in these parameters. In anaesthetized dogs, coronary artery occlusion did not substantially modify heart rate.

Table 2. Haemodynamic changes during a 25 min occlusion of the LAD

Haemodynamic parameters	Control		PC		PN		UAC		UA+PC		UA+PN	
	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change	Baseline	Max. change
SABP (mmHg)	128 ± 6	-17 ± 1[a]	122 ± 5	-10 ± 1[ab]	125 ± 6	-11 ± 1[ab]	133 ± 3	-13 ± 1[a]	121 ± 7	-13 ± 3[ab]	128 ± 3	-13 ± 3[a]
DABP (mmHg)	85 ± 4	-15 ± 1[a]	84 ± 3	-10 ± 1[ab]	85 ± 4	-9 ± 2[ab]	87 ± 3	-13 ± 3[a]	81 ± 5	-10 ± 2[ab]	89 ± 1	-15 ± 5[a]
MABP (mmHg)	99 ± 5	-16 ± 2[a]	97 ± 4	-10 ± 1[ab]	98 ± 5	-10 ± 1[ab]	102 ± 3	-13 ± 2[a]	95 ± 5	-12 ± 3[ab]	102 ± 2	-14 ± 4[a]
LVSP (mmHg)	127 ± 5	-18 ± 2[a]	112 ± 5	-11 ± 11[ab]	127 ± 6	-13 ± 2[ab]	128 ± 4	-18 ± 5[a]	124 ± 6	-12 ± 2[ab]	129 ± 2	-15 ± 3[a]
LVEDP (mmHg)	3.8 ± 0.4	8.0 ± 0.8[a]	3.9 ± 0.4	5.3 ± 0.1[ab]	3.4 ± 0.4	5.0 ± 0.5[ab]	2.8 ± 0.4	7 ± 1[a]	3.6 ± 0.3	6.5 ± 0.8[a]	3.4 ± 0.5	7.8 ± 1.4[a]
+dP/dt$_{max}$ (mmHg·s^{-1})	2780 ± 132	-558 ± 100[a]	2441 ± 180	-380 ± 51[ab]	2742 ± 168	-439 ± 53[a]	2880 ± 170	-516 ± 79[a]	2575 ± 52	-380 ± 72[ab]	2564 ± 172	-658 ± 126[a]
-dP/dt$_{max}$ (mmHg·s^{-1})	2086 ± 72	-611 ± 83[a]	1998 ± 146	-276 ± 49[ab]	1868 ± 83	-326 ± 50[ab]	2209 ± 68	-583 ± 103[a]	2110 ± 52	-133 ± 43[ab]	2065 ± 107	-568 ± 114[a]
HR (beats·min^{-1})	154 ± 3	1 ± 2	140 ± 6	1 ± 3	150 ± 7	2 ± 2	152 ± 9	2 ± 2	160 ± 7	-1 ± 1	155 ± 5	3 ± 2

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.* Data are means ± s.e.m., [b]$P<0.05$ compared to the control and [a]$P<0.05$ compared to the baseline value.

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

These are illustrated in Figures 3 and 4. Occlusion of the LAD for 25 min resulted in high number of VPBs (289 ± 74) and many episodes of VT (11.0 ± 3.6) that occurred in 93% of the control dogs (Figure 3). Furthermore, in this group VF was apparent in 50% of the animals during the occlusion, and no dog survived the combined occlusion and reperfusion insult (Figure 4).

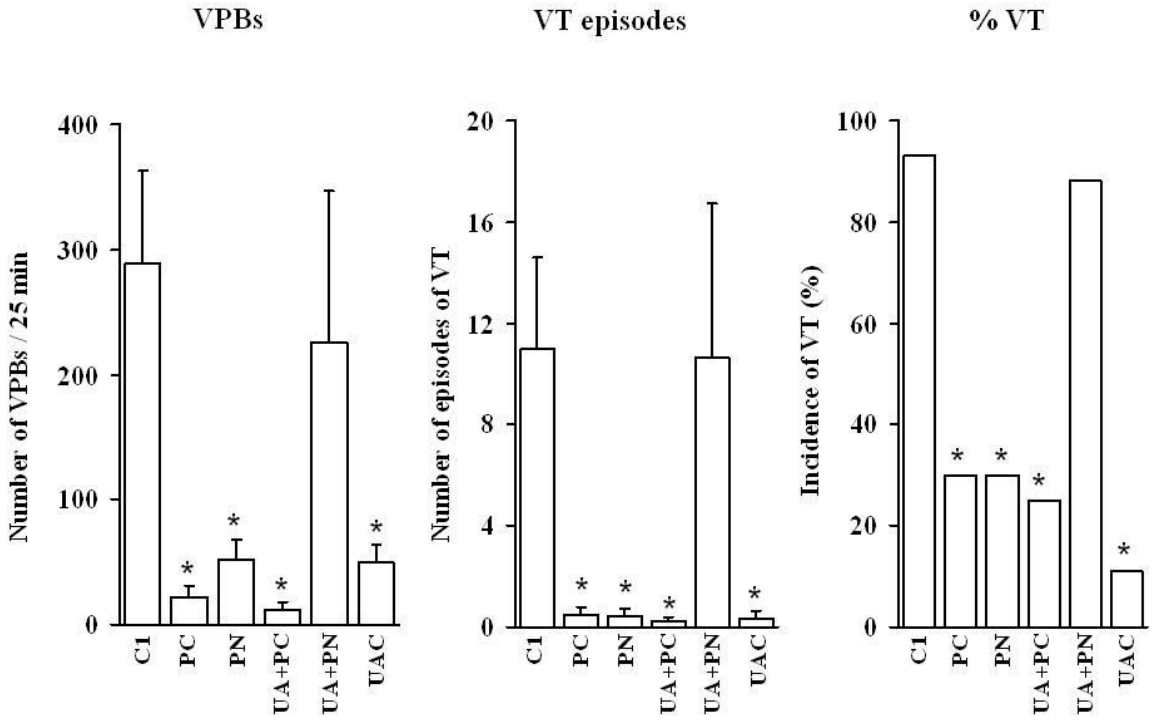

Figure 3. The total number of VPBs, the incidence and the number of episodes of VT during a 25 min coronary artery occlusion in control, preconditioned and peroxynitrite treated dogs, both in the presence (UA, UA+PC, UA+PN) and in the absence of UA (C1, PC, PN). Values are means ± s.e.m., *P<0.05 vs. C1.

Both PC and the infusion of PN significantly decreased the severity of the ischaemia-induced ventricular arrhythmias and increased survival following reperfusion (Figures 3 and 4). Interestingly, the administration of UA did not influence the antiarrhythmic effects of PC, but almost completely abolished the PN-induced protection. Thus, survival from the combined I/R insult was similar in the PC dogs, no matter whether UA was given or not (63% vs. 60 %, respectively), whereas in the PN-treated dogs, in the presence of UA, survival reduced from 50% to 25%. Surprisingly, UA itself also resulted in protection against the acute I/R-induced

ventricular arrhythmias. Compared with controls, in dogs given UA alone there were less numbers of ectopic beats (50 ± 14) and episodes of VT (0.3 ± 0.3), as well as lower incidences of VT (11%) and VF (0%) during the occlusion, and survival was increased from 0% to 55%.

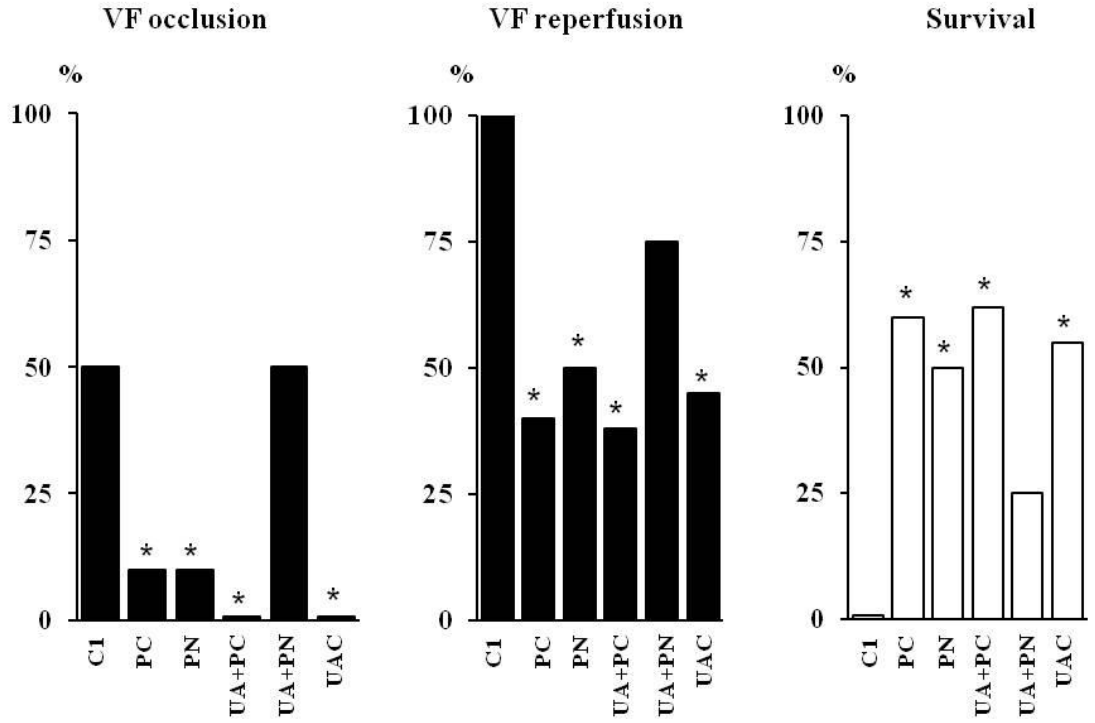

Figure 4. The incidence of VF during occlusion and reperfusion, as well as survival from the combined I/R insult. Values are means ± s.e.m., *P<0.05 vs. C1.

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

This was assessed by two parameters; i.e. changes in epicardial ST-segment (Figure 5A) and in the degree of inhomogeneity of electrical activation (Figure 5B). In control dogs occlusion of the LAD resulted in significant increases in both the epicardial ST-segment and the degree of inhomogeneity, which were especially pronounced during the first 5 min of the occlusion. In contrast, these indices of ischaemia severity were significantly less pronounced in the PC and in the PN-treated dogs. Whereas in PC dogs the administration of UA did not substantially modify the epicardial ST-segment changes and the degree of inhomogeneity, in the PN-treated dogs the anti-ischaemic effects were abolished by UA. Uric acid given alone also exhibited an anti-ischaemic effect.

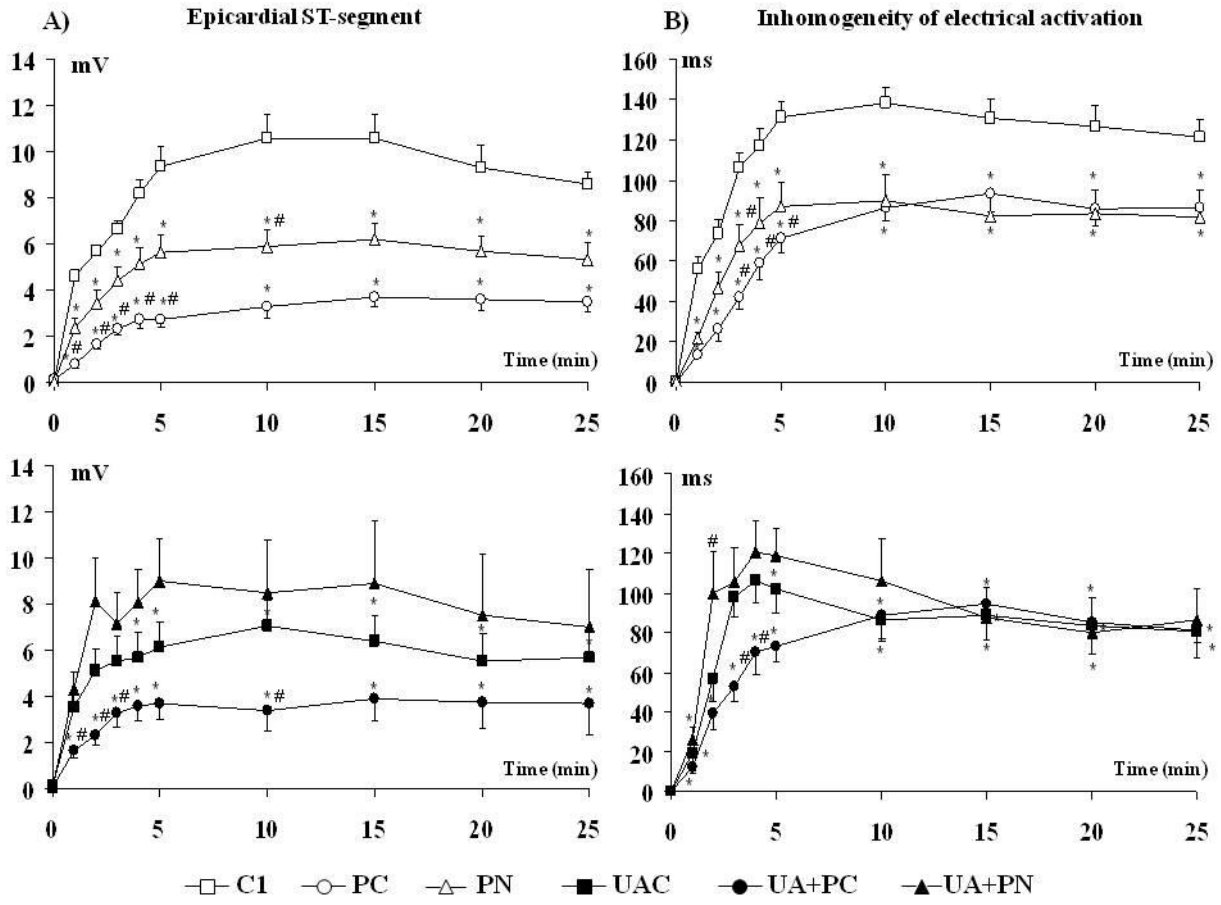

Figure 5. Changes in the epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 25 min occlusion of the LAD. Values are means ± s.e.m., *P<0.05 vs. C1 and $^{\#}$P<0.05 vs. baseline.

3.1.5. Changes in plasma NOx concentrations during a 25 min occlusion of the LAD

There were no significant differences among the groups in the baseline plasma levels of NOx (Figure 6). Both the PC occlusions and also the similar periods of PN infusion increased the level of NO metabolites (Figure 6A), and this was maintained over the entire prolonged period of ischaemia (Figure 6B). This was in contrast with the control dogs, in which NOx levels were markedly reduced by the end of occlusion period (Figure 6B). Uric acid given alone did not influence NOx levels either before or during the sustained ischaemia, and it also failed to modify NO bioavailability in the PC dogs. Uric acid, however, abolished the PN-induced increase in NOx both prior to and during the 25 min of the occlusion.

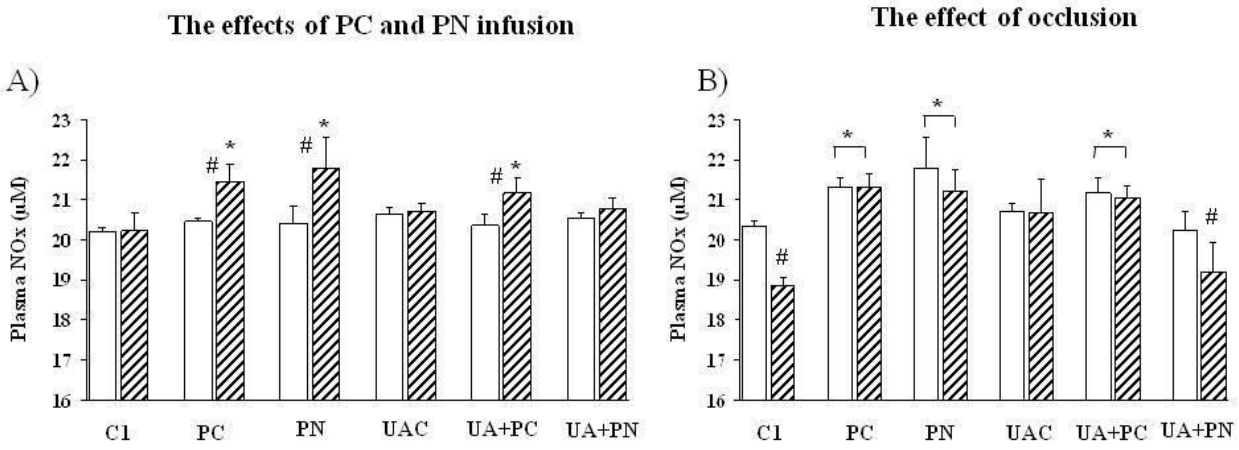

Figure 6. Plasma NOx levels after PC and infusion of PN, as well as following a 25 min LAD occlusion (shaded bars). Values are means ± s.e.m., *P<0.05 vs. C1, and #P<0.05 vs. baseline and/pre-occlusion values.

3.1.6. Myocardial superoxide production following a 25 min occlusion and reperfusion

Myocardial superoxide production was determined 2 min after reperfusion (Figure 7). Compared with the sham controls (C2) a 25 min I/R insult markedly elevated superoxide production, which was significantly attenuated by PC and the administration of PN. Urate itself also decreased the generation of ROS and abolished the PN-induced reduction in ROS formation. Urate, however, did not modify the effect of PC on superoxide production.

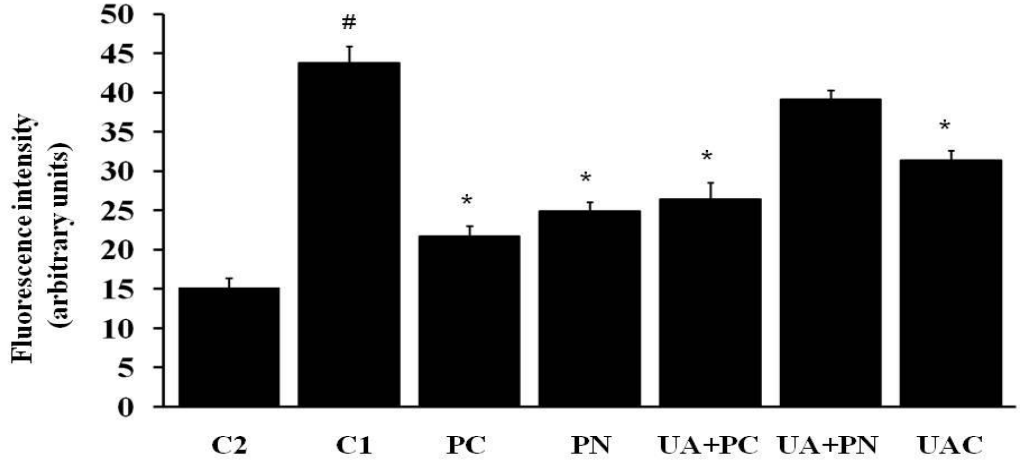

Figure 7. Changes in superoxide production, after a 25 min I/R insult in control (C1) preconditioned (PC) and PN treated dogs in the absence and in the presence of UA (UAC, UA+PC, UA+PN).Values are means ± s.e.m., *P<0.05 vs. C1 and #P<0.05 vs. C2.

3.1.7. Changes in myocardial nitrotyrosine production

This was determined in tissue samples both after the application of PC and PN (Figure 8A) and following the prolonged I/R insult (Figure 8B). Compared with the saline infused controls (C2), both PC and the infusion of PN significantly increased myocardial NT formation. This effect was abolished in the presence of UA (Figure 8A). Occlusion and reperfusion of the LAD for 25 min markedly increased NT production, which was significantly less marked both in the PC and the PN-treated animals. Although UA itself reduced NT formation that resulted from a prolonged period of I/R, it only abolished the effect of PN on NT production (Figure 8B).

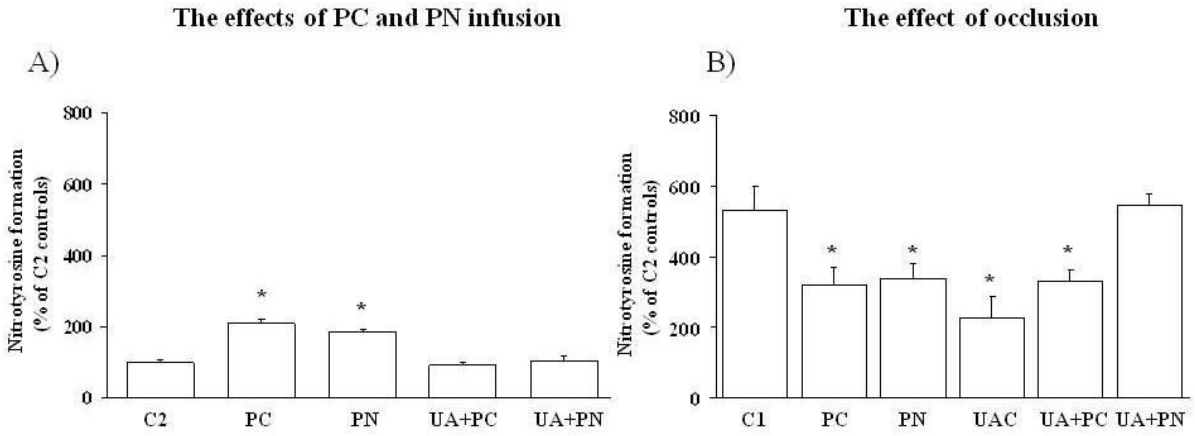

Figure 8. Myocardial nitrotyrosine formation following PC and the infusion of PN infusion, as well as a 25 min occlusion of LAD. Values are means ± s.e.m., *P<0.05 vs. C1 or C2 control groups.

3.2. Study II. Examination of changes in NOS activity during myocardial ischaemia

The objective of this study was to examine, whether changes in the plasma concentrations of NO metabolites (nitrate and nitrite) during coronary artery occlusion are due to a time-course alteration in NOS activity.

3.2.1. NOS enzyme activity following PC and a prolonged period of LAD occlusion

The changes in NOS activation are illustrated in Figure 9. Compared to the sham controls (C0), in dogs subjected to ischaemia there was a marked increase in NOS activity occurring in samples taken 5 min after the onset of the occlusion (C1). However, when the ischaemia was maintained for 25 min, the activation of NOS was markedly reduced by the end of the occlusion period (C2). The PC procedure (two 5 min occlusions with a 5 min reperfusion interval in between) itself increased the activity of NOS (PC1), but this returned to normal 5 min later (PC2). In these PC dogs, however, the NOS enzyme was rapidly activated again, when the animals were subjected to prolonged ischaemia, and this activation continued over the entire 25 min occlusion period (PC4).

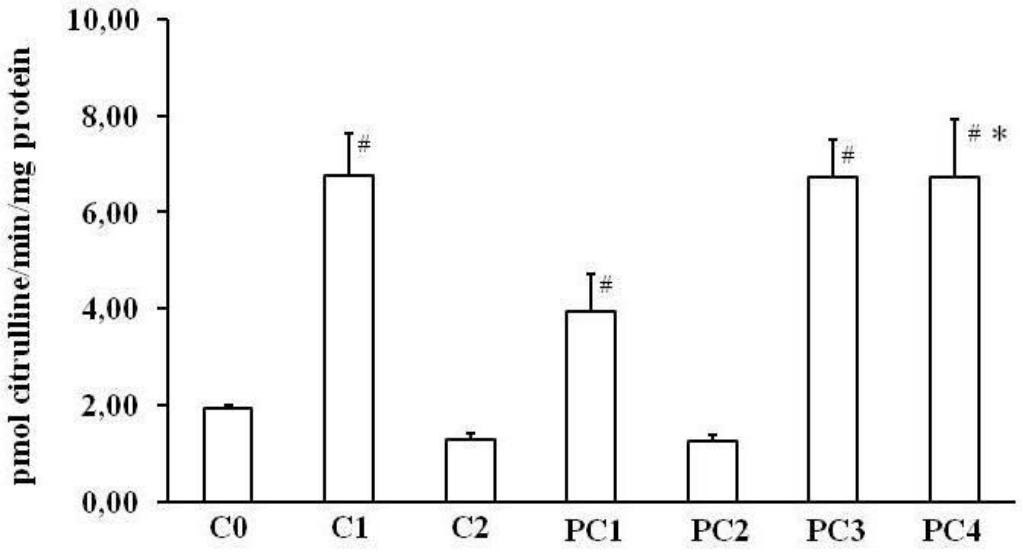

Figure 9. Time course changes in total NOS activity in control and in preconditioned dogs. Values are means ± s.e.m., *P<0.05 vs. C2 and $^{\#}$P<0.05 vs. C0.

3.2.2. Changes in plasma nitrate/nitrite (NOx) levels following preconditioning and during sustained ischaemia

The NOx levels changed almost parallel with the activation of NOS (Figure 10). The baseline plasma level of NO metabolites in the blood of the coronary sinus was 20.3 ± 0.1 μmol/L (data obtained at this time point [C0] from control and PC dogs, were summed). In control dogs, occlusion of the LAD resulted in a significant increase in NOx that occurred in samples taken at 7 min of ischaemia (C1). After this, the concentration of the NO metabolites started to decline, and by the end of the 25 min occlusion (C2), it became significantly lower than the initial baseline value. In contrast, NOx levels elevated following the PC procedure (PC1 and PC2), and these were maintained or even further increased during the subsequent prolonged period of the occlusion (PC3 and PC4).

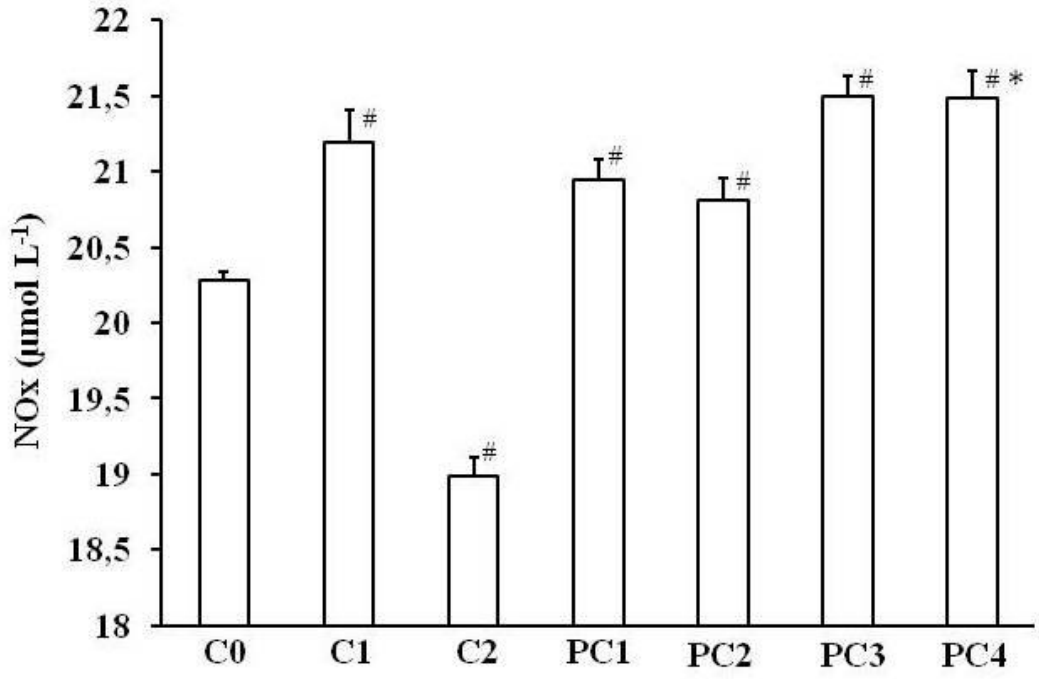

Figure 10. Time course changes in plasma nitrate/nitrite (NOx) concentrations in control and in preconditioned dogs. Values are means ± s.e.m., *P<0.05 vs. C2 group and #P<0.05 vs. C0 group.

3.2.3. Changes in myocardial superoxide production following the PC procedure and during a 25 min LAD occlusion

Compared to the sham controls, the production of superoxide was significantly increased in dogs that had been subjected to a 25 min occlusion (C2). This result indicates that a marked generation of ROS may occur already during the later period of the ischaemia (Figure 11). Although the PC procedure itself increased the generation of superoxide (PC1 and PC2), the superoxide production was less pronounced during the prolonged occlusion in these dogs than in the controls (C2 compared with PC4; $P<0.05$).

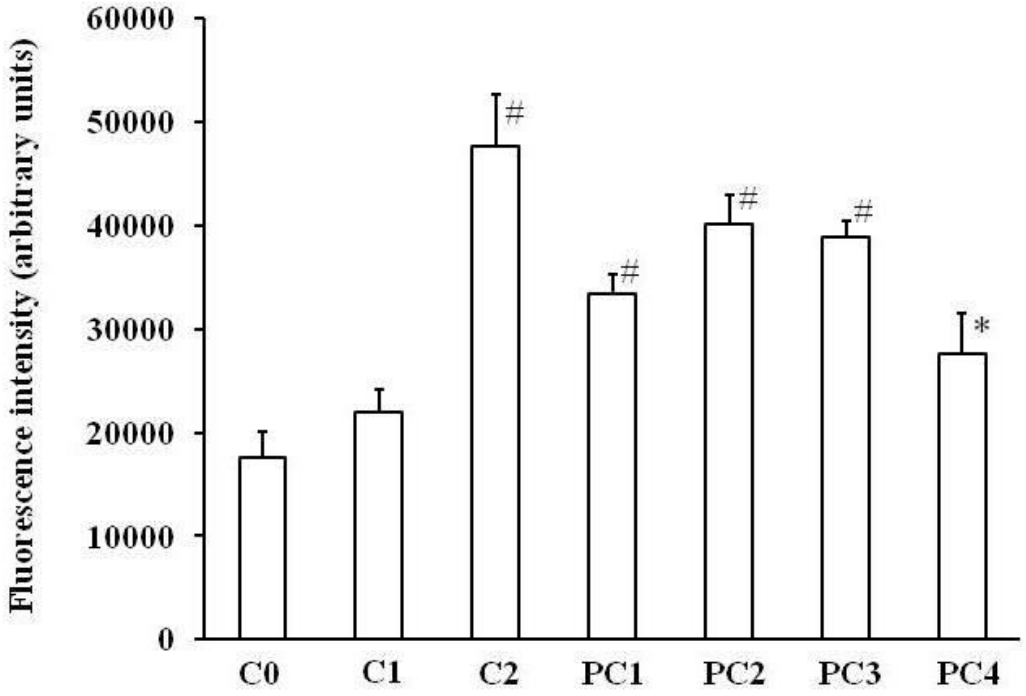

Figure 11. Myocardial superoxide production in control and in preconditioned dogs. Values are means ± s.e.m., $^{\#}P<0.05$ vs. C0 group and $*P<0.05$ vs. C2 group.

3.2.4. Changes in nitrotyrosine formation in control and in preconditioned dogs

For the assessment of peroxynitrite formation, the generation of nitrotyrosine was determined in myocardial tissue samples taken at various time points from control and PC dogs (Figure 12). Compared with the sham controls, occlusion of the LAD significantly increased NT formation (C0 compared with C2; $P<0.05$). There was also an enhanced NT production following the PC procedure (C0 compared with PC1 and PC2; $P<0.05$), but the amount of this stable product of NO and superoxide reaction was not substantially changed during the subsequent, more prolonged period of occlusion (PC2 compared with PC3 and PC4; ns), nor significantly different from that obtained in the controls (C2 compared with PC4; ns).

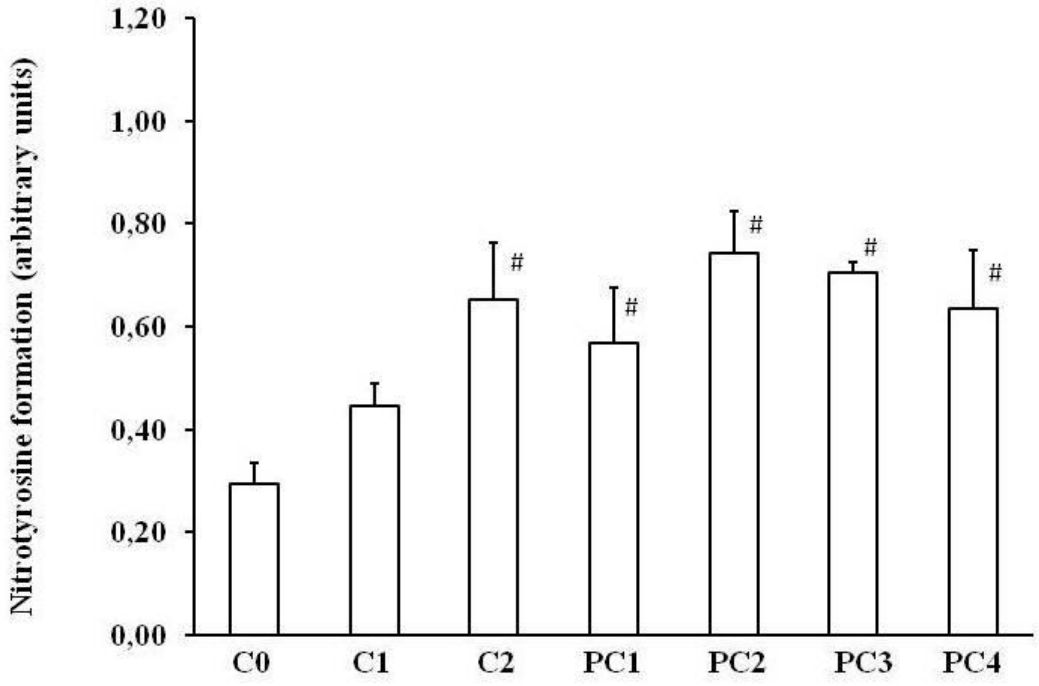

Figure 12. Nitrotyrosine formation in control and in preconditioned dogs. Values are means ± s.e.m., $^{\#}P<0.05$ vs. C0 group.

4. DISCUSSION

New findings

Study I.

1. We have demonstrated that peroxynitrite is produced during preconditioning, induced by brief periods of ischaemia and reperfusion insults. This peroxynitrite formation, however, most probably does not play an important role in the induction of the antiarrhythmic effect of preconditioning, since the protection is still present, if peroxynitrite is scavenged by uric acid, during the PC procedure.
2. In contrast, the cardioprotective effects of the exogenously applied peroxynitrite are completely abolished by the administration of urate.
3. We have also pointed out that uric acid itself may provide an antiarrhythmic effect. This effect may be associated with the antioxidant properties of urate.

Thus our conclusion from Study I is that although peroxynitrite in low (100 nM) concentration can induce a preconditioning-like antiarrhythmic protection, the endogenously formed peroxynitrite is not necessary for the initiation of the PC-induced protection against arrhythmias.

Study II.

The results of this study provided evidence that NO bioavailability during a prolonged (25 min) coronary artery occlusion is tightly associated with the function of NOS enzyme. This observation is supported by the facts that

(i) a prolonged period of ischaemia decreases the activity of NOS (perhaps by uncoupling of the enzyme), resulting in reduced NO and increased superoxide and nitrotyrosine productions,

(ii) preconditioning results in rapid activation of NOS, and this increase in NOS activity persists during the subsequent more prolonged period of ischaemia, resulting in better NO bioavailability and suppressed oxygen radical productions. We suggest that these effects are certainly playing a role in the antiarrhythmic effect of PC.

The findings, summarized in the present thesis, result from two separate studies.

In **Study I**, which aimed to examine the trigger role of PN in the antiarrhythmic effects of PC and PN infusion, we confirmed our previous results that the administration of the nanomolar concentration of peroxynitrite directly into the coronary circulation results in similar antiarrhythmic effect that can be achieved with ischaemic preconditioning (Kiss et al., 2010). This protection is manifested not only in a reduction in the severity of arrhythmias, but also in an attenuation of ischaemic changes, preservation in NO bioavailability during ischaemia and suppression of oxidative stress products, mainly superoxide, during the early phase of the reperfusion. Since there is some evidence that during the short periods of ischaemia and reperfusion insults (i.e. preconditioning), peroxynitrite may be generated from the reaction of NO and superoxide (Altug et al., 2000, 2001), we have raised the question whether in our canine model, PN can be formed in sufficient concentration during the PC stimulus, to trigger and thereby contribute to the antiarrhythmic protection. Therefore, we designed studies in which uric acid (UA), a relatively selective scavenger of PN was infused prior to and during the PC protocol and also the infusion of PN. We have found that in the presence of UA, although both the PC and the exogenous PN-induced increase in NT formation was abolished, the antiarrhythmic effect that resulted from PC remained to be unaffected. This was in contrast with the protection resulted from PN infusion; here scavenging of the exogenously administrated PN by urate, the antiarrhythmic and the other cardioprotective (anti-ischaemic, NO preserving, superoxide suppressing, etc.) effects were completely abolished. Thus, we conclude that although PN can induce cardioprotection, and that PN is generated during the PC procedure, this endogenously formed PN is not necessary for the induction of the PC-evoked cardioprotective (antiarrhythmic) effects.

Despite the similarities between the PC and PN-induced protection, there might be dissimilarities as well, in particular as regards the underlying mechanisms. For example, in this canine model it has been found previously that mitoK_{ATP} channels play a role in the antiarrhythmic effect of PC, since the protection was abolished by the mitoK_{ATP} channel blocker 5-hydroxydecanoate (Végh et al., 2002). In contrast, in the same model, 5-HD failed to inhibit the antiarrhythmic protection, resulted from exogenous PN administration (Kiss et al., 2008). It might well be that there are differences between PC and the application of PN regarding the signalization that lead to cardioprotection. For example, it has been shown that

PN can directly activate downstream targets of PC signalling pathways, including protein kinase C (Agbani et al., 2011). There might be also species differences that would explain the divergent results. In this respect the studies of Altug and colleagues (Altug et al., 2001) are particularly interesting. They showed that in the rat isolated heart preparations, uric acid abolished both the PC and the PN-induced cardioprotective effects (Altug et al., 2001), from which they concluded that PN can serve as a trigger for PC. In this study, however, neither the generation of NO, nor the production of superoxide and peroxynitrite were determined.

One explanation for the difference might be related to the strength of the PC stimulus used in the various experimental setting; i.e. whether a certain PC protocol is able to provide sufficient stimulus for the formation of endogenous PN. One of our previous studies (Hajnal et al., 2004) showed that in anaesthetized dogs a single 5 min period of occlusion/reperfusion was insufficient to produce detectable changes in superoxide production. In this model at least two 5 min PC cycles had to be applied in order to obtain a detectable increase in blood superoxide concentration (Hajnal et al., 2004). It is also likely that the PC procedure not only generates ROS, but stimulates the free radical scavenging systems, such as the activity of manganese superoxide dismutase (Hoshida et al., 1993) as well. This may also account for the rapid elimination of superoxide formation, in particular, if the strength of the PC stimulus is not strong enough.

Whatever supposition is true, it seems from our studies that the two 5 min period occlusion and reperfusion insults that we used for preconditioning, stimulates the generation of NO and also of superoxide (Kiss et al., 2010), thus it is more than likely that these radicals react and form peroxynitrite. Indeed, our Study II provided an evidence for this, showing that the two 5 min occlusion/reperfusion insults increased myocardial nitrotyrosine levels (Figure 12). Nevertheless, uric acid, a scavenger of PN failed to modify the antiarrhythmic and the anti-ischaemic effects of PC. In contrast, urate completely abolished the protective effects that resulted from PN administration. Therefore, we think that in our canine model, the endogenous PN formation does not play a pivotal role in the antiarrhythmic effect of ischaemic PC.

We propose that in this model of ischaemia/reperfusion, other substances that are generated and released during the PC procedure may play a more essential role. The most likely candidate for this is nitric oxide. We have a surmountable amount of evidence that NO plays

an important trigger and mediator role in PC, induced either by brief coronary artery occlusions (Végh et al., 1992b; Végh and Parratt, 1996), cardiac pacing (Kis et al., 1999a,b) or heavy physical exercise (Babai et al., 2002).

There is also evidence that NO, among the other effects, plays an important role in the regulation of superoxide production that occurs after a prolonged period of ischaemia and reperfusion insult (Kiss et al., 2010). This effect can explain that both myocardial superoxide and NT formation were markedly reduced in the PC dogs, but also in dogs that had been given PN exogenously. However, urate in a dose of 0.2 mg/kg/min only abolished the effects of the exogenously administered PN without substantially modifying the superoxide and NT reducing effects of PC. We suppose that the loss of the PN-induced protection is largely due to the fact that PN has been scavenged by UA already during the infusion period.

To evaluate the selectivity of urate for peroxynitrite, especially under *in vivo* conditions, is rather difficult. The production of endogenous PN can only be indirectly assessed by measuring nitrotyrosine, which is a stable product of the reaction between PN and tyrosine residues of proteins. Nitrotyrosine is generally accepted as a biomarker of endogenous PN formation (Skinner et al., 1998). Nevertheless, it is also likely, that PN is not the solely substrate of UA (Teng et al., 2002; Squadrito et al., 2000). This assumption is supported by the results which showed that when UA had been given alone prior to a 25 min occlusion and reperfusion, there was also a marked reduction in superoxide production (Figure 7). There are of course, a number of other possible scavengers, such as ebselen (Parnham and Siesh, 2000) and metalloporhyrins (Crow, 1999; Klassen and Rabkin, 2009; Daiber et al., 2000), which could have been used in our experiments, but these scavengers are also not selective and specific for PN; they can inhibit a wide range of enzymes or interact with other bio-molecules (Crow, 1999; Ferdinandy et al., 2000; Lauzier et al., 2007).

Interestingly UA alone, administered prior to a prolonged period of I/R insult provided protection against arrhythmias and reduced the formation of the reperfusion induced oxidative and nitrosative stress products. Although the precise mechanism by which urate exerts protection is not fully elaborated, it is likely that such a protective effect can be associated with the antioxidant properties of urate. There is well documented in the literature, that the endogenously formed urate accounts for approximately 60 % of plasma total antioxidant capacity (Ames et al., 1981; Robinson et al., 2004), since it is able to react with different

oxygen derived free radicals, such as singlet oxygen, hydroxyl radical, oxo-heme oxidants (Ames et al., 1981), as well as peroxyl radicals (Robinson et al., 2004). Furthermore, urate is widely used for scavenging NO-derived free radicals, such as peroxynitrite, generated either endogenously or administered exogenously (Kooy et al., 1994; Skinner et al., 1998; Altug et al., 2001; Kuzkaya et al., 2003, 2005). It can also trap peroxynitrous acid, the decomposition product of peroxynitrite (Santos et al., 1999).

In summary, the results of Study I demonstrated that the exogenously administered PN can provide similar protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias that can be observed with ischaemic PC. This PN-induced protection was, however, abolished by the simultaneous administration of uric acid. In contrast, although the PC procedure induces endogenous PN formation, the antiarrhythmic effect of PC was not substantially modified by the PN scavenger urate. These results strongly indicate that the endogenously generated PN from the PC stimulus is not required for the development of PC-induced protection.

In **Study II** we aimed to examine whether the increase in NO bioavailability during ischaemia in PC dogs is due to a NOS-dependent or a NOS-independent mechanism.

We stared from those previous observations that in dogs subjected to a 25 min coronary artery occlusion and reperfusion, the concentration of NO, assessed by measuring plasma nitrate/nitrite levels in the blood of the coronary sinus, changed in a biphasic manner; i.e. there was an apparent increase in NOx that occurred soon after (around 7 min) the commencement of the occlusion, followed by a continuous decrease up to the end of the 25 min period of ischaemia (Kiss et al., 2010). In this study we have also pointed out that in dogs subjected to PC the NOx levels were preserved over the entire prolonged period of occlusion (Kiss et al., 2010). Since in this study NOS enzyme activity was not measured, we designed experiments in which primarily the activity of NOS was assessed in parallel with changes in plasma NOx, as well as myocardial superoxide and NT levels in control and in PC dogs.

The results show that total NOS activity was significantly increased following a 5 min period of coronary artery occlusion (Figure 9; C1), indicating a rapid NOS activation and NO formation (Figure 10; C1) following ischaemia. This finding well accords with those previous observations, which suggested that ischaemia rapidly activates NOS (Depré et al., 1997), due

to the changes in cytosolic calcium levels and enzyme phosphorylation (Govers and Rabelink, 2001). In contrast, if the ischaemia was maintained for 25 min, the activity of NOS started to decrease, and there was a parallel reduction in the plasma NOx levels (Figure 9 and 10; C2).

Thus it seems likely that the decrease in NO bioavailability during a prolonged occlusion can be associated with a reduction in NOS activation, but it cannot be ruled out that the formation of peroxynitrite, resulting from the combination of NO with superoxide, may also play a role. Indeed, the present study clearly demonstrated that in control samples both the superoxide and NT levels were markedly elevated by the end of the sustained ischaemia (Figure 11 and 12; C2), confirming those previous findings, which showed that superoxide can be generated even during ischaemia (Vanden Hoek et al., 1997; Becker et al., 1999), especially when the NOS enzyme uncouples because of the limited supply of oxygen, substrate and cofactors. This uncoupling would lead to decreased NO and, in parallel, increased superoxide production by NOS (Xia et al., 1998; Dumitrescu et al., 2007).

The present study also showed that PC stimulates NOS; but interestingly the activity of NOS following the two 5 min occlusions (PC1 group) was not as marked as that could be observed after a single 5 min occlusion (C1 group). An explanation for this difference in NOS activity between these groups may lie in the reperfusion interval, which elapses between the first and the second PC occlusions, suggesting that the NOS enzyme is not only rapidly activated by ischaemia (Depré et al., 1997), but it can also rapidly be deactivated when the ischaemia ceases. This assumption is confirmed by the results obtained from samples of PC2 dogs. These clearly show that NOS activity returned to the normal value after 5 min of the release of the second PC occlusion. Interestingly, despite this decrease in NOS activation, the plasma NOx seems to be maintained over the entire PC procedure. There was also an apparent increase in superoxide and nitrotyrosine production during the consecutive 5 min periods of preconditioning occlusion and reperfusion insults, supporting the role of these nitrogen and oxygen radicals in the initiation of the PC-induced protection (Végh et al., 1992b; Tanaka et al., 1994; Pain et al., 2000; Ferdinandy and Schulz 2003).

We assume these radicals generated during the PC procedure may have also a regulatory effect on NOS activity, whereby they contribute to the normalization of NOS activity soon after the release of the second PC occlusion. Indeed, studies, examining endothelial NOS activity and NO bioavailability in relation to normal and impaired vascular function (Govers

and Rabelink, 2001; Harrison 1997; Peterson et al., 1999; Guzik et al., 2002; Chen et al., 2008), have revealed that the ischaemia and reperfusion-induced free radical products by acting on various regulatory domains of NOS, are able to modify NOS activity in order to keep the balance between NO and superoxide production (Stuehr et al., 2004; Chatterjee and Catravas, 2008). For example, it has been shown that an excess in superoxide production would deactivate NOS, resulting in reduced free NO levels and subsequent peroxynitrite formation (Yaqoob et al., 1996). Similarly, under certain conditions the excessively generated NO can regulate, through a negative feedback (auto-inhibitory) mechanism the subsequent enzymatic synthesis of NO (Rengasamy and Johns 1993; Kotsonis et al., 1999, 2000). As to whether in our experimental circumstances these radicals are, indeed, involved in the regulation of NOS is not known, what is certain that 5 min after the release of the second PC occlusion the activity of NOS has returned to the normal value, whereas the concentration of NO, superoxide and nitrotyrosine is remained to be still elevated.

When the PC dogs were subjected again to occlusion (5 min or 25 min), the activity of NOS and simultaneously, the NOx levels were rapidly increased (PC3 group), but, in contrast to the controls, these were maintained or even further increased by the end of the 25 min occlusion (Figure 9 and 10; C2 compared with PC4; $P<0.05$). Conversely, the superoxide production during the prolonged occlusion was substantially less marked in the PC dogs than in the controls (Figure 11), whereas such a reduction in the NT production was not apparent (Figure 12). The reason for this might be that since NT is a stable product, the NT production determined at the end of the prolonged ischaemia, has already contained the amount of the previously formed peroxynitrite as well. Nevertheless, our results, obtained from the parallel measurement of NOS activation and products, which is an advantage of this study, clearly demonstrated that PC preserves the function of NOS and, as a result of it, increases NO bioavailability and decreases superoxide production during a subsequent prolonged period of occlusion. This finding confirms the previous observations in rat isolated hearts that PC prevents the loss of NOS enzymes and enhances NOS activity during ischaemia and reperfusion (Muscari et al., 2004).

There is some evidence coming mainly from *in vitro* studies that phosphorylation of NOS at various sites can rapidly modify NOS activity (Mount et al., 2007). For example, it has just recently been reported that in isolated rat hearts, PC, through the stimulation of the Akt-PKA

pathway, activates eNOS via serine 1176 phosphorylation (Yang et al., 2013). Also, post-translational modifications, such as protein-protein interactions, have been suggested as important mechanisms in NOS regulation (Gratton et al., 2000). Despite all attempts, the precise mechanism by which PC preserves the function of NOS has not yet been fully elucidated and, indeed, it was not the objective of the present study either. The main focus of this study was a systematic evaluation of NOS activation with a simultaneous measurement its products during ischaemia and following PC in an *in vivo* large animal model. Considering the data of our own and of the others, we can speculate that PC perhaps by preventing the uncoupling of NOS during ischaemia would influence the function of the enzyme. It is well known that NOS primary produces NO in the presence of adequate supply of substrate and cofactors, such as tetrahydrobiopterin (BH4), NADPH, FAD and flavin mononucleotide (Cosentino and Lüscher, 1999). Among these BH4 seems to be an essential cofactor for NOS to produce NO (Xia et al., 1998) by stabilizing NOS dimmers (reductase and oxygenase domains; Moens and Kass, 2006), increasing substrate affinity to the enzyme (Kotsonis et al., 2000) and inhibiting NOS-mediated superoxide production (Xia et al., 1998). There is also evidence that under ischaemic conditions the bioavailability of BH4 is reduced and this, rather than the inadequate substrate availability (Chatterjee and Catravas, 2008), results in uncoupling of NOS leading to superoxide rather than to NO production (Vasquez-Vivar et al., 1998). Indeed, it has been found that in diseases associated with impaired endothelial function, such as hypertension, diabetes, etc., the supplementation of BH4 improved endothelial function (Heitzer et al., 2000). Furthermore, in anaesthetized rabbits, PC decreased infarct size and increased BH4 concentrations after a 30 min coronary artery occlusion (Vladic et al., 2011), which suggests that PC by elevating BH4 levels prevents the uncoupling of NOS and preserves the function of NOS to produce NO even during a prolonged ischaemic period. Although in the present study the concentrations of BH4 were not determined, it is reasonable to hypothesise that a similar mechanism may play a role in our dog model. If so, then this would certainly explain the observed increase in NOS activity and NO synthesis, as well as the decrease in superoxide production in the PC dogs compared with controls.

In summary, the results of Study II demonstrate that in anaesthetized dogs, a short period of ischaemia rapidly activates the NOS enzyme and results in increased NO generation. In

contrast, a longer period of ischaemia decreases NOS activity and the NO bioavailability, with a parallel increase in superoxide and peroxynitrite production. This effect may associate with the uncoupling of NOS, although in the decrease of NO, the formation of peroxynitrite may also play a role. This study also shows that PC induced by brief occlusion and reperfusion insults enhances NOS activity and NO synthesis, but it also elevates the production of ROS; all these radicals are thought to play a trigger role in the PC–induced protection. Although we do not know how these radicals, generated during the PC procedure, modify NOS activity, what is certain, PC preserves the function of NOS, during prolonged occlusion and results in enhanced NO and suppressed superoxide production. We assume that in our model this mechanism plays an important role in the antiarrhythmic effects of PC.

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ANNEX

Reprints of full papers

I. **Juhász L,** Kiss A, Nyeső E, Kovács M, Seprényi G, Kaszaki J, Végh Á. Is there a trigger role of peroxynitrite in the anti-arrhythmic effect of ischaemic preconditioning and peroxynitrite infusion? *European Journal of Pharmacology (2011)*. 667:306-313.

II. **Juhász L,** Demeter Haludka V, Seprényi Gy, Kaszaki J, Gardi J, Végh Á. Acute inhibition of monoamine oxidase with pargyline does not modify the severity of ischemia and reperfusion-induced ventricular arrhythmias in dogs. *Experimental and Clinical Cardiology (2013)*. 1-7.

III. **Juhász L,** Déri S, Kisvári G, Kiss A, Seprényi G, Gardi J, Végh Á. The effect of ischaemic preconditioning on nitric oxide synthase activity during myocardial ischaemia and reperfusion in anaesthetized dogs. *Current Research: Cardiology (2014)*. 2:73-78.

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