The antiarrhythmic effect of exogenous peroxynitrite and preconditioning: the involvement of nitric oxide, superoxide and peroxynitrite

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LIST OF PUBLICATIONS

Full papers

I. **Kiss A**, Juhász L, Huliák I, Végh Á. Peroxynitrite reduces ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs without involving mitoK_{ATP} channels. *Br J Pharmacol*; 155: 1015-1024, 2008.

II. **Kiss A**, Juhász L, Kupai K, Seprényi Gy, Kaszaki J, Végh Á. The role of nitric oxide, superoxide and peroxynitrite in the antiarrhythmic effects of preconditioning and exogenous peroxynitrite. *Br J Pharmacol;* 160: 1263-1272, 2010.

ABSTARCTS

Abstracts for oral presentations

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

II. **Kiss A**, Juhász L, Huliák I, Ferdinándy P, Végh Á. Az exogén peroxinitrit hatása az okklúziós és reperfúziós arrhythmiákra altatott kutyában. *Cardiologia Hungarica;* 37: 19, 2007.

III. **Kiss A**, Juhász L, Huliák I, Kaszaki J, Seprényi Gy, Végh Á. A peroxinitrit intracoronariás infúziója csökkenti az ischaemia és reperfúzió okozta szuperoxid termelődést altatott kutya modellben. *Cardiologia Hungarica;* 38: 18, 2008.

IV. **Kiss A**, Juhász L, Seprényi Gy, Kaszaki J, Kupai K, Végh Á. A nitrogén monoxid, a szuperoxid és a peroxynitrit szerepe a prekondicionálás és az exogén peroxinitrit antiaritmiás hatásában altatott kutyamodellben. *Cardiologia Hungarica*; 39: 44, 2009.

V. **Kiss A**, Gönczi M, Seprényi Gy, Kaszaki J, Végh Á. A nátrium nitrit ($NaNO_2$) antiaritmiás hatása altatott kutyamodellben. *Cardiologia Hungarica;* 40: 42, 2010.

VI. Gönczi M, **Kiss A**, Végh Á. A nátrium nitrit ($NaNO_2$) a réskapcsolatok befolyásával védelmet nyújt az iszkémia során fellépő aritmiákkal szemben. *Cardiologia Hungarica;* 40: 38, 2010.

VII. Juhász L, **Kiss A**, Nyeső E, Seprényi Gy, Kaszaki J, Végh Á. A prekondicionálás antiaritmiás hatásának vizsgálata peroxinitrit scavanger jelenlétében. *Cardiologia Hungarica;* 40: 41, 2010.

VIII. **Kiss A**, Gönczi M, Seprényi Gy, Kaszaki J, Végh Á. A nátrium-nitrit infúzió hatása az iszémia/reperfúzió indukálta kamrai aritmiákra altatott kutyamodellben. Magyar Élettani Társaság és Magyar Kísérletes és Klinikai Farmakológiai Társaság közös Tudományos konferenciája, Szeged. *Abstract book;* 97: E4, 2010.

IX. Gönczi M, **Kiss A**, Végh Á. A nátrium nitrit ($NaNO_2$) védelmet nyújt az iszkémia-reperfúzió során megjelenő kamrai aritmiákkal szemben a réskapcsolatok befolyásolása által. Magyar Élettani Társaság és Magyar Kísérletes és Klinikai Farmakológiai Társaság közös Tudományos konferenciája, Szeged. *Abstract book;* 97: E5, 2010.

Abstracts for poster presentations

I. **Kiss A**, Juhász L, Huliák I, Ferdinándy P, Végh Á. Peroxynitrite induces an antiarrhythmic effect in anaesthetised dogs. International Society for Heart Research World Congress, Bologna. *J Mol Cell Cardiol;* 42: S9-10, 2007.

II. Juhász L, **Kiss A**, Huliák I, Ferdinándy P, Végh Á. Mito-K_{ATP} channels are not involved in the antiarrhytmic effect of peroxynitrite. International Society for Heart Research World Congress, Bologna. *J Mol Cell Cardiol;* 42: S18-18, 2007.

III. **Kiss A**, Juhász L, Huliák I, Kaszaki J, Seprényi Gy, Végh Á. Exogenous peroxynitrite modulates superoxide generation in anaesthetized dogs. International Society for Heart Research European Section Meeting, Athens. *J Mol Cell Cardiol;* 44: 752, 2008.

IV. **Kiss A**, Juhász L, Kupai K, 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 European Heart Failure & International Society for Heart Research European Section, Nice. *Eur Heart J; Supplements 8 (2)*, 2009.

V. Juhász L, **Kiss A**, Nyeső E, Seprényi Gy, Kaszaki J, Végh Á. The role of peroxynitrite in the induction of antiarrhythmic effect of ischaemic preconditioning in aneasthetised dogs. International Society for Heart Research World Congress, Kyoto. *J Mol Cell Cardiol;* 48: S41, 2010.

VI. Gönczi M, **Kiss A**, Végh Á. The antiarrhythmic protection resulting from sodium nitrite administration involves modulation of gap junctions. International Society for Heart Research World Congress, Kyoto. *J Mol Cell Cardiol;* 48: S102, 2010.

VII. **Kiss A**, Gönczi M, Seprényi Gy, Kaszaki J, Végh Á. The antiarrhythmic effect of sodium nitrite in anaesthetised dogs. International Society for Heart Research World Congress, Kyoto. *J Mol Cell Cardiol;* 48: S162, 2010.

SUMMARY

It is established that reactive oxygen (ROS) and nitrogen (RNS) species, such as superoxide ($O_2^{\cdot}$) and nitric oxide (NO) generated during the preconditioning (PC) procedure are involved in the antiarrhytmic effect of ischaemic preconditioning. There is also evidence that their cardioprotective effects, at least in part, connected with mitochondrial ATP-dependent potassium channels (mitoK_{ATP}). However, the effect of peroxynitrite (PN), formed from $O_2^{\cdot}$ and NO, on the acute ischaemia and reperfusion-induced (I/R) ventricular arrhythmias and the possible role of mitoK_{ATP} channels in this effect has not yet been investigated.

We aimed, therefore, to examine this in an anaesthetised open-chest dog model by the intracoronary infusion of peroxynitrite in the absence and in the presence of the mitoK_{ATP} channels inhibitor 5-hydroxydecanoate (5-HD). Besides the evaluation of severity of ischaemia and of arrhythmias, changes in nitrotyrosine levels (NT; a biomarker of PN formation) following ischaemia and reperfusion were also assessed.

We showed, that compared to the controls, two 5 min periods of the 100 nM concentration of PN, similar to the same periods of preconditioning ischaemia, markedly reduced the severity of arrhythmias and attenuated the generation of NT that resulted from a 25 min occlusion and reperfusion of the left anterior descending coronary artery. Since these effects were not modified by the prior administration of 5-HD, we concluded that, in contrast to PC, the antiarrhythmic effect of PN does not involve the opening of mitoK_{ATP} channels.

However, the observed attenuation in I/R-induced NT formation following PC and PN has promted us to examine, whether this results from a reduced NO or from a reduced $O_2^{\cdot}$ production. Therefore, in another series of experiments we measured changes in plasma and tissue nitrate/nitrite (NOx, a biomarker of NO production), $O_2^{\cdot}$ and NT productions in control, in PC and in PN treated dogs. We have found that in contrast to the controls in which the plasma NOx levels were markedly decreased by the end of the occlusion, in dogs subjected to PC and PN infusion NOx levels were maintained over the entire ischaemic period. Furthermore, both PC and PN significantly reduced the ischaemia and reperfusion-induced $O_2^{\cdot}$, and the subsequent NT formations.

Thus, our conclusion was, that the antiarrhythmic effects of PC and the infusion of PN can be associated with the preservation of NO availability during myocardial I/R. This NO by regulating the reperfusion-induced marked $O_2^{\cdot}$ production can attenuate the severe consequences of a prolonged period of I/R.

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LIST OF ABBREVIATIONS

5-HD: 5-hydroxydecanoate

DABP: diastolic arterial blood pressure

DHE: dyhidroethidum

HR: heart rate

I/R: ischaemia/reperfusion

LAD: left anterior descending coronary artery

LVEDP: left ventricular end-diastolic pressure

LVSP: left ventricular systolic pressure

MABP: mean arterial blood pressure

mitoK$_{ATP}$: mitochondrial ATP-dependent potassium channel

mPTP: mitochondrial permeability transition pore

NAC: N-acetyl-L-cysteine

NO: nitric oxide

NOS: nitric oxide synthase

NOx: nitrate/nitrite

NT: nitrotyrosine

$O_2^{\cdot}$: superoxide

PC: preconditioning

PKC: protein kinase C

PKG: protein kinase G

PN: peroxynitrite

RNS: reactive nitrogen species

ROS: reactive oxygen species

SABP: systolic arterial blood pressure

VF: ventricular fibrillation

VPB: ventricular premature beats

VT: ventricular tachycardia

1. INTRODUCTION

Ventricular arrhythmias resulting from acute myocardial ischaemia and reperfusion (I/R) are the main factor responsible for sudden cardiac death. Although cardiopulmonary resuscitation can extend the window for successfull drug and interventional therapy, sudden cardiac death is still one of the most serious problems facing modern cardiology. Thus, the exploration of mechanisms which are involved in the generation of these serious arrhythmias has a particular importance in the development of novel and effective therapeutical strategies.

In 1986 Murry *et al.* [1] firts described that short periods of complete coronary artery occlusions, substantially reduced the ultrastructual changes that resulted from a subsequent, more prolonged period of ischaemia and reperfusion. They termed this phenomenon as „ischaemic preconditioning" (PC). This form of adaptation to ischaemic stress has been confirmed in many subsequent studies and extended, for example, to an enhanced recovery of myocardial contractile function following reperfusion [2], and, which is probably the most important aspect of this phenomenon, to a marked reduction in the severity of I/R-induced ventricular arrhythmias [3, 4]. The preconditioning-induced increased tolerance of the heart to ischaemic stress can be demonstrated in all species thus far studied (rats, rabbits, dogs, pigs etc.), including man. It has also been described, that the protective effects of PC exist in two separated phases; there is an early or „first window" of protection which appears immediately after the PC procedure but this protection is short lived; it wanes 1 or 2 h after the initial PC stimulus. The protection, however, spontaneously re-appears approximately 24 h later, and this phase is termed as delayed or „second window" of protection. Although the delayed phase of the protection is not as marked as the first one, it may persist for a longer period; in some species, for example, in rabbits, it may last even for 72 h [5].

Although the precise mechanism by which preconditioning induces cardioprotection is still not fully understood, there is strong evidence that endogenous substances, generated and released in the myocardium during preconditioning, are involved [6]. These substances include adenosine [7], bradykinin [8], some prostanoids [9, 10], opioids [11], nitric oxide (NO) [8, 11, 12] and reactive oxygen species (ROS) [13, 14]. These mediators acting on their receptors activate various intracellular signaling pathways which ultimately lead to cardioprotection.

1.1 The antiarhythmic effect of ischaemic preconditioning; the role of nitric oxide

In 1987 Shiki and Hearse [3] showed in anaesthetised rats that two, 5 min periods of coronary artery occlusion, separated by variable reperfusion intervals (from 10 min up to 3 days) markedly reduced the severity of arrhythmias that occurred when the second period of occlusion had been suddenly released. This study provided the first evidence that PC may attenuate the reperfusion-induced ventricular arrhythmias. Two years later, in 1989, our research group presented at the meeting of the British Physiological Society that in anaesthetised rats and dogs short periods of occlusions and reperfusions suppressed those ventricular arrhythmias that resulted from a subsequent, more prolonged period of ischaemia and reperfusion [4]. This study provided the first evidence that PC profoundly protects against the acute ischaemia and reperfusion-induced ventricular arrhythmias [4]. Since then we have been extensively examining the early and the delayed antiarrhythmic effects and the underlying mechanisms of the protection afforded by preconditioning, induced either by short coronary artery occlusions [15], rapid cardiac pacing [16] or physical exicerse [17]. For example, as early as 1989 we proposed that in the preconditioning-induced antiarrhythmic protection endogenous myocardial protective substances are involved. One of these is NO, which role in the PC-induced early and delayed protection we thoroughly examined over the years, up to now.

Nitric oxide, is an endogenous substance which is formed by the conversion of L-arginine to L-citrulline, a reaction catalysed by one of the three nitric oxide systhase (NOS) isoforms; neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). Nitric oxide has many actions in the cardiovascular system; it may act, for example, as a regulator of vascular tone [18], it is able to inhibit platelet aggregation, and to attenuate leukocyte adherence to the endothelium [19].

The first piece of evidence that NO plays a trigger role in the protection associated with ischaemic preconditioning comes from studies in which we demonstrated that the inhibition of L-arginine-NO pathway with L-NAME markedly attenuated the antiarrhythmic effect of preconditioning [20]. Since then the involvement of NO in both the early [20, 21, 22] and the delayed phase of the preconditioning-induced cardioprotection has been confirmed [23, 24]. The implication of NO in cardioprotection is also supported by the fact that drugs which are able to donate NO (i.e. NO donors) provide similar protection against ischaemic injury and arrhythmias as preconditioning itself [25, 26, 27].

There is a number of possible ways by which NO may result in an antiarrhythmic protection. For example, NO inhibits noradrenaline release from sympathetic nerve endings, and enhances, acting on presynaptic muscarinic receptors and via a cGMP-dependent pathway, the effect of vagal nerve stimulation [28, 29, 30, 31, 32]. As we have proposed previously, one possible mechanism for the NO-induced antiarrhythmic protection might be the elevation of cGMP through the stimulation of soluble guanylyl cyclase by NO [21, 22]. This would depress myocardial contractilty and reduce energy demand perhaps by limiting myocardial cAMP levels through the stimulation of a cGMP-dependent phosphodiesterase enzyme [33, 34]. cGMP can also regulate calcium transport through the L-type calcium channels [34] by inhibiting the influx of calcium. The reduction of calcium overload during ischaemia would result in an antiarrhythmic protection [32, 35].

The beneficial effect of NO during ischaemia and reperfusion is, however, not unambiguous. There are studies, derived mainly from *in vitro* experiments, which showed opposite results [36, 37]. For example, Zweier *et al.* [36] found that in the rat isolated heart NO levels, measured by electron spin resonance (ESR), were markedly increased during a prolonged period of global ischaemia. Similarly, Csonka *et al.* [37] suggested that the cardioprotective effects of PC was due to the reduction of this harmful overproduction of NO that resulted from a prolonged period of I/R. In contrast to this, most *in vivo* studies performed mainly in large animals, such as in dogs and pigs, reported that the NO production was significantly reduced during sustained coronary artery occlusion [38, 39, 40], and that when the NO availablity was restored by the administration of L-arginine the protection reinstated [40]. The importance of NO in cardioprotection against ischaemia and reperfusion-induced myocardial injury was also supported by studies which showed that the administration of nitrite, which provides a NOS-independent NO supply during ischaemia, significantly reduced infarct size [41] and oxidative stress [42], suggesting a possibile mechanism of NO regulated mitochondrial ROS production in the cardioprotection [43].

It is also hypothesized that the mitochondrion might be the end-effector of the PC-induced cardioprotection. Indeed, there is evidence that opening of mitoK_{ATP} channel by diazoxide mimics the PC-induced antiarrhythmic effect [44]. Furthermore, both PC and the diazoxide-induced protection was abolished by the administration of the selective mitoK_{ATP} channel blocker, 5-hydroxydecanoate (5-HD) [44, 45, 46]. A more recent study suggested that NO opens mitoK_{ATP} channels through the activation of a cGMP-PKC dependent pathway [47]. Furthermore, the opening of mitoK_{ATP} channels by nicorandil significantly increased the

thresold for the epinephrine-induced arrhythmias in anaesthetised rats; an effect which was also inhibited by the administration of 5-HD and with L-NAME [48]. These studies support the idea that opening of mitoK_{ATP} channels by nitric oxide may play an important role in the cardioprotection.

1.2 The role of reactive oxygen species in preconditioning

The mitochondrion plays also an important role in the generation of ROS, another endogenous substance, which is produced in high amounts during I/R. It is also thought that the generation of ROS is also involved in the cardioprotective effect of ischaemic preconditioning.

ROS are normally produced during aerobic metabolism by the univalent reduction of O_2 to H_2O, whereas superoxide ($O_2^{\cdot}$) radicals are formed. Under aerobic conditions this ROS formation is neutralised by the endogenous ROS scavenging systems, including superoxide dysmutase (SODs), endogenous peroxidases, glutathion, etc. However, under anoxic or ischaemic conditions the capacity of these endogenous ROS scavenger systems is markedly attenuated, leading to reduced myocardial antioxidant defense and the accumulation of ROS. There are, of course, a number of possible ways by which ROS production is increased during I/R. Superoxide is produced preferably at low levels of aerobic mitochondrial metabolism [49], during conversion of hypoxanthine to uric acid which is catalyzed by xanthine oxidase [50], or during the autooxidation of mitochondrial enzimes, such as flavoproteins and catecholamines. Neutrophil infiltration into the microvasculature also enhances ROS production [51]. The cytotoxic effect of ROS is thought to be due to the oxidation and peroxidation of membrane and cellular proteins and lipids. In addition, ROS production increases mitochondrial calcium overload and may result in the opening of mitochondrial permeability transition pore (mPTP), which is involved in myocardial cell death [52].

There is abundant evidence that $O_2^{\cdot}$ production largely contributes to I/R injury. For example, Becker *et al.* [49] showed in isolated cardiomyocytes, that the generation of ROS has already started during the later phase of ischaemia and this, together with the reperfusion-induced marked $O_2^{\cdot}$ production, leads to severe reperfusion injury. In addition, Vanden Hoek *et al*. [53] also showed that PC attenuates the severe consequences of oxidative stress. Recently, oxidative stress is also considered as one of the main causes of cardiac arrhythmias. There is experimental evidence that ROS modify cardiac sympathovagal balance in

cardiomyocytes [54], and provoke fatal ventricular arrhythmias by influencing ion currents and causing action potential abnormalites [55, 56].

The role of ROS in cardioprotection afforded by PC is, however, rather controversial. The reason for this can be related to the model used; ie. as to whether the experiments are performed under *in vitro* or *in vivo* conditions, the dose and route of administration of the antioxidant applied, as well as the intensity of the preconditioning stimulus (i.e. the duration and number of the preconditioning occlusions) [57, 58, 59, 60] which may substantially determine the signaling pathways which lead to cardioprotection [61]. In addition, the possible trigger or mediator role of ROS in PC and their relation to the mitoK_{ATP} channels, is also not fully elucidated [62, 63]. It is suggested that $O_2^{\cdot}$ generated during PC, activates mitoK_{ATP} channels by directly aciting on the sulphydril group of these channel proteins [64]. Furthermore, Lebuffe *et al.* [63] showed that free radicals formed during the PC stimulus activate protein kinases (PKG and/or PKC) and thereby open mitoK_{ATP} channels in the mitochondrial inner membrane. This results in further ROS generation which is then activates various kinases, such as tyrosine kinases, involved in the cardioprotective signaling pathway [47]. However, there is also evidence that the PC procedure-induced $O_2^{\cdot}$ attenuates further mitochondrial ROS production, which results from the subsequent more prolonged ischaemia and reperfusion [65].

Thus, it seems from the abovementioned studies that the exact role and position of ROS in the PC-induced cardioprotective signaling cascade still remains unclear. There is even less information available as concerns the relationship between ROS, mitoK_{ATP} channels and arrhythmias. It is suggested that the antiarrhythmic effect of the opening mitoK_{ATP} channels involves the ROS activated ERK pathway which reduces gap junction permeability by the phophorylation of connexin 43 [66]. Although in a previous study we have found that in anaesthetised dogs the administration of ROS scavenging N-2-mercaptopropionylglycine did not modify the antiarrhythmic effect of PC [60], the role of ROS in the PC-induced cardioprotection cannot be ruled out.

1.3 The role of peroxynitrite in ischaemia and reperfusion

Nitrogen and oxygen derived radicals, generated during ischaemia and reperfusion, are rapidly react and form other highly reactive molecules, such as peroxynitrite (PN) [67, 68]. There is substantial evidence that peroxynitrite is extremely cytotoxic and largely contributes to endothelial dysfunction [69], cell necrosis [70] and to arrhythmias [71, 54].

During that late 90's experimental studies revealed that PN in high nanomolar or low micromolar concentrations result in benefical effects; thus it causes vasodilation, inhibits platelet aggregation and the inflammatory cell adhesion, and protects against ischaemia and reperfusion injury [13]. For instance, the exogenously administered PN preserved myocardial and coronary endothelial function [72], and reduced infarct size [73]. Moreover, in rat isolated hearts the administration of PN, prior to ischaemia, markedly reduced the I/R-induced ventricular arrhythmias [74]. In the same model, peroxynitrite, generated during the brief ischaemia and reperfusion insults (preconditioning), has been found to be involved in the antiarrhythmic effect of preconditioning [75]. Nevertheless, the precise cardioprotective mechanisms by which PN results in cardioprotection is still not fully clear. One possible mechanism by which PN might be cardioprotective is that PN oxidizes thiols, such as glutathione, and forms S-nitrosothiols [76]. These S-nitrosothiols may serve as NO donors and release NO over a long time interval. The release of NO from S-nitrosothiols and the subsequent stimulation of soluble guanylate cyclase [77], as well as the activation of tyrosine kinase [78] pathways are the proposed mechanisms which may have roles in the cardioprotective effects of PN. In addition, similar to NO and $O_2^{\cdot}$, PN can, perhaps by a feedback mechanism, regulate the generation of ROS. There is evidence that PN acts on xanthine oxidase [79] and modifies mitochondrial uncoupling protein activity [43]; both mechanisms lead to a reduction in $O_2^{\cdot}$ production and limit the overproduction of endogenous peroxynitrite.

As it becomes obvious from the studies mentioned above, there is much controversy as regards the importance of NO, $O_2^{\cdot}$ and PN in ischaemia and reperfusion injury, as well as the involvement of these radicals in cardioprotection. One of the explanations of this confusion, apart from the great variety of models and experimental conditions used, might be that in most of the studies NO, $O_2^{\cdot}$ and PN were measured separately; indeed, only a few attempts have been made to assess the production of these radicals simultaneously during ischaemia and reperfusion [80, 81]. Therefore we designed studies which allowed us to obtain information about the generation of these substances from our established *in vivo* canine model of ischaemia and reperfusion.

1.4 Aims

The following questions were raised and accomplished in two sepatare studies, according to the enclosed publications.

In the first series of the experiments we examined the effect of peroxynitrite on the ischaemia and reperfusion-induced arrhythmias. The following questions were raised:

(1) whether in anaesthetised dogs, brief (5 min) periods of the low, 100 nM concentration of peroxynitrite, infused locally into the coronary circulation modifies ischaemic changes and the acute ischaemia and reperfusion-induced ventricular arrhythmias?

(2) if so, whether this effect, similar to preconditioning, involves the activation of mitoK_{ATP} channels? For this purpose 5-hydroxydecanoate (5-HD), a relatively selective blocker of mitoK_{ATP} channel was used and administered prior to coronary artery occlusion in a dose of 150 $\mu g\ kg^{-1}min^{-1}$.

Since these studies clearly demonstrated that PN, similar to PC, results in a marked antiarrhythmic effect and a reduction in endogenous peroxynitrite production following the prolonged ischaemia and reperfusion, we designed a second series of experiments to explore the mechanism of this protection. We raised the question,

(3) whether the attenaution of endogenous peroxynitrite formation in preconditioned and peroxynitrite treated dogs results from a reduction in superoxide or from a reduction in NO production? Therefore, in these experiments, besides the evaluation of severities of ischaemia and arrhythmias, we measured plasma nitrate/nitrite levels, superoxide and nitrotyrosine productions in blood and tissue samples taken from control dogs, and from dogs which were subjected to either preconditioning or infused with peroxynitrite prior to the prolonged ischaemia and reperfusion.

2. MATERIALS AND METHODS

2.1 Experimental animals

Adult mongrel dogs of either sex with a mean body weight of 20.5 ± 3.3 kg were used. 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 Institute of Health (NIH publication no. 85-23, revised 1996).

2.2 Surgical interventions

The anaesthesia was introduced with an intravenous bolus injection of sodium pentobarbitone (30 mg kg^{-1}). Under this light anaesthesia the right femoral artery, for the measurement of arterial blood pressure {systolic (SABP), diastolic (DABP), and mean (MABP)} and the right femoral vein for further anaesthetic administration (chloralose: 60 mg kg^{-1} and urethane: 200 mg kg^{-1}) were catheterized. Through the left carotid artery another polyethylene catheter was introduced into the cavity of the left ventricle for the measurement of the left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures. From the pressure curve changes in positive and negative dP/dt_{max} were determined. The arterial catheters were connected through transducers (Statham P23XL) to SYSTEM6, a six channel haemodynamic apparatus (Triton Technology, USA). All these haemodynamic parameters, together with a chest II lead electrocardiogram were registered on an eight channel Medicor R81 recorder.

Dogs were intubated and ventilated with room air (Harvard Respirator, USA) at a rate and volume sufficient to maintain the normal values of arterial blood gases and pH (Radiometer ABL 505, Denmark). Body temperature was monitored from the mid-oesophagus and maintained, by a heating pad, around 37 ± 0.5°C.

A thoracotomy was performed in the fifth intercostal space and the pericardium was excised. The anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. In some dogs, a small side branch of the LAD, proximal to the occlusion site, was also prepared and catheterized for the administration of peroxynitrite, saline or 5-HD (Sigma, MO).

2.3 Measurement of ischaemia severity

The severity of myocardial ischaemia was assessed by changes in the epicardial ST-segment and in the degree of inhomogeneity of electrical activation using a composite electrode positioned within the potentially ischaemic area, as described previously [15]. This electrode gives a summarized recording of R-waves from 24 epicardial measuring points. In the adequately perfused and oxygenated myocardium all sites are activated almost 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 inhomogeneity of conduction. We expressed this as the greatest delay in activation (ms) within the ischaemic area. The composite electrode also contains four unipolar electrodes by which epicardial ST-segment changes are measured within the ischaemic area. This was expressed in mV.

2.4 Assessment of ventricular arrhythmias

Ventricular arrhythmias were determined from the chest lead II ECG and evaluated according to the Lambeth conventions [15]. In brief, during the coronary artery occlusion the number of ventricular premature beats (VPBs), the incidence and the number of tachycardiac episodes (VT, defined as four of more VPBs at a rate faster than the sinus rythm) as well as the incidence of ventricular fibrillation (VF) were determined. During reperfusion only the incidence of VF was measured, and dogs that alive 1-2 min after reperfusion was considered to be survivors. In dogs survived reperfusion the hearts were arrested by an intravenous bolus injection of potassium chloride (KCl), excised rapidly and myocardial samples were taken for *in vitro* analyses.

2.5 Synthesis of peroxynitrite

Peroxynitrite, the reaction product of superoxide and nitric oxide was synthetised from acidified nitrite and hydrogen peroxide according to the method of Beckman *et al.* [82]. In brief, an aqueous solution of $NaNO_2$ (0.83 g) was mixed with a solution containing of H_2O (3.8 ml), HNO_3 (11.1 M) and H_2O_2 (8.2 M), and immediately quenched with NaOH (4.2 M). All reactions were performed on ice. Powdered MnO_2 was added to remove the excess of H_2O_2, and then the solution was kept in dark for about 5 min. The mixture was filtered and the final concentration of the aliquot peroxynitrite was measured spectrophotometrically (peak absorbance at 302 nm wave length). The stock solutions (50-150 mM) were aliquoted and

stored at -80^{o}C away from light. Before each experiment the absorbance of peroxynitrite was again measured and the concentration was adjusted to 100 nM with pH 8.4 saline. The pH of saline solution was corrected by an appropriate volume of 0.1 M NaOH [73].

2.6 Determination of nitrotyrosine formation

In these studies nitrotyrosine (NT), a marker of peroxynitrite formation, was assessed by two methods; Western immunoblot analysis and enzyme-linked immunosorbent (ELISA) assay, in tissue samples taken from the ischaemic myocardium 1-2 min after reperfusion. The samples were frozen immediatly in liquid nitrogen and stored at -80°C.

2.6.1 Determination of nitrotyrosine formation by Western immunoblot

Samples were powered and homogenized in ice-cold buffer (HEPES 10 mM, sucrose 0.32 mM, EDTA 0.1 mM, DTT 1.0 mM, trypsin inhibitor 10 μg mL^{-1}, leupeptin 10 μg mL^{-1}, aprotinin 2.1 μg mL^{-1}, PMSF 10 μg mL^{-1}, at pH 7.2). Tissue homogenates were centrifugated for 10 min at 4^{o}C at 10000g. The protein concentration of the supernatants was determined by a bicinchonic acid protein assay kit (Sigma, MO). To determine protein nitrotyrosine levels, 20 μg of the total protein was loaded onto 8% polyacrylamide gel. The electrophoresis was performed at 100 V, 400 mA, for 120 min, and the proteins were transfered into nitrocellulose membrane by electroblotting (40 V, 400 mA, 120 min). Unspecific binding capacity of the membrane was blocked by incubation overnight with TBS Tween-20 solution (the volume fraction is 0.05) containing 5% non-fat milk powder, at 4^{o}C. Then, the membrane was incubated for 90 min, at room temperature with the primary antibody (monoclonal anti-nitrotyrosine, diluted to 1:1000, MAB5404, Chemicon, Millipore Corp., USA). After the subsequent incubation with the secondary antibody (horseradish peroxidase-conjugated secondary antibody (rabbit anti-mouse IgG, diluted in 1:1000) for 50 min at room temperature, the membrane was developed with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare, UK) and exposed to X-ray film and scanned. Density of the nitrotyrosine bands were calculated by integrating the area (in pixels) and expressed in arbitrary units by means of the Quantity One (Bio-Rad Laboratories, USA) software.

2.6.2 Determination of nitrotyrosine formation by ELISA

Myocardial tissue samples were homogenized and centrifuged as described above. The supernatants were collected and incubated overnight with anti-nitrotyrosine rabbit IgG (Cayman Chemical; USA) and with nitrotyrosine acetylcholinesterase tracer in precoated

(mouse anti-rabbit IgG; Cayman Chemical; USA) microplates, followed by the development with Ellman's reagent. Nitrotyrosine content was normalized to protein content of cardiac homogenate and expressed in ng mg^{-1} protein.

2.7 Measurement of plasma and tissue nitrate/nitrite levels

Plasma nitrate/nitrite (NOx) concentrations were determined by means of Griess reaction modified by Moshage *et al.* [83]. Blood samples, taken from the coronary sinus at various time intervals (*Figure 1*), were centrifuged with 10000g for 15 min at 4°C. The plasma was mixed with β-NADPH, FAD, nitrate reductase (Sigma, MO) and incubated for 30 min at 37°C. Following the enzymatic reduction of nitrate to nitrite, the Griess reagent was added to the mixture and incubated for additional 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 (μM L^{-1}) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma, MO).

Tissue NOx was measured in samples taken from the ischaemic myocardium. Tissue blocks were homogenised in phosphate buffer (pH 7.4) containing Tris-HCl (50 mM), EDTA (0.1 mM), dithiotretol (0.5 mM), phenylmethylsulfonyl fluoride (0.1 mM), soybean trypsin inhibitor (10 μg ml^{-1}) and leupeptin (10 μg ml^{-1}). The homogenates were centrifuged (20 000g, for 15 min at 4°C) and the supernatants were collected. The total nitrate/nitrite was determined as outlined above. NOx levels were expressed in nmol mg^{-1} protein.

2.8 Assessment of superoxide generation following ischaemia and reperfusion

Two or three myocardial tissue blocks were excised from the ischaemic area within 2 min of the reperfusion and were embedded in optimal cutting temperature (OTC) compounds and cryosections (20 μm) were produced. Superoxide production was determined using dihydroethidium (DHE; Sigma, MO) fluorescence staining [84]. DHE reacts with superoxide anions to form ethidium bromide, which in turn intercalates into DNA to provide nuclear fluorescence as a marker of superoxide generation. The sections were stained with DHE (1μM), dissolved in PBS (pH 7.4) and incubated at 37°C for 30 minutes in a dark humidified chamber. Negative controls were obtained by blocking the reaction with N-acetyl-L-cysteine (NAC, 100 mM; Sigma, MO), a scavenger of ROS. Fluorescence signals were analyzed by confocal microscopy. Ten to fifteen serial images of the stained samples were captured by

Olympus FV1000 confocal laser scanning microscope with standard parameter settings. The fluorescence intensity of DHE stained specimens was quantitatively analyzed by ImageQuant software (Molecular Dynamics) as follows: 15 equally sized elliptical areas covering the positive signal resulted from staining was randomly selected on each image; the backgrounds of the selected areas were eliminated by threshold set up. Similar process was performed on negative control specimens. The fluorescence intensities/pixel values of the randomly selected areas were quantified and were expressed in arbitrary units.

2.9 Evaluation of the area at risk

At the end of the experiments, in some dogs of each group the heart was stopped by intravenous injection of KCl and was removed. The risk area following coronary artery occlusion was determined by injecting patent blue V dye into the re-occluded coronary artery and was expressed as a percentage of the left ventricular wall together with the septum as previously described [15].

2.10 Experimental protocol

This is shown in *Figure 1*. Nine groups of randomly selected dogs were used. In all groups a 30 min recovery period was allowed to stabilize after surgery. In some groups pH 8.4 saline (the dissolvent of peroxynitrite [73]) was infused at a rate of 0.5 ml min^{-1} locally, into a side branch of the LAD over a period of 10 min in order to evaluate the effects of alcalinic saline solution on the haemodynamic parameters. Seven dogs served as sham-operated controls (SO). These dogs were instrumented, infused locally with normal saline for 30 min and, without subjecting them to ischaemia, they were euthanised and samples were taken for the determination of tissue NOx levels, O_2^- and NT formation.

There we two control groups. In the one group (C1; n = 12) the dogs were given intracoronary infusion of normal saline (pH 7.4) over a period of 30 min, just prior to the 25 min occlusion of the LAD. This group served as controls for 5-HD administration. In the other control group (C2; n = 14) pH 8.4 saline was infused also by the intracoronary route (rate: 0.5 ml min^{-1}) two times for five minutes, 5 min before the 25 min occlusion and reperfusion of the LAD. Another groups of animals (n = 13) PC was induced by two 5 min occlusions of LAD, 5 min prior to the prolonged ischaemia. In four out of these animals the hearts were stopped immediately after the PC procedures and tissue samples were taken, whereas the remaining nine dogs were subjected to the prolonged I/R insult. Peroxynitrite was

given in 34 dogs in a concentration of 100 nM (chosen as one tenth of the intravenous concentration used previously *in vivo*; [73]), and infused directly into the coronary circulation (rate: 0.5 ml min^{-1}) two times for 5 min. In four dogs out of these 34 animals after cessation of the second period of the PN infusion, the hearts were stopped for tissue sampling. In 19 dogs, 5 min after stopping the infusion of PN the LAD was occluded for 25 min followed by reperfusion. In 11 dogs, 10 min prior to and over the administration of PN, 5-HD was also given in intracoronary infusion either in a dose of 150 µg kg^{-1} min^{-1} (n = 8) or 300 µg kg^{-1} min^{-1} (n = 3). In three additional dogs only 150 µg kg^{-1} min^{-1} 5-HD was infused over a period of 30 min. Blood samples (BS) were taken from the coronary sinus at various time intervals as illustrated in *Figure 1* (see ↓). Tissue samples (TS) were collected from the ischaemic region of the left ventricle, 1-2 min after reperfusion (see ↑ in Figure 1).

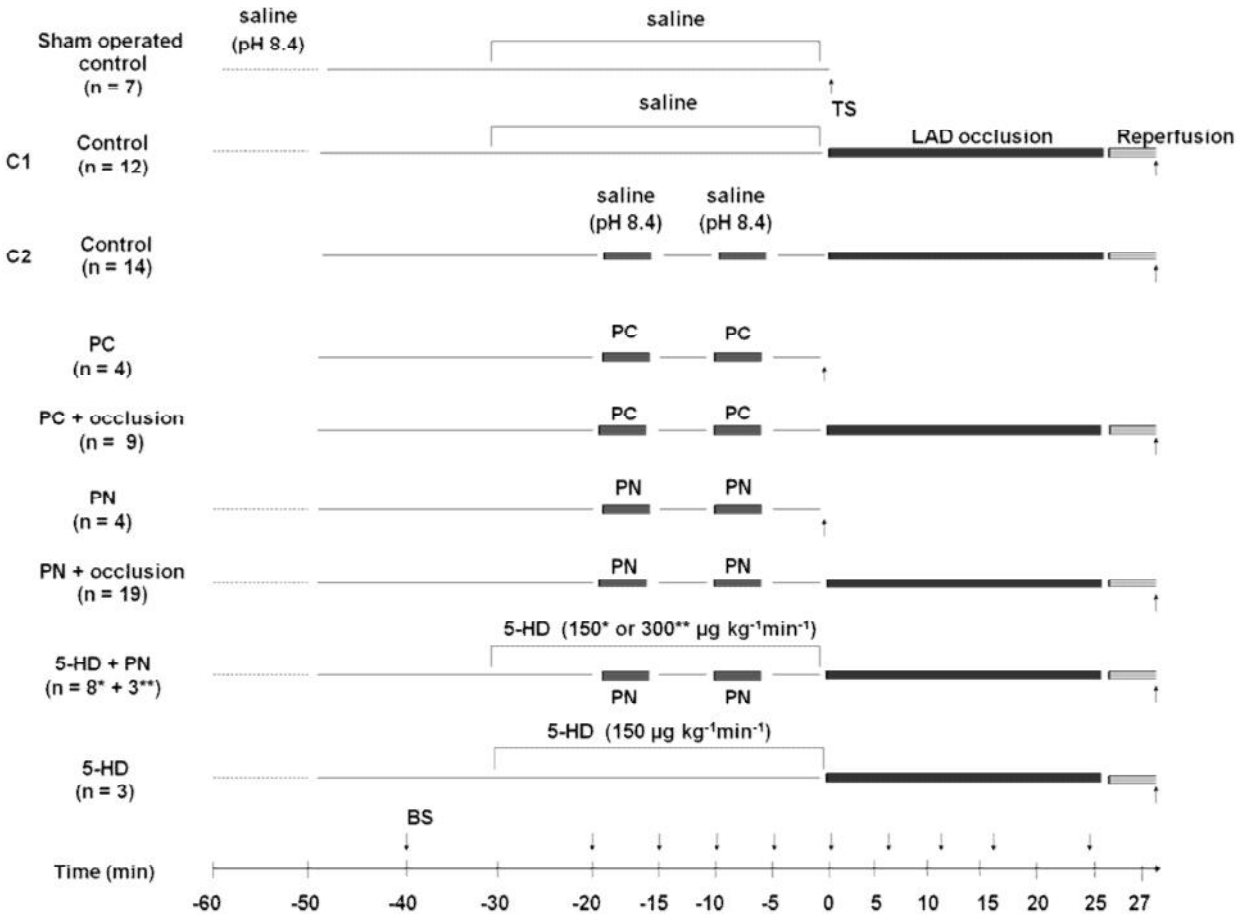

Figure 1.
Experimental protocol for the evaulation of the antiarrhythmic effect of preconditioning and peroxynitrite infusion and for the determination of changes in nitric oxide, superoxide and nitrotyrosine formation.

2.11 Statistical analysis

All data 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 $P < 0.05$.

3. RESULTS

3.1 Haemodynamic changes following the administration of saline, PN, 5-HD and 5-HD + PN, as well as during the PC procedure

There were no significant changes in any of the haemodynamic parameters following the intracoronary infusions of alcalinic saline (pH 8.4), PN, 5-HD and 5-HD + PN (*Table 1)*. The table also shows the haemodynamic alterations that occurred during PC occlusions. There were significant reductions in arterial blood pressure, LVSP and in the positive and negative dP/dt_{max}, whereas LVEDP was significantly increased.

3.2 Haemodynamic changes during a 25 min occlusion of the LAD

These are summarized in *Table 2*. In all groups the occlusion-induced haemodynamic changes were similar, except that in dogs given PN and 5-HD + PN, as well as preconditioned prior to the prolonged ischaemia with brief periods of occlusions the elevation of LVEDP and decreases of the positive and negative dP/dt_{max} were significantly less marked than in the controls (*Table 2*). In anaesthetised dogs the occlusion of the LAD resulted in no substantial changes in HR (*Table 2*).

3.3 The severity of ventricular arrhythmias during coronary artery occlusion

These are illustrated in *Figure 2* and *3*. In control dogs, occlusion of the LAD resulted in a high number of VPBs (around 300), many separate episodes of VT (around 10) and a high incidence of VT (96%; *Figure 2*). Furthermore, nearly 50% of the animals died in VF during occlusion and no control dog survived reperfusion (*Figure 3*). In contrast, in dogs subjected to PC the number of VPBs (25 ± 12) as well as the incidence (33%) and the number of episodes of VT (0.6 ± 0.3) were markedly reduced. The most striking effect was, however, that no preconditioned dog fibrillated during occlusion and 60% of the PC animals survived

reperfusion. Similary, in dogs given PN prior to the prolonged ischaemia the total number of VPBs (87 ± 24), the number (1.9 ± 0.8) and the incidence of VT (33%) were markedly suppressed. Furthermore, only one dog out of 19 dogs fibrillated during the LAD occlusion, irrespective whether or not they had been given 5-HD. Thus, survival from the combined ischaemia and reperfusion insult was 50% in the peroxynitrite treated dogs and 62 % when 5-HD was also administered. These results indicate that 5-HD in a dose of 150 μg kg^{-1} min^{-1}, which by itself did not modify arrhythmia severity during coronary artery occlusion (VPBs: 325 ± 22, VT episodes: 5 to 7 per dog. VF: 1 out of 3 dogs; data obtained from 3 observations), failed to modify the antiarrhythmic effect of PN. Similarly, the higher dose of 5-HD (300 μg kg^{-1} min^{-1}) given to another three PN-treated animals was unable to abolish or even attenuate the marked antiarrhythmic effect of PN (VPBs: around 98 ± 6, no VT or VF occurred in these three dogs, data are not illustrated).

3.4 Changes in the severity of ischaemia following coronary artery occlusion

The severity of myocardial ischaemia was assessed by changes in the epicardial ST-segment (*Figure 4A*) and in the degree of inhomogeneity of electrical activation (*Figure 4B*). In control dogs, the epicardial ST-segment rapidly increased especially during the initial 5 min period of the occlusion, and this was maintained throughout the whole ischaemic period (*Figure 4A*). In contrast, both the brief local infusions of PN and the PC occlusions significantly attenuated the prolonged ischaemia-induced ST-segment elevations. The administration of 5-HD did not modify the reduction in epicardial ST-segment that resulted from PN administration. The degree of inhomogeneity of electrical activation (*Figure 4B*), which is usually around 50 to 60 ms in the normal non-ischaemic heart, was markedly increased to around 170 ms when the LAD was occluded for 25 min. Both PC and infusion of PN markedly reduced the development and degree of inhomogeneity over the entire occlusion period. Administration of 5-HD did not substantially influence this peroxynitrite-induced reduction of inhomogeneity.

3.5 Area at risk

There were no significant differences in the area at risk between those five groups where this was measured. Thus, the risk area was 34.3 ± 1.4% (n = 5) in the controls, 35.2 ± 2.2% (n = 3) in the controls given 5-HD, 34.6 ± 1.2% (n = 3) in the peroxynitrite group, 34.2 ± 1.3% (n = 3) in the 5-HD (150 μg kg^{-1} min^{-1}) + PN group, 33.8 ± 2.6% (n = 3) in the PN treated dogs also given 5-HD in a dose of 300 μg kg^{-1} min^{-1}.

***Table 1*. Haemodynamic changes following the administration of saline, PN, 5-HD[1] = 150 µg kg^{-1} min^{-1}; 5-HD[2] = 300 µg kg^{-1} min^{-1}) and PC occlusions**.

	Control (saline pH 8.4)		PN		5-HD		5-HD+PN[1]		5-HD+PN[2]		PC	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
SABP (mm Hg)	131 ± 7	4 ± 3	135 ± 5	-4 ± 1	139 ± 6	1 ± 2	127 ± 6	6 ± 2	137 ± 3	3 ± 3	129 ± 5	-11 ± 2$^{\#}$
DABP (mm Hg)	88 ± 6	1 ± 2	90 ± 4	-4 ± 1	83 ± 6	1 ± 1	84 ± 6	4 ± 1	100 ± 5	1 ± 1	88 ± 3	-9 ± 2$^{\#}$
MABP (mm Hg)	103 ± 6	3 ± 2	105 ± 3	-4 ± 1	98 ± 5	1 ± 1	98 ± 6	4 ± 1	107 ± 3	2 ± 2	84 ± 4	-11 ± 1$^{\#}$
LVSP (mm Hg)	134 ± 6	4 ± 3	136 ± 4	-5 ± 2	139 ± 5	1 ± 2	133 ± 5	3 ± 1	137 ± 2	3 ± 3	119 ± 5	-12 ± 2$^{\#}$
LVEDP (mm Hg)	2.8 ± 0.4	0 ± 0	3.2 ± 0.3	0.1 ± 0.1	2.4 ± 0.3	0.2 ± 0.1	2.8 ± 0.4	0.1 ± 0.2	3 ± 1	0.5 ± 0.5	3.5 ± 0.3	4.3 ± 0.9$^{\#}$
+*d*P/*d*t (mm Hg s^{-1})	2912 ± 12	37 ± 48	2591 ± 82	-150 ± 40	3211 ± 212	19 ± 81	3061 ± 208	80 ± 73	2975 ± 75	75 ± 75	2610 ± 214	-478 ± 101$^{\#}$
-*d*P/*d*t (mm Hg s^{-1})	2241 ± 70	24 ± 12	1814 ± 63	-85 ± 26	2438 ± 260	19 ± 36	2659 ± 219	19 ± 36	2385 ± 240	62 ± 18	1954 ± 96	-339 ± 77$^{\#}$
HR (beats min $^{-1}$)	153 ± 6	2 ± 2	153 ± 4	0 ± 1	167 ± 7	0 ± 2	166 ± 8	-1 ± 1	163 ± 3	3 ± 1	146 ± 5	1 ± 3

Values are means ± s.e.m.; calculated from n = 3-26 observations, $^{\#}P < 0.05$ vs. pre (baseline) values.

***Table 2*. Haemodynamic changes following LAD occlusion**.

	Control (saline pH 8.4)		PN		5-HD		5-HD + PN		PC	
	Baseline	Occlusion	Baseline	Occlusion	Baseline	Occlusion	Baseline	Occlusion	Baseline	Occlusion
SABP (mm Hg)	129 ± 5	-12 ± 2$^{\#}$	131 ± 6	-12 ± 5$^{\#}$	132 ± 10	-11 ± 3$^{\#}$	133 ± 7	-9 ± 2$^{\#}$	122 ± 5	-10 ± 2$^{\#}$
DABP (mm Hg)	88 ± 4	-13 ± 1$^{\#}$	86 ± 5	-8 ± 2$^{\#}$	95 ± 5	-12 ± 3$^{\#}$	88 ± 6	-8 ± 2$^{\#}$	84 ± 4	-11 ± 2$^{\#}$
MABP (mm Hg)	100 ± 4	-12 ± 1$^{\#}$	101 ± 5	-9 ± 2$^{\#}$	133 ± 9	-12 ± 1$^{\#}$	103 ± 6	-8 ± 2$^{\#}$	101 ± 4	-10 ± 3$^{\#}$
LVSP (mm Hg)	133 ± 5	-13 ± 3$^{\#}$	129 ± 6	-9 ± 2$^{\#}$	137 ± 6	-10 ± 1$^{\#}$	137 ± 6	-11 ± 2$^{\#}$	121 ± 6	-11 ± 2$^{\#}$
LVEDP (mm Hg)	2.5 ± 0.6	12.0 ± 0.7$^{\#}$	3.6 ± 0.3	7.5 ± 0.7$^{\#}$*	2.5 ± 0.5	8.5 ± 0.5$^{\#}$	3.1 ± 0.4	6.5 ± 0.6$^{\#}$*	4.0 ± 0.5	5.1 ± 0.9$^{\#}$*
+*d*P/*d*t (mm Hg s^{-1})	2846 ± 323	-600 ± 139$^{\#}$	2794 ± 94	-560 ± 37$^{\#}$	3233 ± 167	-556 ± 102$^{\#}$	3195 ± 236	-370 ± 46*	2435 ± 178	-394 ± 60$^{\#}$*
-*d*P/*d*t (mm Hg s^{-1})	2586 ± 211	-538 ± 92$^{\#}$	1950 ± 76	-315 ± 47$^{\#}$*	1959 ± 150	-581 ± 81$^{\#}$	2445 ± 130	-285 ± 34$^{\#}$*	1976 ± 146	-282 ± 44$^{\#}$*
HR (beats min $^{-1}$)	157 ± 5	-2 ± 2	148 ± 5	8 ± 3	160 ± 11	-2 ± 3	164 ± 9	2 ± 2	143 ± 5	2 ± 3

Values are means ± s.e.m.; calculated from n = 3-26 observations. Occlusion values represent maximum changes occurring during the occlusion period,
$^{\#}P < 0.05$ vs. baseline value; *$P < 0.05$ vs. control group.

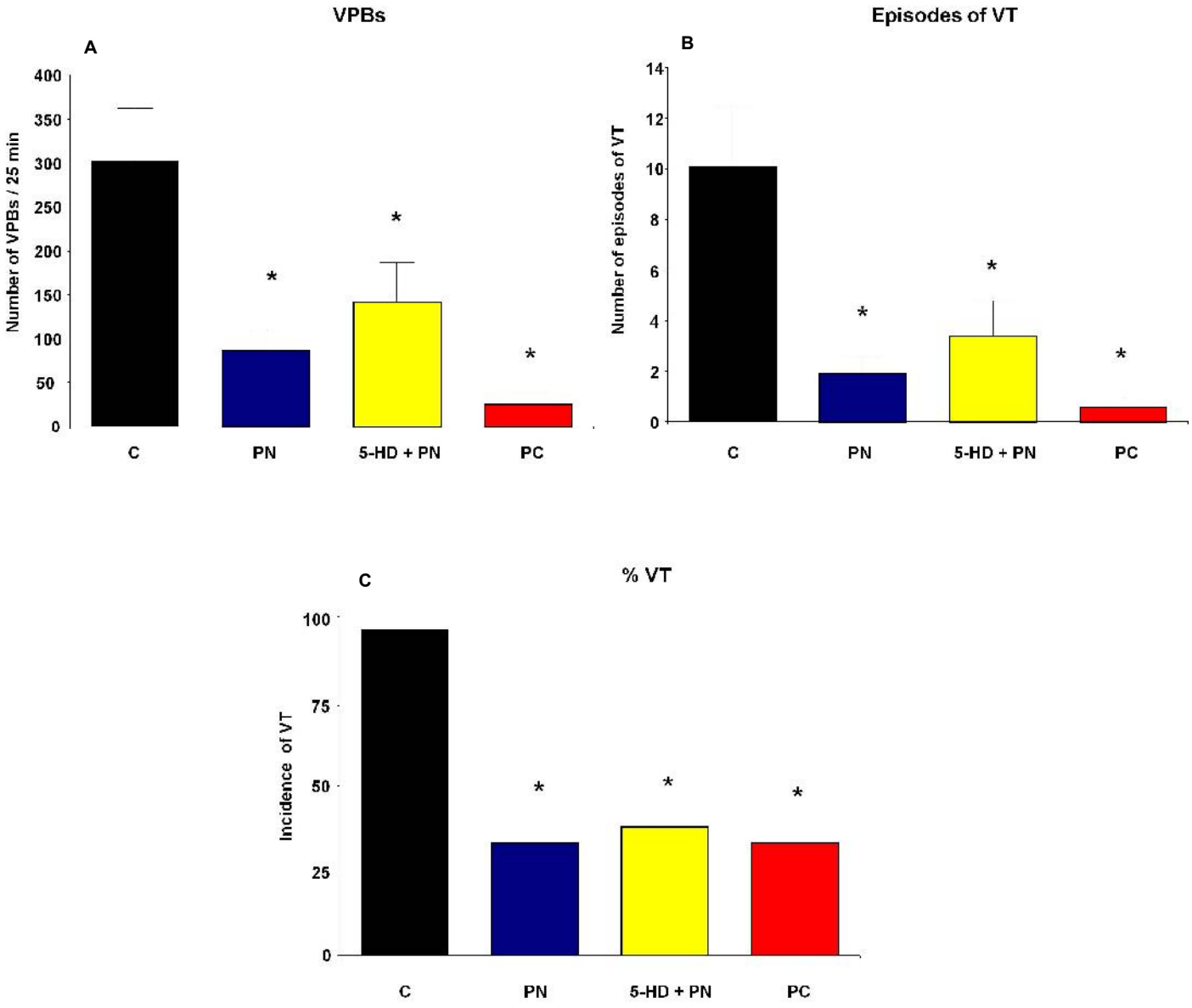

Figure 2.

The total number of VBPs (A), the number of episodes (B) and the incidence of VT (C) during a 25 min occlusion of LAD. Values are means ± s.e.m.; calculated from n = 8-26 observations; **P* < 0.05 vs. controls.

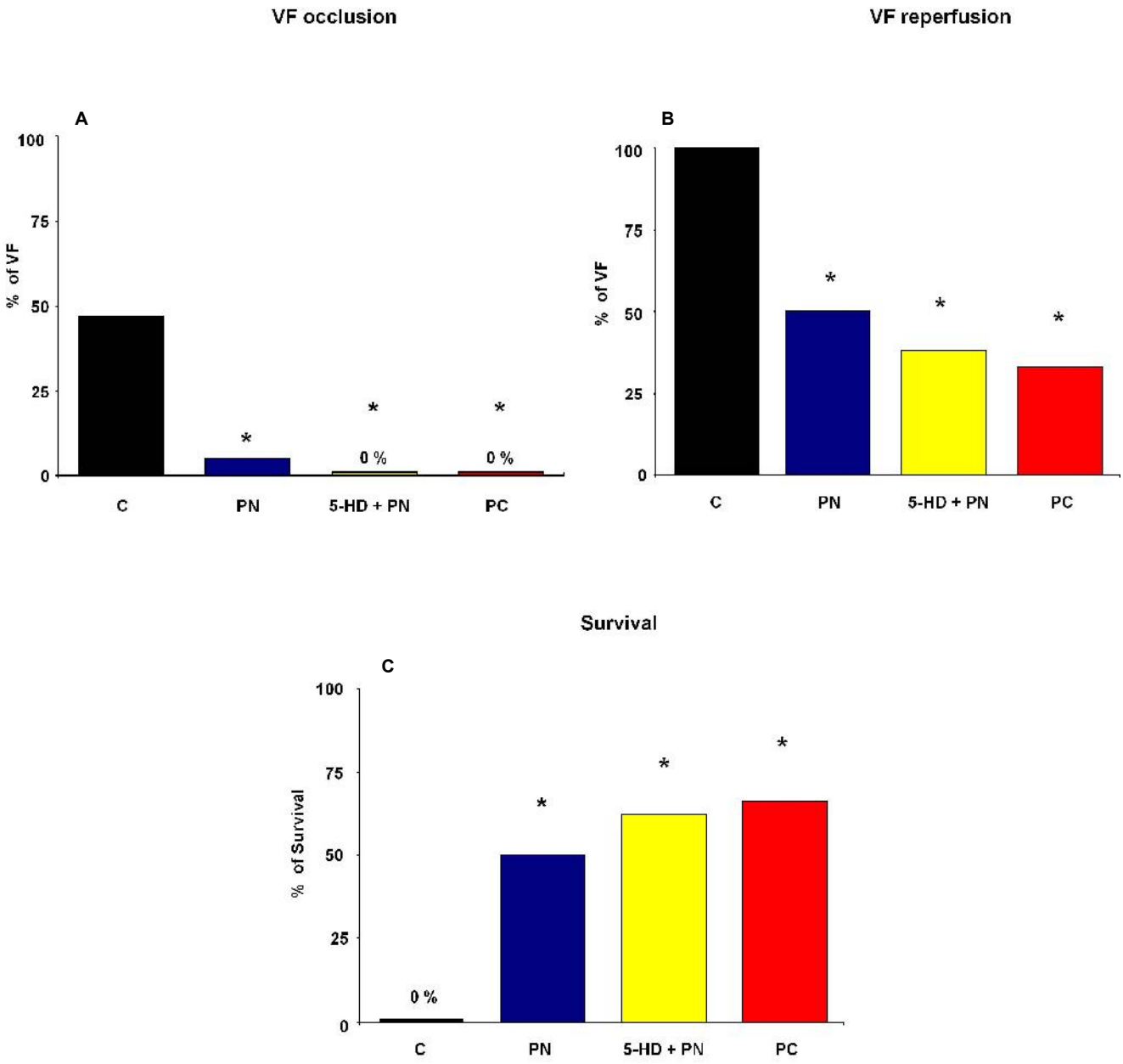

Figure 3.

The incidence of VF (A; B) and survival (C) following a 25 min occlusion and reperfusion of the LAD. Values are calculated from n = 8-26 observations; **P* < 0.05 vs. control group using the Fischer exact test.

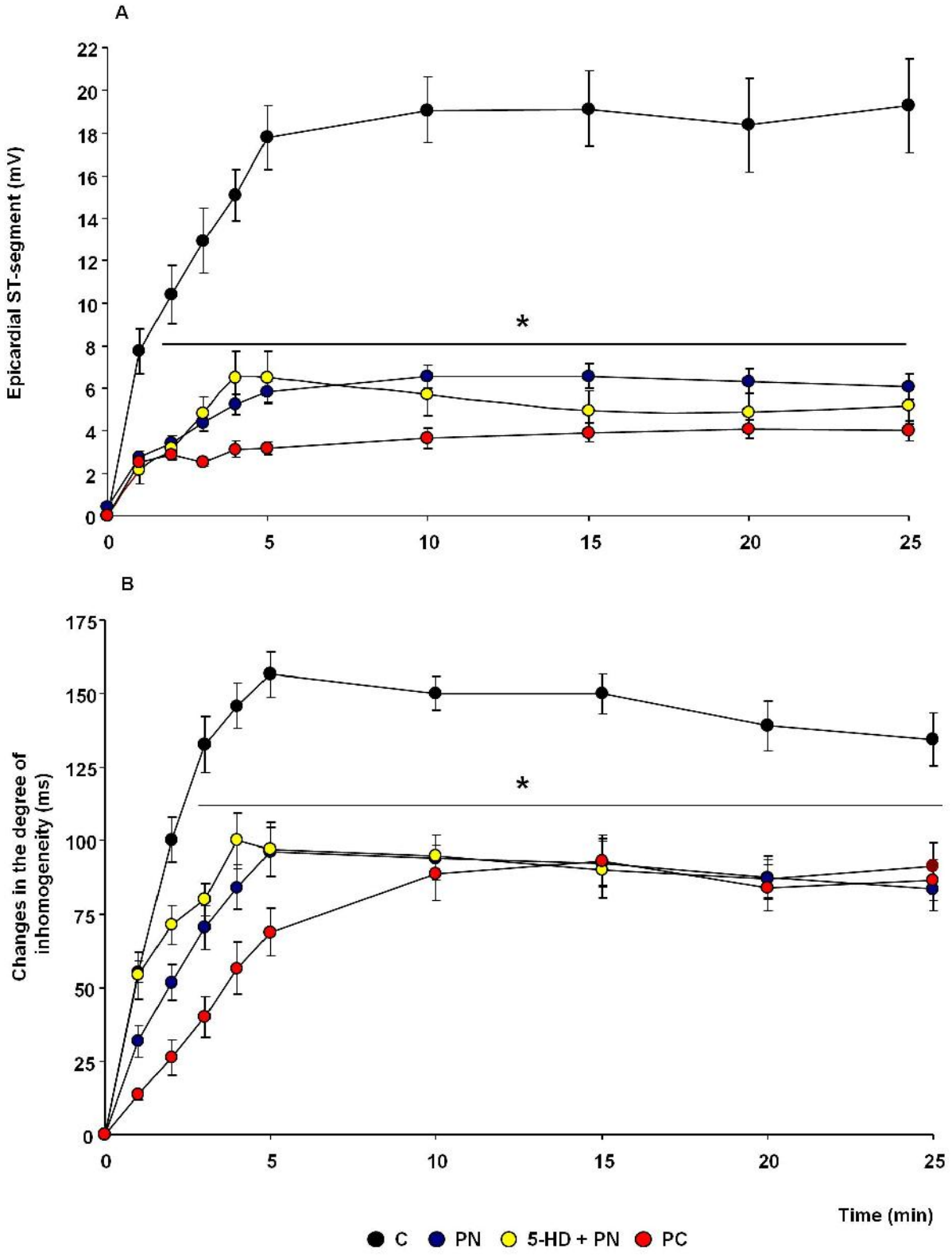

Figure 4.

Changes in epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 25 min occlusion. Values are means ± s.e.m., calculated from n = 8-26 observations; **P* < 0.05 vs. the control goup.

3.6 Changes in plasma nitrate/nitrite levels during occlusion and reperfusion

Blood samples were taken from the coronary sinus in order to determine the time course changes in plasma nitrate/nitrite (NOx) levels during the experiment. The results are illustrated in *Figure 5*. In the control group, occlusion of the LAD resulted in an initial increase in NOx levels reaching the maximum at around 7 min of the occlusion. After this, NOx levels started to decline and by the end of the occlusion period they were significantly lower than the initial, baseline values (18.8 ± 0.2 vs. 20.3 ± 0.3 μmol L^{-1}). In contrast, both PC and PN elevated NOx levels which were particularly marked following the first period of the PC occlusion (from 20.4 ± 0.2 to 21.8 ± 0.3 μmol L^{-1}) and of PN infusion (from 20.2 ± 0.2 to 21.5 ± 0.3 μmol L^{-1}), and these were maintained at a significantly higher level than in the controls over the entire prolonged occlusion. When the myocardium was reperfused NOx levels significantly increased in each group almost in a same extent.

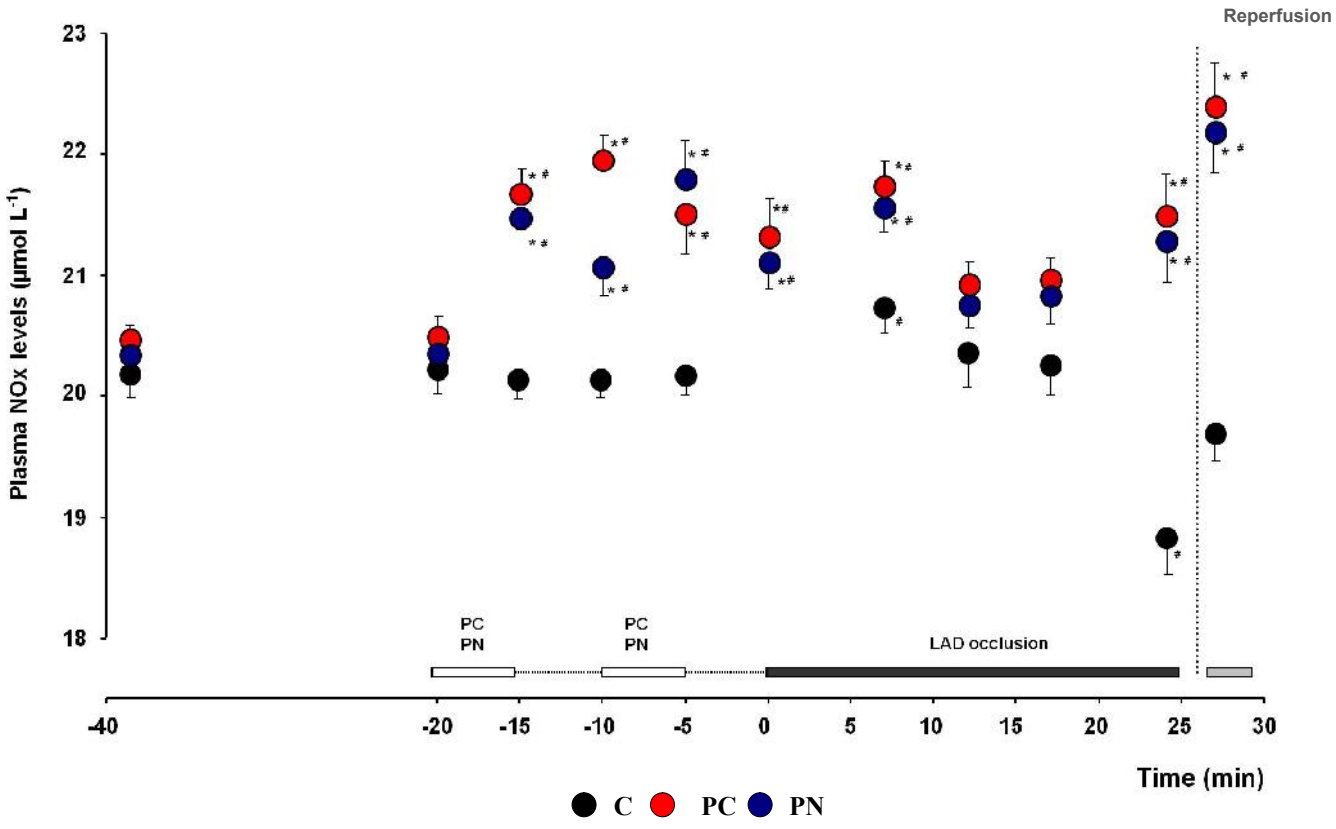

Figure 5.

Changes in plasma nitrate/nitrite (NOx) levels following preconditioning and the administration of peroxynitrite as well as during the combined ischaemia and reperfusion insult. Values are means ± s.e.m., calculated from n = 9-14 observations; $^{\#}P < 0.05$ vs. baseline value; $^{*}P < 0.05$ vs. the control group.

3.7 Changes in myocardial nitrate/nitrite levels following ischaemia and reperfusion

The myocardial NOx content was assessed in tissue samples taken from the ischaemic myocardium within 1-2 min of the reperfusion. The results are illustrated in *Figure 6*. There were no significant changes in tissue NOx levels following a 25 min occlusion and reperfusion, compared to the sham-operated controls (3.1 ± 0.2 vs. 3.4 ± 0.2 nmol mg^{-1} protein). In contrast, in dogs subjected either to PC or infused with PN the myocardial NOx content was markedly increased after the combined prolonged period of I/R insult (4.4 ± 0.2 and 4.0 ± 0.1 nmol mg^{-1} protein, respectively).

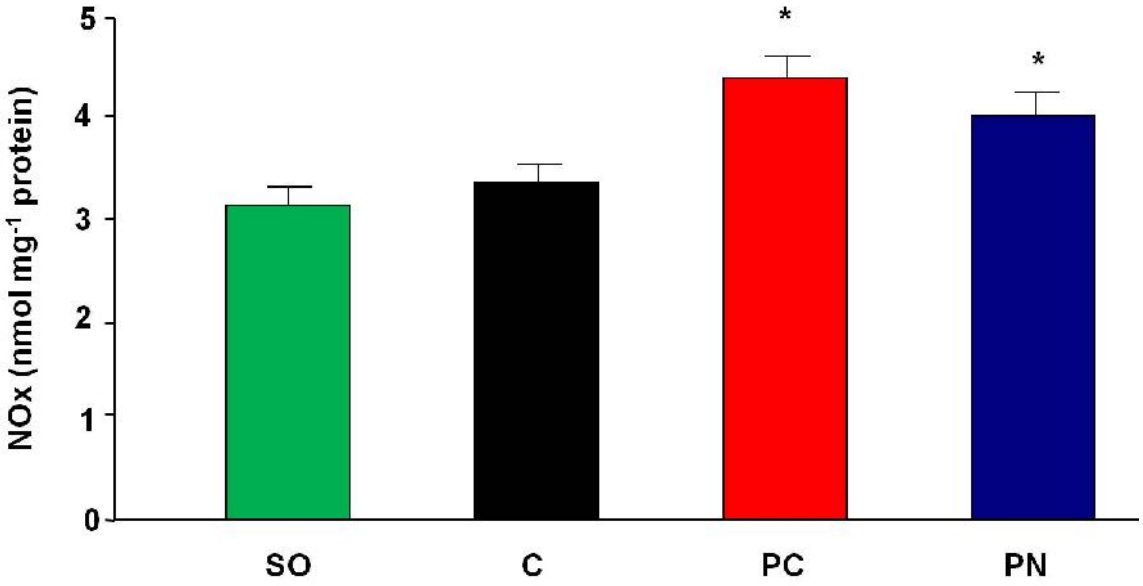

Figure 6.

Tissue NOx levels following a 25 min coronary artery occlusion and reperfusion. Values are means ± s.e.m., calculated from n = 4-14 observations; *$P < 0.05$ vs. the control group.

3.8 Changes in superoxide production following occlusion and reperfusion

The generation of superoxide was measured in myocardial tissue samples taken from the ischaemic myocardium 1-2 min after reperfusion (*Figure 7*). In control dogs subjected to I/R there was a significant increase in myocardial superoxide production compared to the sham-operated controls (40.1 ± 1.8 vs. 14.7 ± 1.5 arbitrary units). Both PC and PN significantly reduced this I/R-induced superoxide production (21.4 ± 1.4 and 24.5 ± 2.2 arbitrary units, respectively). The figure also shows representative fluorescence pictures from each group.

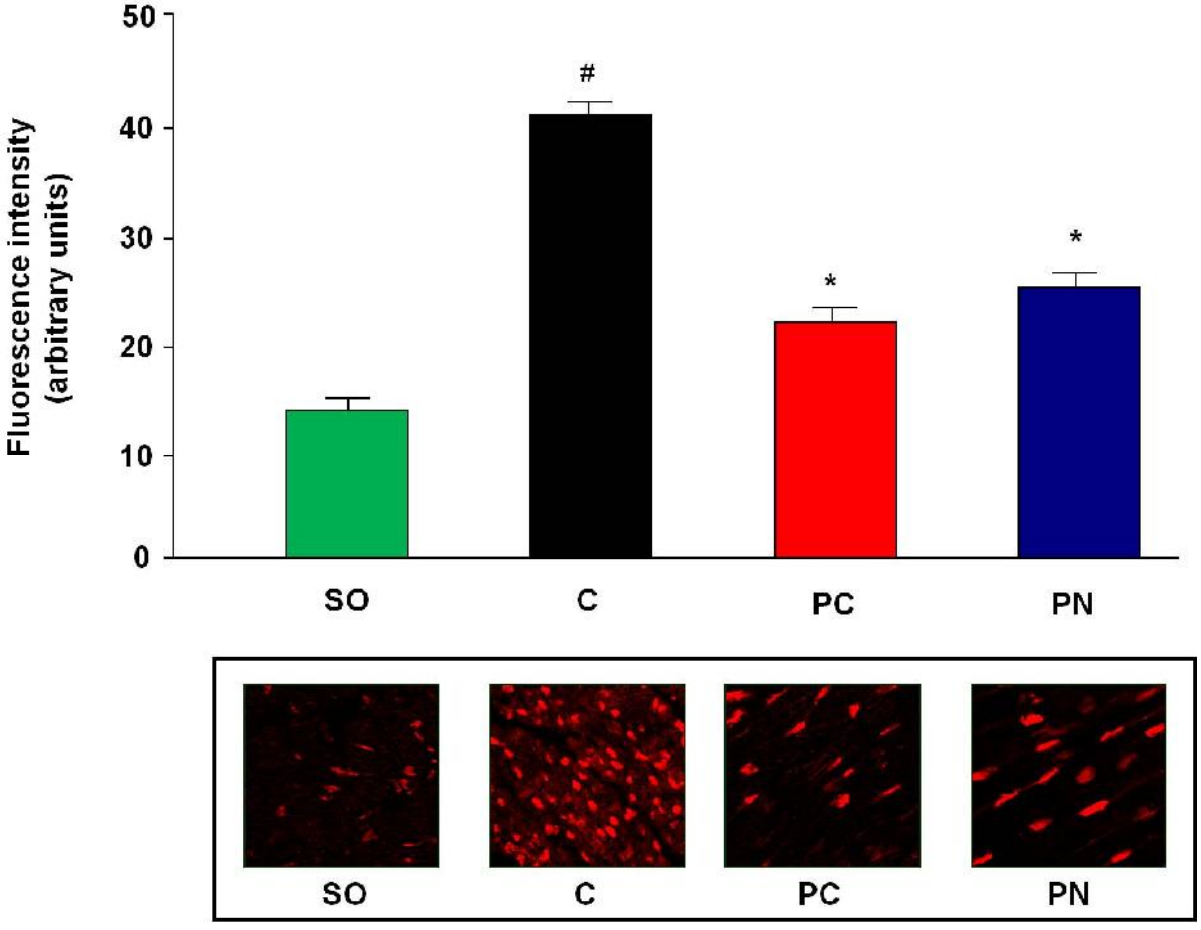

Figure 7.

Changes in tissue superoxide production following ischaemia and reperfusion in control dogs and in dogs subjected either to PC or treated with PN. Values are mean ± s.e.m., calculated from n = 4-14 observations. $^{\#}P < 0.05$ vs. sham-operated controls; $^{*}P < 0.05$ vs. the ischaemic control group.

3.9 The generation of nitrotyrosine following ischaemia and reperfusion

In these experiments two methods were used to assess NT formation, a biomarker of peroxynitrite formation. In the first study Western-blot was used and the detected NT was expressed in arbitrary units (*Figure 8A*). In the second study NT formation was measured by ELISA, and the NT content was expressed in ng mg^{-1} protein (*Figure 8B*).

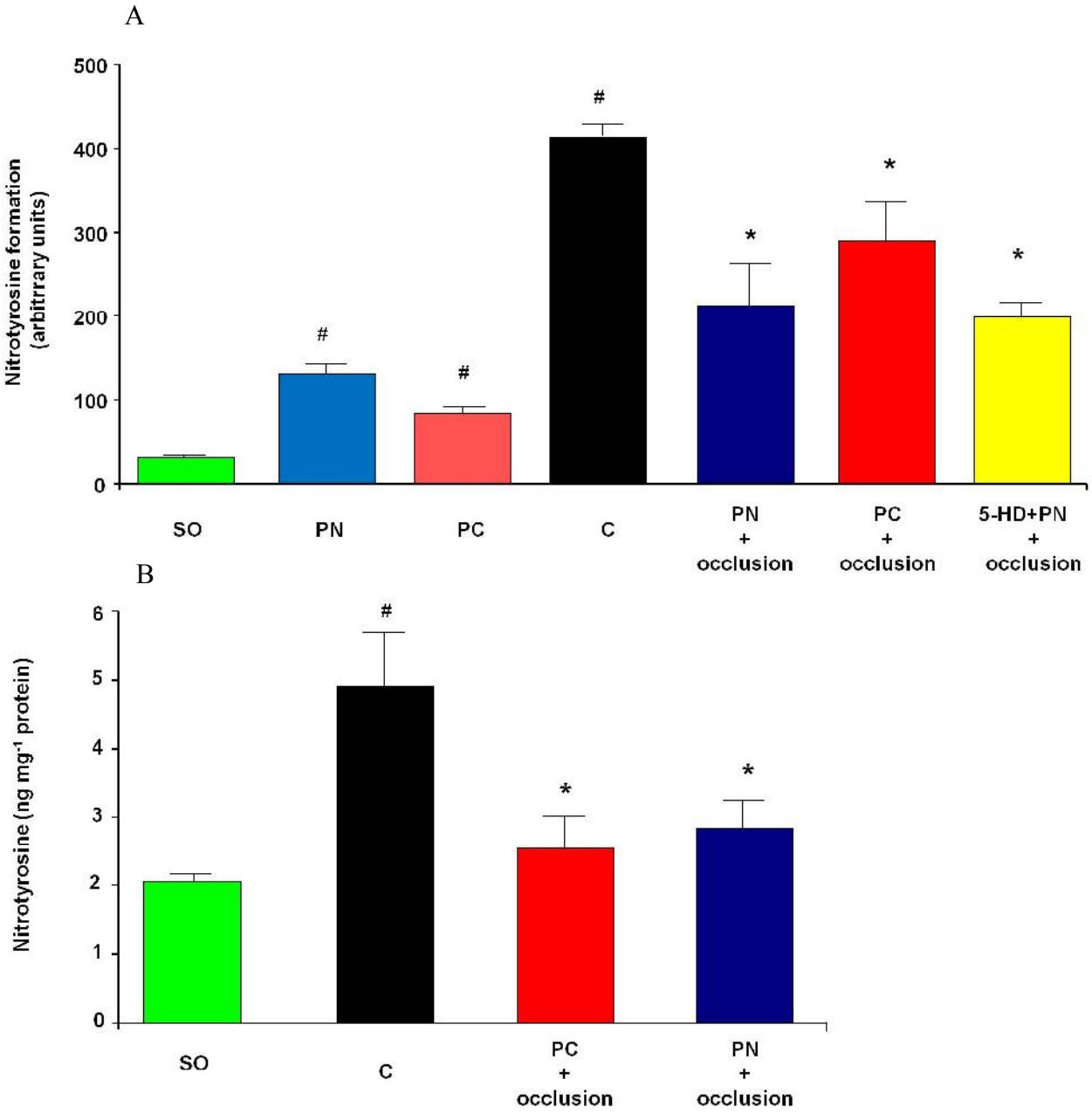

Figure 8.

Changes in nitrotyrosine production, determined by Western blot (A) and by ELISA (B) following PC and the treatment with PN, as well as after a 25 min occlusion and reperfusion of the LAD. Values are means ± s.e.m., calculated from n = 4-14 observations; $^{\#}P < 0.05$ vs. sham-operated controls; $^{*}P < 0.05$ vs. ischaemic control dogs.

Compared to the sham-operated dogs, both the PC procedure and the administration of PN resulted in a slight but significant increase in NT content of the myocardial tissue, indicating the presence of endogenous PN formation following the two brief periods of PC occlusions and PN infusions (*Figure 8A*). However, in those dogs that were subjected to a prolonged period of I/R, compared to the sham-operated controls, there were marked elevations in NT production, no matter which method had been used for its evaluation. For example, using ELISA the NT content was 2.0 ± 0.1 ng mg^{-1} protein in the sham-operated controls and this was increased to 4.9 ± 0.8 ng mg^{-1} protein in dogs that were subjected to a 25 min occlusion and reperfusion challenge. Both PC and the infusion of PN significantly reduced NT production (2.5 ± 0.5 and 2.8 ± 0.4 ng mg^{-1} protein; respectively) that resulted from this combined I/R insult (*Figure 8B)*. The suppression in NT formation that resulted from PN infusion was not influenced by the prior administration of 5-HD *(Figure 8A*).

4. DISCUSSION

4.1 New findings

1. Our results provided evidence that in anaesthetised open chest dogs, brief periods of 100 nM concentration of peroxynitrite infusion, administered directly into the coronary artery markedly suppresses the occurrence and severity of ventricular arrhythmias, and the generation of endogenous peroxynitrite, that result from a subsequent, more prolonged period of ischaemia and reperfusion. These effects were similar to those obtained with preconditioning induced by similar periods of occlusion and reperfusion.

2. We have also pointed out that, in contrast to preconditioning, the antiarrhythmic effect of peroxynitrite does not involve the opening of mitoK_{ATP} channels, since 5-HD, a relatively selective inhibitor of these channels, did not modify the protection afforded by the administration of PN.

3. We showed, however, that the marked decrease in endogenous PN formation both following PC and PN is due to a reduced superoxide rather than to a reduced NO production, as has been previously suggested. This is supported by the fact that in anaesthetised dogs both PC and the adminstration of PN increased, or at least, preserved NO availability during coronary artery occlusion. We propose that the preservation of NO production during ischaemia plays a mandatory role in the antiarrhythmic protection, perhaps by attenuating the marked superoxide production and the formation of peroxynitrite following reperfusion.

4.2 The role of nitric oxide, superoxide and peroxynitrite in ischaemia and reperfusion

Although several studies examined the role of NO, $O_2^{\cdot}$ and PN in the heart, there is still ongoing dabate whether these substances have beneficial or harmful effects under conditions of myocardial ischaemia and reperfusion [13]. In addition, their involvement in cardioprotection is even more controversial. The reasons of the conflicting data include the variety of species, models and experimental conditions are used, the time of sampling and the methods by which these radicals are determined, etc. For example, PN generated following a prolonged period of ischaemia and reperfusion is generally considered to be cytotoxic and largely contributes to the development of ischaemia/reperfusion injury. This is certainly true, but recent findings also show that PN administered in low, nano or micromolar concentrations, is able to produce cardioprotective effects [72, 73]. This concept is supported

by our results, i.e. a low amount of peroxynitrite, generated during preconditioning or administered in intracoronary infusion, attenuates the severity of ischaemic changes, as assessed by a reduction in epicardial ST-segment and in the degree of inhomogeneity, and markedly suppresses the ventricular arrhythmias which occur during a subsequent, prolonged period of ischaemia and reperfusion. These results are in a good aggrement with those found in rat isolated hearts examining the antiarrhythmic effect of the low concentration of the exogenously administered peroxynitrite [75]. This study has also pointed out that peroxynitrite generated during the preconditioning stimulus is likely involved in the PC-induced antiarrhythmic protection [75].

Athough the precise mechanism by which PN results in antiarrhythmic effect is not fully understood; there are proposals which may explain the cardioprotective (antiarrhythmic) effects. First, PN may serve as an NO donor [76]. We know that drugs which able to donate NO, such as nicorandil [85], isosorbide mononitrate [26] and sodium nitroprusside [27], can reduce the severity of ischaemia and reperfusion-induced arrhythmias in the anaesthetised canine. Thus, PN, if posesses NO donor properties, can release NO in cardiac myocytes and thereby increases NO availability in the heart. This NO activates various intracellular cascades, including the stimulation of sGC [86], and the subsequent elevation of cGMP [21, 22], which is considered as a primary action of NO-induced vasodilatation [18, 86] and reduction in calcium overload [33, 34]. This mechanism is certainly involved in the antiarrhythmic protection, since inhibition of sGC with methylene blue abolished the PC-induced antiarrhythmic effect in dogs [35], whereas the preservation of myocardial cGMP levels by zaprinast reduced the incidence of the reperfusion-induced ventricular fibrillation in rat isolated hearts [30]. NO may also regulate, through a cGMP-mediated pathway, the effects of the autonomic nervous system; i.e. it inhibits the release of noradrenaline from nerve endings [29; 32], and enhances the effect of vagal nerve stimulation [31]. Both effects may also account for an antiarrhythmic and/or an antifibrillatory effect [31].

Second, in cardiomyocytes PN, like NO, may lead to cardioprotection by nitrosylation of thiols [87, 88, 89, 90, 91] and increasing the levels of S-nitrosothiols [76, 91]; an efffect which is independent form the activation of guanylyl cyclase. These nitrosothiols, such as S-nitrosoglutathione, may serve as NO reservoirs and release NO over a prolonged period of time [91, 92, 93]. Furthermore, the S-nitrosylation of various proteins and ion channels, such as the cardiac sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a), the mitochondrial F1-ATPase α1 subunit and the L-type Ca^{2+} channel α1 subunit, are thought to play role in the cardioprotective effects of preconditioning and peroxynitrite. For example, the increased

SERCA2a activity improves Ca^{2+} uptake into the sarcoplasmic reticulum, whereas the inhibition of L-type Ca^{2+} channel reduces Ca^{2+} influx during ischaemia and reperfusion; both mechanisms lead to a reduction in Ca^{2+} overload [87, 88] and to the suppression of arrhythmias [41].

Third, PN, similar to NO, may act on the mitochondrion and regulate $O_2^{\cdot}$ production. It is established that NO activates protein kinases (PKG, PKC) and opens mitoK_{ATP} channels which are thought to play a pivotal role in the preconditioning-induced protection [94, 95, 63, 96]. Indeed, our as well as the others experiments showed that closing mitoK_{ATP} channels by 5-HD prior to the preconditioning procedure completely abolished the antiarrhythmic effect of preconditioning [44, 47, 97]. However, our present results clearly indicated, that in contrast to PC, the antiarrhythmic effect of PN not involves mitoK_{ATP} channels. Neither the 150 μg kg^{-1} min^{-1} nor the higher, 300 μg kg^{-1} min^{-1} doses of 5-HD were able to abolish or attenuate the antiarrhythmic effect of PN. Thus, we concluded that mitoK_{ATP} channels are not necessary be involved in the protective effects of PN, and there are certainly exist other mechanisms by which PN attenuates the the prolonged ischaemia and reperfusion-induced superoxide production. Indeed, there is evidence that PN has a direct effect on the electrotransport chain and the uncoupling proteins [43] which results in a reduction in mitochondrial $O_2^{\cdot}$ generation.

We think, that one of the most important findings of our studies was that both PC and the infusion of PN markedly reduced the endogenous PN formation that resulted from a prolonged period of ischaemia and reperfusion. Since peroxynitrite is know as a toxic metabolite which largely contributes to the myocardial injury [13, 71, 98, 99, 100], any intervention which attenuates peroxynitrite production expected to be cardioprotective. Preconditioning can certainly be such an intervention. For example, Novalija *et al.* [101] showed, albeit in a different species, that PC significantly reduced the generation of peroxynitrite resulted from ischaemia and reperfusion.

Although we do not know the exact mechanisms by which PC and also the exogenous administration of peroxynitrite are able to reduce the endogenous peroxynitrite formation, it is almost certain from our studies, that this is resulting from a reduction superoxide, rather than from a reduction in NO production. In contrast to the in *vitro studies* [36, 37] in which the generation of NO was markedly increased during myocardial ischaemia [36] and PC by reducing this overproduction of NO [37] decreased LDH release and the incidence of reperfusion-induced VF [37], we have found that NOx levels, after an initial increase, were markedly declined. Such a decrease in NO production during ischaemia was confirmed by other studies [38, 39, 40]. For example, Stevens *et al.* [38] demonstrated that in anaesthetised

pigs preconditioning by two cycles of 5 min LAD occlusion prior to a 60 min regional ischaemia markedly decreased the interstitial nitric oxide metabolites compared to the controls. Similary, Engelman *et al.* [40] have found that in an open-chest working pig heart model, the release of NO significantly decreased throughout the entire ischaemia and reperfusion insult. This decrease in NO production *in vivo* during myocardial ischaemia seems quite obvious since the availability of those cofactors which are necessary for NOS activity and NO production, such as L-arginine, NADPH, tetrahydrobioptherin (BH4), molecular oxygen, is limited during a prolonged period of ischaemia [102]. This is supported by the fact, that the administration of L-arginine, the precursor of nitric oxide, reduced the ischaemia-induced arrhythmias and lipid peroxidiation [40]. However, in contrast to these abovementioned studies [39, 40], we observed a transient increase in NO metabolites which occurred soon after (around 7 min of the occlusion) the commencement of the coronary artery occlusion. Since we have not measured NOS activity, we can speculate that this initial increase in NOx levels results from the stimulation of NOS, as that there is some evidence for an early (within 5 min) activation of NOS enzyme by ischaemia [103]. The other possible explanation for this transient increase in NO production might be that we measured plasma NOx levels in blood samples taken from the coronary sinus. Since this collects blood from both the ischaemic and non-ischaemic areas, an elevation in NOx may derive from a compensatory increase in NO production occurring within the non-ischaemic myocardium. Such an elevation in NOx levels within the non-ischaemic area has been demonstrated by others as well [38, 39]. Whatever the precise mechanism, we think, that this transient increase in the coronary sinus NOx levels may represent an important compensatory mechanism; ie. the enhanced NO production within the normal region attempts to compensate for the loss of NO production within the severely injured myocardium.

In contrast to the results obtained from the control dogs that were subjected simply to a prolonged period of ischaemia and reperfusion, both preconditioning and the administration of peroxynitrite increased NOx levels prior to, and maintained NO availability during the subsequent, prolonged ischaemic challenge. Han *et al.* [22] have also found an increase in NO production following the PC occlusions. This increase in NO production both following PC and the administration of PN may result from the upregulation of eNOS [104, 105] and the subsequent preservation of eNOS protein levels [106] or/and from the stimulation of an AMP-dependent protein kinase which leads to further activation of NOS [107]. The preservation of structures responsible for NO release has also proposed to be a mechanism in the PC-induced cardioprotective and antiarrhythmic effect [108]. All these studies provide a strong evidence

for the cardioprotective role of NO that is formed and released in the myocardium following preconditioning, and perhaps also when NO is donated by the administration of peroxynitrite. There is substantial evidence that NO donors effectively prevent ATP depletion, calcium overload and arrhythmias [27]. For example, we have evidence that in anaesthetised dogs SNP has an antiarrhythmic effect, and this, at least in part, involves the modulation of gap junctions [27]. Similarly, Kumar *et al.* [30] showed in pigs that nitroglycerin suppressed arrhythmogenesis.

We think, that we provided a stonge evidence that the antiarrhythmic effect of preconditioning and peroxynitrite results from the maintenance of NO availability during myocardial ischaemia and reperfusion. In contrast to the ischaemic conditions, under which NO availability continuously decreases, both the PC procedure and the administration of PN increase NO production, and this is maintained and provide adequate NO availability during the subsequent more prolonged ischaemic insult. We also suggest that this preserved NO production during ischaemia regulates superoxide production which may already occur during the later period of the coronary artery occlusion [109, 110] but certainly extremely increased after reperfusion of the ischaemic myocardium.

There is accumulating evidence that NO reduces ROS production [55, 111] during ischaemia and reperfusion and thereby attenuates the severity of ischaemia and of arrhythmias [53, 112, 113]. Among the mechanisms by which NO regulates ROS formation the inhibition of xanthine oxidase [114] and the modulation of mitochondrial respiratory chain [113] are thought to have particular importance. Similar to nitric oxide, peroxynitrite, formed in biological systems can also regulate xanthine oxidase [79] and mitochondrial uncoupling protein activity [43], resulting in a reduction in $O_2^{\cdot}$ production which further limits peroxynitrite formation. Although we do not know whether peroxynitrite itself or through the release of NO results in cardioprotection it is clear from these studies that both possibilities, through the regulation of superoxide production, can lead to a reduction in nitrotyrosine formation.

In summary, we designed studies to examine the possible antiarrhythmic effect of low concentration of peroxynitrite, administered locally into the coronary circulation. We showed that peroxynitrite similar to preconditioning reduces the severe consequences of the acute ischaemia and reperfusion; and of particular importance, significantly suppresses the generation of endogenous peroxynitrite. However, in contrast to preconditioning, these cardioprotective effects of peroxynitrite do not require the opening of mitoK_{ATP} channels.

Since both preconditioning and peroxynitrite significantly reduced nitrotyrosine formation, a biomarker of endogenous peroxynitrite production, in another study we examined whether this is due to a reduction in nitric oxide or in superoxide production. Our results clearly showed that both PC and PN result in an inrease in the concentration of NO metabolites and provide an increased or, at least, maintained NO availability during ischaemia. We propose, that this preserved NO availability has particular importance in the antiarrhythmic effect of preconditioning and peroxynitrite, through the reduction of oxidative stress.

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7. ANNEX

Reprints of full papers

I. **Kiss A**, Juhász L, Huliák I, Végh Á. Peroxynitrite reduces ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs without involving mitoK_{ATP} channels. *Br J Pharmacol*; 155: 1015-1024, 2008.

II. **Kiss A**, Juhász L, Kupai K, Seprényi Gy, Kaszaki J, Végh Á. The role of nitric oxide, superoxide and peroxynitrite in the antiarrhythmic effects of preconditioning and exogenous peroxynitrite. *Br J Pharmacol;* 160: 1263-1272, 2010.