FURTHER EVIDENCE FOR THE ROLE OF NITRIC OXIDE IN
THE ANTIARRHYTHMIC EFFECTS OF ISCHAEMIC
PRECONDITIONING: THE EFFECT OF PEROXYNITRITE AND
CHANGES IN NOS-DEPENDENT NO PRODUCTION

Summary of PhD Thesis

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PUBLICATIONS

The results summarized in the present Thesis are based on the following publications:


Other publications:


INTRODUCTION

Study I.

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 WHO, both in men and women the ischaemia-induced sudden cardiac death results in more than 7 million deaths per year in the world. 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 (PC). It is now 28 years that PC, as one of the endogenous adaptive phenomena, was first described by Murry and colleagues 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. 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 I/R-induced ventricular arrhythmias and improves the restoration of contractile dysfunction following reperfusion. 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. It is proposed that these substances, via receptor-dependent and receptor-independent mechanisms, may activate various signalling pathways and modify end-effectors, leading ultimately to cardioprotection. The first endogenous substances that were associated with the PC-induced cardioprotection were prostacyclin, bradykinin and adenosine. More recent evidence suggests that peroxynitrite (PN), a by-product of the reaction of nitric oxide (NO) and superoxide, may also play a trigger role in the PC-induced antiarrhythmic protection. In rat isolated hearts the inhibition of PN formation by the administration of uric acid (UA) during the PC procedure, markedly attenuated the antiarrhythmic effect of PC. Similarly, the protection resulted from the exogenous administration of PN was abolished in the presence of UA. 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. This protection was similar to that seen with ischaemic PC, or after the administration of NO donors. 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.

**Study II.**

The first evidence that NO may play both a trigger and mediator role in the PC-induced antiarrhythmic protection came out from the previous studies of our laboratory. These showed that in anaesthetized dogs the protection against ventricular arrhythmias was abolished by the administration of the nitric oxide synthase (NOS) enzyme inhibitor, L-NAME. More recently, it has also become evident that under ischaemic conditions NO can be produced by an enzyme-independent way as well. For example, in rat isolated heart preparations, a considerable increase in NO production was detected, suggesting that in the absence of adequate oxygen supply NO may preferably generate from nitrite, rather than synthesized by NOS.

Our own previous studies, measuring the metabolites of NO (plasma nitrate/nitrite; NOx) in the blood of the coronary sinus, 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. However, after this the NOx levels started to markedly decline up to the end of the occlusion period. This was in contrast with dogs subjected to PC, where the plasma NOx levels were elevated during the PC procedure, and were maintained even during the entire prolonged period of occlusion.

Although it seems more than likely that PC may influence NOS activity and expression, there is a lack of evidence whether the preservation of NO bioavailability following PC would result from the influence of the PC stimulus on the enzyme-dependent or/and an enzyme-independent NO formation. It is also not clear, what the time-course changes are in the generation of other substances, such as superoxide and PN. These substances, are also generated during PC, and their formation can be modified by NO.
AIMS OF STUDY I & STUDY II

The objective of Study I was to examine whether peroxynitrite (PN) generated during the PC stimulus, may act as a trigger for the PC-induced antiarrhythmic protection. Thus, 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.

The experiments involved in Study II 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.

METHODS

1. Experimental animals & surgical procedures

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 i.v). 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). Polyethylene catheters 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.

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. Assessment of ventricular arrhythmias

The number and severity of ventricular arrhythmias were assessed from the chest lead II electrocardiogram, according the Lambeth conventions. 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 and survival were determined.

3. 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, using a composite electrode positioned within the ischaemic area. This electrode gives a summarised recording of R waves from 24 epicardial measuring points. During normal myocardial perfusion, 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 milliseconds. The composite electrode also contains four unipolar electrodes by which changes in epicardial ST-segment were evaluated (in mV) within the ischaemic zone.

4. Determination of plasma nitrate/nitrite levels

Plasma nitrate/nitrite (NOx) concentrations were measured by means of Griess reaction. Blood samples, collected from the coronary sinus at different time points, were centrifuged at 10 000g for 15 min at 4°C. The plasma was mixed with β-NADPH, FAD, nitrate reductase 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 NaNO₂ and NaNO₃.
5. Assessment of myocardial superoxide (O$_2^-$) generation

Superoxide generation 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. 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), served as 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 and expressed in arbitrary units.

6. Determination of nitrotyrosine formation

Myocardial nitrotyrosine (NT), a biomarker of peroxynitrite formation was evaluated by Western blot. Tissue samples were taken from the ischaemic area within two minutes of the reperfusion. 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), and then with horseradish peroxidase-conjugated rabbit anti-mouse IgG (in a dilution of 1:1000, P0161) as a secondary antibody. For visualisation, the membrane was developed with an enhanced chemiluminescence kit, exposed to X-ray film and scanned. The intensity of bands was determined using ImageJ software, and expressed in percentage of the sham-operated dogs. Equal protein loading was confirmed by monitoring GAPDH.

7. Assessment of nitric oxide synthase activity (NOS)

In Study II this was performed using a NOS assay kit (Cayman Chemical), 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, 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™) 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.

8. Experimental protocols

**Protocol for Study I.** Dogs were randomly divided into eleven groups. After completing surgery, 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.

**Protocol for Study II.** In study II, 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 with 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 were also taken from the coronary sinus at various time points of the experiments to measure plasma NOx levels.

RESULTS

I/1. Occlusion of the LAD for 25 min resulted in high number of VPBs and many episodes of VT that occurred more than in 90% of the control dogs. 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. 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. Both PC and the infusion of 100 nM PN significantly decreased the severity of the ischaemia-induced ventricular arrhythmias and increased survival following reperfusion. Moreover, severity of ischaemia was significantly less pronounced in these dogs. Administration of UA did not influence the antiarrhythmic and anti-ischaemic effects of PC, but almost completely abolished the PN-induced protection. Surprisingly, UA itself also resulted in protection against the acute I/R-induced ventricular arrhythmias and exhibited an anti-ischaemic effect.

I/2. Both the PC procedure and the administration of PN significantly increased the formation of NT (a biomarker of PN production), which were almost completely abolished in the presence of UA. A 25 min occlusion and reperfusion of the LAD markedly increased the production of NT, which was significantly less pronounced both in the PC and the PN-treated animals. Urate itself also reduced the NT formation, but it only abolished the effect of PN on NT production.

I/3. Both the PC occlusions, and also the similar periods of PN infusion, increased the level of NO metabolites, and this was maintained over the entire prolonged period of ischaemia. This was in contrast with the control dogs, in which NOx levels were markedly reduced by the end of occlusion period. 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.

I/4. The combined I/R insult markedly elevated superoxide production, which was significantly attenuated both 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.

II/1. 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).

II/2. The NOx levels changed almost parallel with the activation of NOS. Occlusion of the LAD resulted in a transient but significant increase in NOx (C1), followed by a continuous decrease up to the end of the 25 min occlusion (C2). In contrast, NOx levels were markedly 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).

II/3. Superoxide production was elevated 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. 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 (PC3 and PC4) than in the controls (C2).

II/4. Compared with the sham controls, occlusion of the LAD significantly increased NT formation (C2). There was also an enhanced NT production following the PC procedure (PC1 and PC2), but the amount of this stable product of NO and superoxide reaction was not substantially changed during the subsequent, more prolonged period of occlusion (PC3 and PC4), nor significantly different from that obtained in the controls (C2).
DISCUSSION

New findings

Study I.

1. We have demonstrated that although ischaemic preconditioning (2 x 5 min occlusion of the LAD) in anaesthetized dogs, induces detectable PN production, this PC-induced PN formation most probably does not play an important trigger role in the antiarrhythmic effect of PC, since the protection against arrhythmias is still present, if PN is scavenged by uric acid, during the PC procedure.

2. In contrast, the antiarrhythmic effect of the exogenously administered PN is completely abolished by the administration of UA.

3. We have also pointed out that UA itself may also provide protection against arrhythmias, which effect can be associated with the antioxidant properties of UA.

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

(a) a prolonged period of ischaemia decreases the activity of NOS, resulting in reduced NO and increased superoxide and NT productions,

(b) PC 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.

Although in the presence of UA 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 (Study I). This was in contrast with the protection resulted from PN infusion; here scavenging of the exogenously administrated PN by UA, the antiarrhythmic and the other cardioprotective (anti-ischaemic, NO preserving, superoxide suppressing) 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 protective 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, it has been found previously that mitoK<sub>ATP</sub> channels play a role in the antiarrhythmic effect of PC, whereas inhibition of these channels with 5-HD fails to modify the antiarrhythmic protection resulted from administration of PN. Furthermore, it has been also shown that PN can directly activate downstream targets of PC signalling pathways, including PKC.

Another explanation for the difference might be related to the strength of the PC stimulus used under *in vivo* and *in vitro* conditions; i.e. whether a certain PC protocol is able to provide sufficient stimulus for the formation of endogenous PN. Our previous study showed that 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. It is also likely that the PC procedure not only generates ROS, but stimulates the free radical scavenging systems (MnSOD) which may also account for the rapid elimination of superoxide. We propose that in this model of I/R, other substances that are generated and released during the PC procedure may play a more essential role, such as NO.

It is well documented in the literature, that UA accounts for approximately 60 % of plasma total antioxidant capacity, since it is able to react with oxygen derived radicals, such as singlet oxygen, hydroxyl radical, oxo-heme oxidants as well as peroxyl radicals. It can also trap peroxynitrous acid, the decomposition product of PN. The precise mechanism by which UA exerts antiarrhythmic effect is not fully elaborated, it is likely that such a protective effect can be associated with the antioxidant properties of UA.

The results of *Study II* show that NOS activity was markedly increased following a 5 min period of coronary artery occlusion (C1), indicating a rapid NOS activation and NO formation following ischaemia. This finding accords with those previous observations, which suggested that ischaemia rapidly activates NOS, due to the changes in cytosolic calcium levels and enzyme phosphorylation. 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 (C2). The reduction 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 PN, 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, confirming those previous findings, which showed that superoxide can be generated during ischaemia, especially when NOS uncouples because of the limited supply of oxygen, substrate and cofactors.

Study II also showed that PC stimulates NOS; but interestingly the activity of NOS following the two 5 min occlusions (PC1) was not as marked as that could be observed after a single 5 min occlusion (C1). An explanation for this difference may lie in the reperfusion interval, which elapses between the first and the second PC occlusions, suggesting that the NOS is not only rapidly activated by ischaemia, but it can also rapidly be deactivated when the ischaemia ceases. This assumption is confirmed by the results in 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 NT production during the consecutive 5 min periods of preconditioning occlusion and reperfusion insults, supporting the role of these radicals in the initiation of PC. Moreover, 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. For example, it has been shown that an excess in superoxide production would deactivate NOS, resulting in reduced free NO levels and subsequent PN formation. Similarly, under certain conditions the excessively generated NO can regulate, through a negative feedback mechanism the subsequent enzymatic synthesis of NO.

When the PC dogs were subjected again to occlusion, the activity of NOS and simultaneously, the NOx levels were rapidly increased (5 min; PC3), but, in contrast to the controls, these were maintained or even further increased by the end of the 25 min occlusion. Conversely, the superoxide production during the prolonged occlusion was substantially less marked in the PC dogs than in the controls, whereas such a reduction in the NT production was not apparent. 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 availability and decreases superoxide production during a subsequent
prolonged period of occlusion.

There is some evidence coming mainly from in vitro studies that phosphorylation of eNOS at various sites (serine 1176) can rapidly modify NOS activity. Also, post-translational modifications, such as protein-protein interactions, have been suggested as important mechanisms in NOS regulation.

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. We can speculate that PC perhaps by preventing the uncoupling of NOS during ischaemia would influence the function of the enzyme. NOS primary produces NO in the presence of adequate supply of substrate and cofactors, such as tetrahydrobiopterin (BH4), NADPH, FAD and flavin mononucleotide. Among these BH4 seems to be an essential cofactor for NOS to produce NO by stabilizing NOS domains, increasing substrate affinity and inhibiting NOS-mediated superoxide production. There is also evidence that under ischaemic conditions the bioavailability of BH4 is reduced and this, rather than the inadequate substrate availability, results in uncoupling of NOS leading to superoxide rather than to NO production. In anaesthetized rabbits, PC increased BH4 levels after a 30 min coronary artery occlusion, 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.
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