Pharmacological approaches to the possible involvement of nitric oxide in ischaemia and reperfusion-induced myocardial injuries

PhD thesis

Katalin György, MD

Department of Pharmacology and Pharmacotherapy
Albert Szent-Györgyi Medical University, Szeged

Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moléculaires UMR CNRS 7034, Université Louis Pasteur, Strasbourg

1999

Summary

Nitric oxide (NO) is an endogenous protective substance involved both in the early and delayed cardioprotective effects of ischaemic preconditioning. However, there is recent evidence that NO may also play a role in myocardial injury associated with reperfusion of the ischaemic myocardium. According to a current hypothesis, peroxynitrites formed from the concomitant generation of NO and superoxide anions during reperfusion, may activate cytotoxic mechanisms, including poly(ADP-ribose) polymerase (PARP) and this leads to intracellular energy depletion and ultimately to cell death. The main objective of this thesis was to determine whether pharmacological modulation of cardiac NO, or suppression of PARP activity, can reduce injury induced by myocardial ischaemia/reperfusion.

For this purpose we examined: (1) the effects of intracoronary infusions of isosorbide-2-mononitrate (a NO donor), levcromakalin (a K_{ATP} channel opener) and nicorandil (a NO donor and K_{ATP} opener) on ventricular arrhythmias induced by ischaemia and reperfusion in anaesthetised dogs; (2) the cardioprotective effects of monophosphoryl lipid A (MLA) against ventricular arrhythmias in a dog model of ischaemia-reperfusion and in rat isolated, Langendorff perfused hearts; (3) NO production in rat isolated hearts by EPR spectroscopy following MLA treatment and the effects of MLA on vascular reactivity; (4) the possible involvement of PARP in ischaemia and reperfusion-induced myocardial injury in mice.

We showed that pharmacological agents described as NO donors (nicorandil and isosorbide-2-mononitrate), administered intracoronarily, before and throughout coronary occlusion, reduce the number of ventricular premature beats, the number of episodes of ventricular tachycardia, the incidence of ventricular tachycardia and ventricular fibrillation and increase survival following an ischaemia-reperfusion insult in anaesthetised dogs.

We have also demonstrated that MLA induces delayed cardioprotection in dogs and rats against ischaemia/reperfusion induced arrhythmias and ventricular dysfunction. In dogs, the protective effect was only slightly attenuated with aminoguanidine, but the antiarrhythmic protection was completely abolished when aminoguanidine was given toghether with meclofenamate, an inhibitor of the cyclooxygenase enzyme. In rats, the protective effect of MLA could be reversed by various iNOS inhibitors (aminoguanidine, L-NIL) when they were administered at the same time with MLA, but failed to influence the protection when these inhibitors were given just prior to occlusion. In rat hearts, MLA pretreatment increased NO levels, analysed with EPR spectroscopy. This increased formation of NO occured earlier than the manifestation of the cardioprotection but NO could not be detected when the protective effect was apparent. Furthermore, MLA did not affect the reactivity to noradrenaline of aortic rings taken from rats. There were no differences in the recovery of contractile function, heart rate or coronary flow of the hearts taken from PARP +/+ and PARP -/- mice.

Our results are consistent with the hypothesis that myocardial NO elevation before an ischaemic episode protects the heart against ischaemia and reperfusion-induced injury. Such a protection can be obtained with pharmacological agents, which donate NO, or induce iNOS. In this latter case, a delayed cardioprotection can be obtained and is probably due to the capacity of iNOS derived NO to initiate adaptative mechanisms in cardiac tissue which may exert a protective effect against ischaemia and reperfusion–induced injuries. Concerning the molecular mechanisms which underly reperfusion-induced contractile dysfunctions, it appears that PARP enzyme activity does not play an obligatory role.

Contents

List of publications

Full papers

1. **K. György**, B. Muller, A. Végh, A. L. Kleschyov, J.-C. Stoclet. Triggering role of nitric oxide in the delayed protective effect of monophosphoryl lipid A in rat heart. *Br. J. Pharmacol.* **1999**; 127: 1892-1898
2. J.C. Stoclet, B. Muller, **K. György**, R. Andriantsitohaina, A.L. Kleschyov. The inducible NO-synthase in vascular and cardiac tissue. *Eur. J. Pharmacol.* **1999**, 375: 139-155
3. A. Végh, **K. György**, J.GY. Papp, G.T. Elliott, J.R. Parratt - Delayed protection against ventricular arrhythmias by monophosphoryl lipid A in a canine model of ischaemia and reperfusion. *Eur.J.Pharmacol.* **1999**; 382: 81-90
4. B. Muller, A.L. Kleschyov, **K. György**, J.-C.Stoclet. Inducible NO synthase activity in blood vessels and heart: new insight into cell origin and consequences. *Phys. Res.* **1999**; (in press)
5. A. Végh, J. Gy. Papp, **K. György,** K. Kaszala, J. R. Parratt: Does the opening of ATP-sensitive K^+channels modify ischaemia-induced ventricular arrhythmias in anaesthetised dogs ? *Eur. J. Pharmacol.* **1997**; 333 (1): 33-38
6. A. Végh, **K. György**, J. Gy. Papp, J.R. Parratt. Nicorandil suppressed ventricular arrythmias in a canine model of myocardial ischaemia. *Eur. J. Pharmacol.* **1996**; 305:163-168

Quotable abstracts

7. **K. György**, B. Muller, A. Végh, A. L. Kleschyov, J.-C. Stoclet. Nitric oxide triggers the MLA-induced delayed cardioprotection in the rat heart. *J.Cell.Mol.Cardiol.* **1999**, 31(6):A97
8. **K. György**, B. Muller, A. Végh, A. L. Kleschyov, J.-C. Stoclet. The role of nitric oxide in monophosphoryl lipid A-induced cardioprotection in the rat heart. *Fund. Clin. Pharmacol.* **1999**, 13 (1): 292
9. **György K.**, Muller B., Kleschyov A., Végh Á., Stoclet J.-C., Papp J. Gy. A nitrogén monoxid szerepe a monophosphoryl lipid A indukálta késõi védõhatásban patkányszív modellen. Cardiologia Hungarica Suppl. **1999/2**
10. **K. György**, Á. Végh, M. A. Rastigar, J.Gy. Papp, J.R. Parratt. 2-isosorbide mononitrate reduces the consequences of ischaemia in anaesthetised dogs. *J.Cell.Mol.Cardiol* **1998**, 30: A82
11. **K. György**, Á. Végh, J.Gy. Papp, J.R. Parratt Effects of isosorbide-2-mononitrate on ischaemia and reperfusion-induced ventricular arrhythmias in anaesthetised dogs. Cardiologia Hungarica Suppl.**1998**/1
12. **K. György**, Á. Végh, J.Gy. Papp, J.R. Parratt. Opening of K_{ATP} channel with L-Cromakalim before an ischaemic episode does not modify the severity of ischaemia reperfusion-induced ventricular arrhythmias. Journal für Kardiologie **1997**, 2
13. **K. György**, A. Kis, Á. Végh, J.Gy. Papp. Haemodynamic effects of intravenous Levosimendan during oral metoprolol treatment in anaesthetised dogs. *J.Cell.Mol.Cardiol* **1997**/5

1. Introduction

Ischaemic heart disease is one of the most serious problems facing modern cardiology and remains one of the leading causes of death in many developed countries. Myocardial ischaemia, described as an imbalance between energy demands and metabolic substrate supply (including O_2) of the heart, occurs when a coronary artery is partially or fully obstructed. Ischaemia results in hypoxia and this implies depletion of high energy stores (glycogen, ATP, phosphocreatine) and the accumulation of potentially harmful anaerobic catabolites (1-4). These cellular alterations induce disturbances in the activity of ion channels and membrane pumps. In the absence of reperfusion, pronounced ischaemia inevitably leads to myocardial necrosis. While readmission of blood to the ischaemic myocardium is required to avoid the development of irreversible ischaemic damage, such reperfusion may also lead to extension of ischaemia-induced injuries (5-6). Morphological alterations, increased resting tension, increased enzyme leakage (7-8) can occur as a result of reperfusion which can also precipitate severe ventricular arrhythmias, including lethal ventricular fibrillation (9).

1.1. Nitric oxide production and its effects on the heart

Nitric oxide (NO) produces negative inotropic, chronotropic and positive lusitropic effects in the heart and decreases cardiac O_2 consumption. Besides being a potent vasodilator, NO inhibits platelet aggregation and leucocyte adhesion to the endothelium. Formation of endogenous NO from L-arginine in the presence of O_2 and NADPH, is catalyzed by a family of enzymes termed NO-synthases (NOS). In the heart, NOS enzymes have been identified in myocytes, vascular endothelial cells, endocardium, intracardiac nerves and macrophages (10). The neuronal (nNOS or NOS-1) and endothelial (eNOS or NOS-3) forms are called constitutive enzymes, because they function to maintain physiological relevant concentrations of NO, although their expression may vary. A third isoform (iNOS or NOS-2) can be induced by a variety of stimuli (11). Exogenous NO may derive from the degradation (spontaneous or enzyme-mediated) of NO donor drugs such as glycerin trinitrate, isosorbide mononitrate, sydnonimines, nicorandil, etc.

Various molecular mechanisms are responsible for the effects of NO in cardiac tissue. Activation of guanylate cyclase by NO leads to an increase in intracellular cGMP levels which, in turn, activate cyclic GMP-dependent protein-kinases, activate or inhibit cyclic nucleotide phosphodiesterases (PDE 2 and 3, respectivelly) (12) and which also interact with various proteins, including ion channels. Cyclic GMP-independent effects of NO in the heart include reactions leading to S-nitrosation of critical thiol groups of protein (13) or to the generation (via reaction with superoxide anion) of potentially cytotoxic products like peroxynitrite and hydroxyl radicals. Some of the actions of NO have an established therapeutic value (e.g. cGMP elevation in angina), whereas others are potentially dangerous e.g. cardiac hydroxyl formation during reperfusion of the ischaemic myocardium (14).

1.2. Role of NO in myocardial ischaemia-reperfusion

Although the role of NO in ischaemia/reperfusion injury has been studied intensively, because of the great variety of experimental conditions used it is still difficult to conclude whether NO is protective or injurious to the ischaemic/reperfused heart (15). A beneficial role of NO derives from those observations which showed that marked protection results from the administration of agents, which would be expected to elevate NO in cardiac tissue. These findings suggested that ischaemia-induced injury could be attributed to a reduced formation or activity of NO and that the development of reperfusion injury can be prevented with NO-replacement therapy (16). However, in some studies, blockade of NO-synthase with L-arginine analogs did not affect ischaemia and reperfusion-induced damages (17, 18). Furthermore, in several instances, these inhibitors even improved reperfusion function (19, 20, 21), suggesting that NO production might be deleterious to the reperfused heart. This detrimental effect of NO can be attributed to the enhanced formation of the potentially cytotoxic peroxynitrite (14).

1.3. Ischaemic preconditioning

An endogenous protective phenomenon of the heart termed «ischaemic preconditioning» was described by Murry and colleagues in 1986. They showed that short ischaemic periods increase the ability of the heart to tolerate the severe consequences of subsequent, prolonged

ischaemia. Preconditioning can limit myocardial infarct size (22, 23, 24), markedly reduce the incidence and severity of ventricular arrhythmias both during ischaemia (25, 26, 27) and reperfusion (28, 26, 29), enhance postischaemic functional recovery (30, 31, 32) improve metabolic disturbances that are associated with ischaemia (33) and protect the endothelium (34). These protective effects of preconditioning seem to be present in all species studied; dogs, rats, rabbits, pigs and also in humans (35). Apart from multiple, short coronary artery occlusions, preconditioning can be induced by rapid cardiac pacing (36), partial occlusion of the coronary artery (37), alterations in myocardial energy supply-demand balance (38), or by the administration of several substances („pharmacological preconditioning") such as prostacyclin (39), adenosine (40), ACE inhibitors (41), interferon χ (42), bacterial endotoxin (43) and monophosphoryl lipid A (44).

The protective effects of preconditioning appear in two distinct phases. The first phase, «classical preconditioning», occurs within minutes following the preconditioning stimulus; although this protection is extremely pronounced it is transient, lasting only for 30 min to 2 h (22). The second, "delayed phase" (45, 46, 47), also known as the «second window of protection», appears 12-24 h after preconditioning stimulus and lasts for at least 72 h.

The molecular and cellular basis for each form of protection is still uncertain, but there is strong evidence that endogenous diffusible mediators, released during preconditioning, such as adenosine, bradykinin, NO, free radicals and prostanoids are involved in both phases of this adaptative response. For example, Downey and his collegues proposed that the early release of adenosine and the consequent activation of protein kinase C (mediated trough the A1 receptor subtype) is involved in the cardioprotective effect of preconditioning in rabbits (48). There is also evidence that bradykinin-induced generation of NO (mediated by the activation of the B2 receptors) and the subsequent stimulation of cGMP synthesis (49) contributes to the antiarrhythmic effect of preconditioning in dogs. Since complex cross-talks exist between these different signalling pathways, it is difficult to describe these mechanisms in a comprehensive manner. The distal effectors of both the early and late forms of the protection are unclear. Delayed preconditioning may involve up-regulation of protective systems (anti-oxidant enzymes, heat shock proteins) (50, 51). There is also pharmacological evidence implicating an effector role for ATP-sensitive K^+ channels in both phases of protection (52, 53, 54). In normally oxygenated myocytes these channels are in a closed state but during ischaemia, when

the intracellular ATP concentration falls and ischaemic metabolites accumulate, the probability of their opening increases. This leads to loss of intracellular K^+, shortening of the action potential duration and the development of re-entry arrhythmias. On the other hand, shortening of the action potential duration may abolish arrhythmias due to abnormal impulse generation.

1.3.1. The role of NO in the first phase of preconditioning

Végh and colleagues (1992) (26) proposed for the first time that the L-arginine nitric oxide pathway is involved in the cardioprotective effects of ischaemic preconditioning. In anaesthetised dogs, the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), given either before preconditioning or just before the prolonged coronary occlusion, markedly attenuated the antiarrhythmic effects of ischaemic preconditioning. Similarly, the local, intracoronary infusion of methylene blue (an inhibitor of both L-arginine-NO synthesis and of the guanylyl cyclase enzyme) totally abrogated the protection induced by short coronary occlusions against ventricular arrhythmias (55). The antiarrhythmic effects of preconditioning induced by rapid cardiac pacing was also prevented by L-NAME administration (56). It was concluded from these results that NO, acting through the guanylyl-cyclase-cGMP system, is a mediator of the antiarrhythmic effects of classical preconditioning (26).

It has been proposed that as a result of ischaemia, the heart can release potentially protective substances (57), among which is NO. As a valid approach to therapy of ischaemia and reperfusion-induced injury it was suggested that the enhancement, by drug intervention, of those protective substances produced by the cardiovascular system would be beneficial under these conditions. To prove, or to strenghten this hypothesis it needs to be shown that, when given exogenously (e.g. locally into the coronary circulation) in amounts similar to those that might be released, these substances exert an antiarrhythmic effect. In isolated rat hearts, the administration of NO donors (glyceryl trinitrate, SIN-1) prior to the ischaemia, mimicked the protective effects of ischaemic preconditioning against reperfusion induced arrhythmias (58). However, when the administration of these drugs was restricted to the reperfusion period, the incidence and the duration of sustained ventricular fibrillation was actually increased.

Since both NO and K_{ATP} channels seem to play a role in the cardioprotective effects of ischaemic preconditioning, we were interested to analyse whether the effect of classical

preconditioning against ventricular arrhythmias induced by ischaemia-reperfusion could be mimicked pharmacologically in anaesthestized dogs. Therefore, in the first part of the study the potential contribution of NO was evaluated by comparing the effects of isosorbide mononitrate (a NO donor), levcromakalin (a K_{ATP} channel opener) and nicorandil (a NO donor and K_{ATP} opener). These drugs were administered by local intracoronary infusion prior to, and during ischaemia.

1.3.2. The role of NO in the delayed phase of preconditioning

It has been known for several years that the principal component of bacterial endotoxin, lipopolysaccharide (LPS) may protect the myocardium against ischaemic damage, an effect which occurs several hours after LPS administration and which is comparable to the late phase of preconditioning (43). Similar protection can also be induced by TNF α, a pro-inflammatory cytokine (59). The mechanism of protection induced by these agents was suggested to involve the induction of iNOS activity in various cells types (60). The involvement of iNOS induction in the delayed cardioprotective effect of preconditioning is also likely, since dexamethasone, a drug which among many pharmacological properties inhibits iNOS induction, was able to attenuate the cardioprotective effects of LPS (61). More recent reports suggest that administration of various, reasonably selective inhibitors of iNOS (aminoguanidine, S-2(aminoethyl-isothiourea)) before ischaemia, abolished the delayed protection against ventricular arrhythmias (62, 63), myocardial infarction (64, 65) and myocardial stunning (66) whether preconditioning was induced either by short coronary occlusions or by rapid cardiac pacing. These results suggest that NO derived from iNOS may play a role in delayed cardioprotection afforded by preconditioning.

The mechanisms of iNOS induction in delayed cardioprotection induced by short coronary occlusion against myocardial stunning were further investigated by Bolli and colleagues (1998) (67). They suggested that the preconditioning stimulus increases eNOS activity. NO, together with reactive oxygen species, would lead to the sequential activation of PKC, tyrosine kinase, NF-kB, which in turn would promote transcription of the iNOS gene (68).

Pharmacological induction of delayed cardioprotection is perhaps the most appealing way to eventually capitalise on the benefits of preconditioning in the clinical setting. Recently, a

relatively non-toxic LPS derivative, monophosphoryl lipid A (MLA) has been developed and examined for this purpose. The structural modifications render MLA devoid of the undesirable ability of LPS to activate neutrophils and induce diffuse coagulopathy (69), but conserve the cardioprotective properties of the parent molecule. Administered as a single dose 24h prior to the cardiac ischaemic insult, MLA reduces reperfusion damage (infarct size, ventricular arrhythmias, myocardial stunning) in dogs (70, 71, 72), rabbits (73, 74) and rats (44, 75). Indeed, MLA-induced cardioprotection was abrogated by nitric oxide synthase inhibitors (76, 77). In addition it was associated with enhanced NOS activity (assessed by citrulline assay) in the heart from MLA-treated rabbits (76) and with the expression of inducible NOS mRNA in rat hearts (77). An obligatory role for iNOS in mediating the cardioprotective effect induced by MLA was recently confirmed by pharmacological inhibition of iNOS and by using gene knockout mice (78).

Therefore in this part of the study, we wished to further evaluate the role of NO produced by iNOS in delayed protection induced by monophosphoryl-lipid A using two experimental models (rat isolated perfused hearts and anaesthetized dogs). In the rat, NO levels within cardiac tissue, and the temporal relationship between myocardial NO production and cardioprotection, were more specifically investigated. Aortic reactivity to noradrenaline after MLA treatment was also examined.

1.4. The role of NO in ischaemia-reperfusion injury

In the absence of L-arginine, the NOS enzymes produce superoxide (O_2^-). These enzymes produce no O_2^- in the presence of saturating concentrations of both L-arginine and tetra-hydrobiopterin (co-factor), but they catalyze the formation of both O_2^- and NO if the concentrations of either compound is not saturating, with the possible formation of peroxynitrites (79). It is now well accepted that reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals and hydrogen peroxide, as well as peroxynitrite, contribute to the injury resulting from reperfusion of the previously ischaemic myocardium (80, 21). The generation of ROS, either during ischaemia or reperfusion, has been directly demonstrated using electron paramagnetic resonance spectroscopy (81), chemiluminescence

(82) and spin trapping (83). Interventions which either attenuate the generation, or reduce the effects of ROS, protect the heart against ischaemia-reperfusion injury (84, 85). ROS cause cell injury by peroxidation of membrane lipids, denaturation of proteins and DNA injury. For instance, exposure of cultured cells to ROS, including peroxynitrite formed from NO and superoxide anions during reperfusion, results in strand breaks in DNA and subsequent activation of poly(ADP-ribose) polymerase (PARP) (86). PARP is a nuclear enzyme which plays an essential role in DNA repair and contributes to the efficient maintenance of genome integrity when cells are challenged by genotoxic agents. Rapid activation of this enzyme depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide (NAD), thus slowing the rate of glycolysis, electron transport and subsequently ATP formation. A decline in the intracellular levels of ATP results in severe cellular dysfunction and ultimately cell death (86). In addition to the direct cytotoxic pathway, it appears that PARP plays an important role in regulating the expression of a variety of genes including the iNOS. Indeed, PARP seems to be required in the pathway leading to the activation of NFkB-mediated gene transcription of iNOS (87).

The administration of 3-aminobenzamide, nicotinamide or several other chemically distinct inhibitors of PARP activity upon reperfusion reduce the infarct size caused by regional myocardial ischaemia and reperfusion in anaesthetized rabbits and rats (88, 89). Inhibitors of PARP activity also attenuate the contractile dysfunction of rabbit and rat isolated hearts subjected to global ischaemia and reperfusion (88, 90). In the rat heart, cardioprotection afforded by PARP inhibitors is accompanied by a preservation of high energy phosphates and cellular NAD levels, suggesting that the mechanism responsible for this cardioprotection may involve prevention of intracellular ATP depletion (90). Thus, it has been proposed that activation of PARP contributes to reperfusion injury and that pharmacological inhibition of PARP activity may be useful in the therapy of cardiac reperfusion injury.

In this work, isolated hearts from mice lacking the PARP gene were used to analyze the role of PARP in ischaemia/reperfusion-induced contractile dysfunction.

1.5. Aims of the study

The main objective of this thesis was to determine whether pharmacological modulation of cardiac NO, or suppression of PARP activity, can reduce the injuries induced by myocardial ischaemia/reperfusion. For this we analysed:

(1) Whether the intracoronary administration of NO donors prior to and during ischaemia exert protective effects against ischaemia-induced ventricular arrhythmias;

(2) The cardioprotective effects of monophosphoryl lipid A administration against ventricular dysfunction and arrhythmias induced by ischaemia and reperfusion in various species (dogs and rats). In these studies we evaluated the i.) role of NO produced by iNOS in a canine model of delayed cardioprotection induced by monophosphoryl-lipid A; ii.) the effects of monophosphoryl lipid A on vascular reactivity;

(3) The possible involvement of PARP in injuries produced by ischaemia and reperfusion.

2. Materials and methods

All animals received humane treatment in compliance with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health and local institutional policy.

2.1. Experiments performed with anaesthetised dogs

2.1.1. General preparation

Mongrel dogs, of either sex, with a body weight in excess of 17 kg (mean 26.7 ± 5.8 kg) were used in these experiments. Dogs were lightly anaesthetised with sodium-pentobarbitone (i.v.), and the anaesthesia was maintained by a mixture of chloralose and urethane (60 and 200 mg/kg i.v., respectively). Dogs were ventilated with room air using a Harvard Respirator at a rate and volume sufficient to maintain arterial blood gases and pH within normal limits (91). The temperature was measured from the oesophagus and maintained by a heating pad between 36.8 and 37.5 °C. Polyethylene catheters were inserted into the right femoral artery for monitoring arterial blood pressure, into the left ventricle (for the measurement of left

ventricular pressure and dP/dt) and into the right femoral vein (for anaesthetic administration). Blood flow was measured on both the anterior descending (LAD) and the left circumflex (LCX) branches of the left coronary artery using Doppler and electromagnetic flow probes, respectively. These flow probes were coupled to a Doppler flowmeter and to a Statham SP 2202 flowmeter. Epicardial ST-segment changes and the degree of inhomogeneity of electrical activation were measured from the left ventricular wall distal to the occlusion site using a composite electrode described previously (Végh et al. 1992). This gives a summarised recording of R-waves from 30 epicardial measuring points. In the adequately perfused and oxygenated myocardium all sites are activated virtually simultaneously, resulting in a large single spike. However, following occlusion, widening and fractionation of the summarized R-waves occurs, indicating that 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. All this parameters together with a limb lead electrocardiogram, systemic arterial (systolic, diastolic, mean), left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures and LV dP/dt were recorded. (Figure 1).

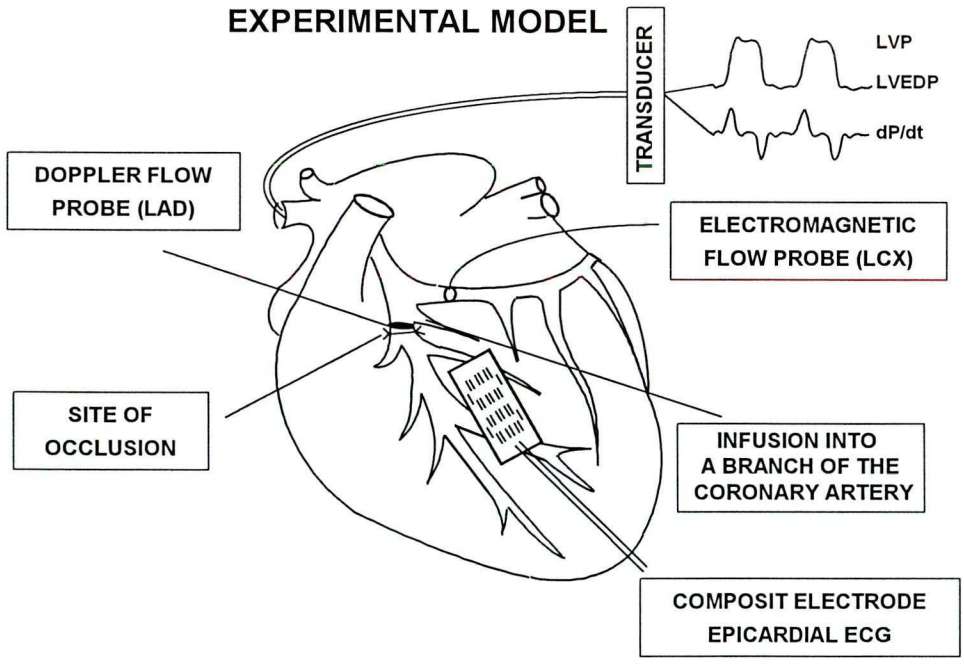

Figure 1. The experimental model in anaesthetised dogs for the measurement of haemodynamic parameters, changes in epicardial ST-segment elevation and degree of inhomogeneity of electrical activation and for the assessment of ventricular arrhythmias.

A thoracotomy was performed at the fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) prepared for the occlusion just proximal to the first diagonal branch. A side branch of this artery, proximal to the occlusion, was catheterised for the intracoronary administration of nicorandil, levcromakalim or isosorbide-2-mononitrate (Figure 1).

2.1.2 Assessment of ventricular arrhythmias and the measurement of the area at risk

Ventricular arrhythmias were assessed according to the Lambeth conventions (92), modified as described previously (91) In brief the total number of ventricular premature beats (VPBs), the incidence and number of episodes of ventricular tachycardia (defined as a run of four or more VPBs at a rate faster than the resting heart rate), and the incidence of ventricular fibrillation (VF) were assessed during occlusion. The reperfusion period was followed for 10 minutes; only the incidence of reperfusion-induced ventricular fibrillation was assessed. Survival indicates those dogs which were in sinus rhythm without any ischaemic alterations on the epicardial or limb lead ECGs for at least 10 minutes after reperfusion.

At the end of the experiments the heart was excised and the LAD suture was again tightened, and methylene blue dye was infused into the occluded coronary artery distal to the site of occlusion. Area at risk was expressed as a percentage of the mass of the left ventricular wall together with the septum.

2.1.3. Experimental protocol

2.1.3.1. Protocol for evaluating the effects of intracoronary infusion of nicorandil, levcromakalim and isosorbide-2-mononitrate in anaesthetised dogs

Nicorandil (2.5 $\mu g\ kg^{-1}min^{-1}$) and isosorbide-2-mononitrate (3 $\mu g\ kg^{-1}min^{-1}$) were administered by means of slow infusion (0.5 ml min^{-1}) started 20 min before the occlusion and maintained throughout the entire occlusion period. Levcromakalim was slowly infused in a total dose of 3 $\mu g\ kg^{-1}$ over a 30 min period before the coronary occlusion. The coronary artery was then occluded for 25 min. Twenty dogs, which were given saline infusion served as controls (Figure 2).

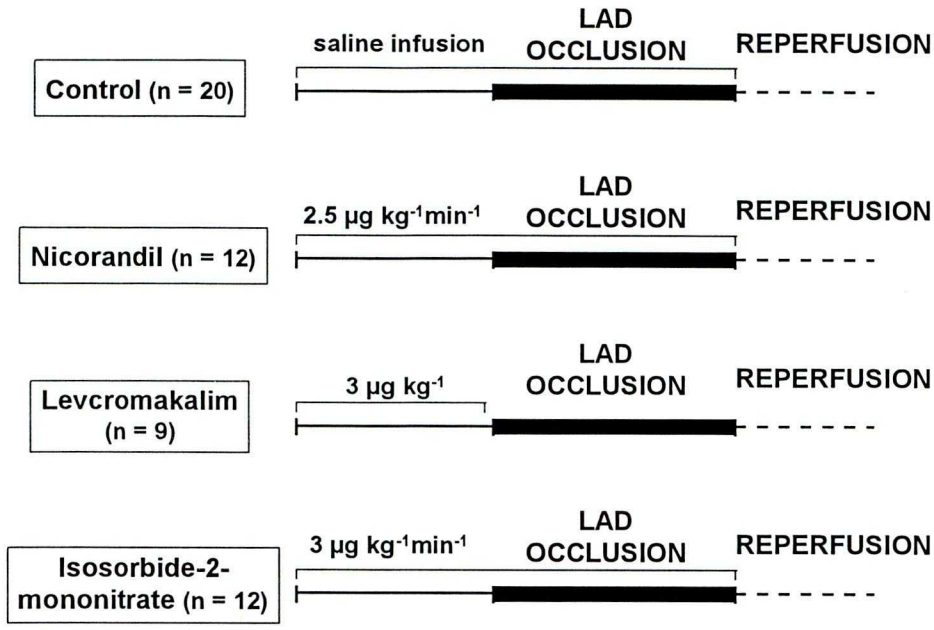

Figure 2. Experimental protocol for evaluating the effects of nicorandil, levcromakalim and isosorbide-2-mononitrate in anaesthetised dogs subjected to ischaemia and reperfusion

2.1.3.2. Protocol for evaluating the delayed protective effects of MLA

MLA was given in doses of 100 μg kg^{-1} (n = 13) and 10 μg kg^{-1} (n = 8) by intravenous injection, 24 h prior to anaesthesia and coronary artery occlusion. Sixteen vehicle-treated dogs served as controls. In two additional groups dogs treated with 100 μg kg^{-1} MLA, either aminoguanidine (50 mg kg^{-1}), a reasonably selective inhibitor of iNOS activity, or aminoguanidine and meclofenamate (2 mg kg^{-1}), an inhibitor of cyclooxygenase were given 30 and 20 min prior to coronary artery occlusion. The doses of aminoguanidine and meclofenamate were selected according to previous studies. For example, 50 mg kg^{-1} completely abolished the protection against ventricular arrhythmias resulting from cardiac pacing, 24 h before an ischaemia-reperfusion insult (62). Similarly, the 2 mg kg^{-1} dose of meclofenamate markedly reduced the antiarrhythmic effect of preconditioning, induced by brief coronary artery occlusion, in anaesthetized dogs (93).

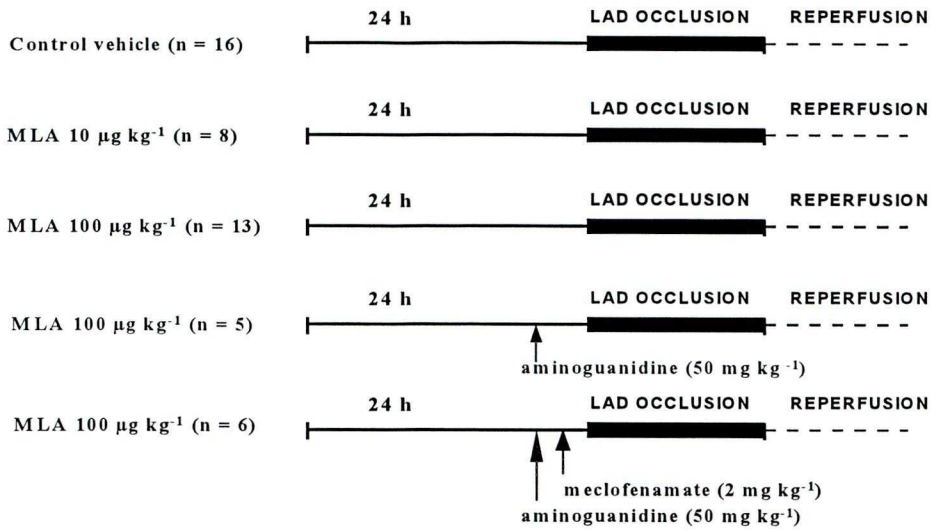

Figure 3. Experimental protocol for evaluating the delayed protective effect of MLA in anaesthetised dogs

2.2. In vitro studies performed in isolated perfused hearts of rats and of mice

2.2.1. Isolated perfused heart preparations from rats and mice

Male Wistar rats (10-12 weeks old, 280-300 g), pretreated 8 or 24 h previously with MLA, were anaesthetised with sodium pentobarbitone (60 mg kg^{-1} i.p.) and heparinized (500 U kg^{-1} i.p.). Hearts were excised, placed in ice-cold Krebs-Henseleit buffer and perfused according to Langendorff, at 37°C and pH 7.4, with Krebs-Henseleit solution containing (mmol l^{-1}) NaCl 118, $NaHCO_3$ 24, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.7 and glucose 10, gassed with 95 %O_2/ 5%CO_2. Perfusion pressure was constant and equivalent to 100 cm H_2O. Under these conditions, the range of coronary flow was about 12-15 ml min^{-1}. The heart was placed in a semi-closed, circulating water warmed (37°C) air chamber. A water filled latex balloon, connected to a pressure transducer was inserted into the left ventricle for the measurement of left ventricular pressure (LVP). The left ventricular end-diastolic pressure (LVEDP) was set to 5 mm Hg by adjusting the volume of the balloon. This balloon volume was maintained over the entire period of the experiment. Cardiac parameters were monitored continuously and included: left ventricular developed pressure (LVDP: difference between left-ventricular peak systolic pressure and end-diastolic pressure), positive and negative first derivatives of LVDP ($\pm dP/dt_{max}$). An epicardial ECG was recorded throughout the experimental period by two electrodes attached directly to the heart.

PARP (+/+) (n=6) and PARP (-/-) (n=6) mice (10-12 weeks, 23-25 g) anaesthetised and heparinised, were used in these experiments. After rapid removal, the hearts were cannulated under iced Krebs-Henseleit buffer (at 4°C) and transferred to the perfusion apparatus. The perfusion solution consisted of modified Krebs-Henseleit buffer. A silk suture was placed through the apex of the left ventricle and connected to a rigid rod which was in direct continuity with a force transducer. The diastolic tension of the suture was adjusted to 1 g during stabilisation period of the heart. The coronary flow was recorded by collecting the effluent over a period of 60 sec. Recovery of contractile force, coronary flow rate and heart rate were measured.

2.2.2.Experimental protocol for the studies performed in isolated perfused hearts of rats and mice

Rats were randomly assigned to one of the following groups as illustrated in Table 1.

In vivo treatment	**Langendorff perfusion**		
I. vehicle control			
II. MLA 0.5 mg^{-1} kg^{-1} 8h			
III. MLA 0.5 mg^{-1} kg^{-1}24h			
IV. MLA 2.5 mg^{-1} kg^{-1} 8h			
V. MLA 2.5 mg^{-1} kg^{-1} 24h	**equilibration**	**ischaemia**	**reperfusion**
VI. vehicle control + AG/24h	30 min	30 min	30 min
VII. MLA 2.5 mg^{-1} kg^{-1} + AG/24h			
VIII. vehicle control + L-NIL/24h			
IX. MLA 2.5 mg^{-1} kg^{-1} + L-NIL/24h			
*X. vehicle control	+ AG		
*XI. MLA 2.5 mg^{-1} kg^{-1} 24h	+ AG		

*Table 1. Experimental protocol for the studies performed in isolated perfused rat hearts. The animals were randomly assigned to one of the following groups: vehicle controls (group I, n = 8); 8 h pretreatment with MLA 0.5 mg kg^{-1} (group II, n = 8); 24 h pretreatment with MLA 0.5 mg kg^{-1} (group III, n = 8); 8 h pretreatment with MLA 2.5 mg kg^{-1} (group IV, n = 8); 24 h pretreatment with MLA 2.5 mg kg^{-1} (group V, n = 8). Further experiments were performed in the following groups, pretreated for 24 h with: aminoguanidine (2 x 300 mg kg^{-1}) + vehicle control (group VI, n = 6); aminoguanidine (2 x 300 mg kg^{-1}) + MLA (2.5 mg kg^{-1}) (group VII, n = 6); L-NIL (10 mg kg^{-1}) + vehicle control (group VIII, n = 4); L-NIL (10 mg kg^{-1}) + MLA (2.5 mg kg^{-1}) (group IX, n = 4); vehicle control (group X, n = 6); MLA (2.5 mg kg^{-1}) (group XI, n = 6). Note *: In these groups, isolated hearts were perfused during the 30 min period that preceded the onset of ischaemia with aminoguanidine (150 μM).*

The concentration of aminoguanidine used in this study was selected as being effective to inhibit iNOS activity in cells or tissue (94, 95). In addition, it corresponded to the highest concentration that did not significantly affect ventricular parameters in hearts removed from vehicle-control animals. Since there was no significant difference in the measured parameters in rats, treated with the vehicle of MLA given 8 or 24h previously (n = 4 in each group), the control vehicle group (group I) includes animals treated with the vehicle of MLA for 8 and 24 h (n = 8). Aminoguanidine (300 mg kg^{-1}, dissolved in saline) was given (i.p.) at the same time as MLA (or vehicle) and 6 h later; L-NIL (10 mg kg^{-1}, dissolved in saline) was given at the same time as MLA (or vehicle). This protocol was selected on the basis of preliminary experiments, which showed that such treatment was able to abolish the EPR signal for NO observed 8 h after MLA injection.

In both cases (rats and mice) hearts were equilibrated for 30 min, then subjected to 30 min global, no-flow ischaemia at 37°C, followed by 30 min reperfusion

2.3. NO spin trapping and electron paramagnetic resonance (EPR) studies

NO spin trapping was performed *in vivo* using Fe-diethyldithiocarbamate (DETC) as a trap following *ex vivo* EPR spectroscopy of the tissue (96, 97). Vehicle or MLA-pretreated rats were injected with the spin trap components, DETC (500 mg kg^{-1}; i.p.) and $FeSO_4.7H_2O$ plus Na-citrate (50 and 250 mg kg^{-1} respectively; s.c.). After 30 min, the animals were decapitated, the hearts were isolated and frozen in liquid nitrogen. EPR spectra were recorded on a Bruker 300E spectrometer with a Dewar flask (Wilmad, USA) at 77K. EPR settings were 10 mW microwave power, 0.61 mT amplitude modulation, 9.47 GHz microwave frequency and 100 kHz modulation frequency. NO spin trapping data was quantified by comparison with paramagnetic signal of dinitrosyl iron-thiosulphate complex of known concentration (97).

2.4. Aortic ring preparations

The thoracic aorta was removed from vehicle or MLA-pretreated rats, cleaned of connective and fat tissues and cut in rings (2 to 3 mm length). In some rings, endothelium was removed by gently rubbing the intimal surface with curved forceps. Rings were mounted under a passive tension of 2 g in organ bath filled with a Krebs solution continuously kept at 37°C and bubbled with 95% O_2 and 5% CO_2. Tension was measured with an isometric force transducer. After a 30 min equilibration period, addition of cumulative concentrations of noradrenaline (NA, 0.1 nM to 1 μM) was performed in the absence or presence of L-N^G-nitroarginine methyl ester (L-NAME, 300 μM, added 15 min before NA). After completion of the concentration-response curve to NA (in rings untreated with L-NAME), acetylcholine (1 μM, to assess the presence or the absence of functional endothelium) or L-arginine (1 mM) was added.

2.5. Drugs

MLA was a kind gift of Ribi ImmunoChem Research Inc. (Montana, USA), pentobarbital was provided by Sanofi Santé Animale (Libourne, France) and L-NIL by Alexis Corporation (Läufelfingen, Switzerland). All the other drugs were obtained from Sigma (Grenoble, France).

2.6. Statistical analysis

All data are expressed as mean ± s.e.mean. In experiments performed in dogs the differences between groups were compared by Student's t-test corrected for multiple comparisons (two way ANOVA), or, for arrhythmias, by the Mann-Whitney U test. In experiments performed in rats one way analysis of variance was carried out to test for differences between the groups. If differences were established, the values of the drug-treated groups were compared wih those of the corresponding control group by a Dunnett's *post hoc* test. For comparison of incidences of arrhythmias (ventricular tachycardia, ventricular fibrillation and survival from the combined occlusion-reperfusion insult) the Fisher exact

probability test was used. Differences between groups were considered significant when P was < 0.05.

3. Results

3.1. Evaluation of the effects of nicorandil, levcromakalim and isosorbide-2-mononitrate in anaesthetised dogs

3.1.1. The haemodynamic effects of intracoronary nicorandil, levcromakalim and isosorbide-2-mononitrate

These data are summarised in Table 1. Intracoronary infusion of levcromakalim or isosorbide-2-mononitrate resulted in a slight reduction in mean arterial blood pressure. There was no significant change in left ventricular dP/dt, LVEDP or heart rate. Nicorandil in this dose had no substantial effect on these haemodynamic parameters.

Table 2.	nicorandil		levcromakalim		isosorbide-2-mononitrate	
	pre-drug	5 min post-drug	pre-drug	5 min post-drug	pre-drug	5 min post-drug
Arterial blood pressure						
systolic (mmHg)	117±9	114±9	113±7	101±9	122±3	107±4*
diastolic (mmHg)	74±5	70±5	74±5	63±4*	84±3	76±3*
mean (mmHg)	88±6	85±6	87±5	76±5*	96±2	86±3*
LVSP (mmHg)	135±9	132±10	130±4	127±4	113±5	103±5*
LVEDP (mmHg)	5±0.3	5±0.4	7±1	9±1	6±1	5±1
LVdP/dtmax (+ve;mmHg/s)	2694±251	2803±297	2667±203	2789±189	2625±229	2721±293
LVdP/dtmax (-ve;mmHg/s)	1915±271	2035±330	2403±128	2744±156	2829±296	2831±328
Heart rate (beats/min)	160±10	167±10	134±7	137±8	130±6	132±6

*$P < 0.05$

Table 2. Haemodynamic effects of nicorandil, levcromakalim and isosorbide-2-mononitrate infusion in anaesthetised dogs

The most pronounced haemodynamic effect of all three drugs was an increase in blood flow in both the anterior descending and circumflex branches of the left coronary artery. This was

more pronounced in the anterior descending branch (which was the artery into the drugs were infused) as illustrated in Table 3.

Table 3.	nicorandil		levcromakalim	
	pre-drug	post-drug	pre-drug	post-drug
LAD diastolic coronary blood flow (cm sec^{-1})	31 ± 5	35 ± 6*	18 ± 4	25 ± 5*
LCX diastolic coronary blood flow (ml min^{-1})	120 ± 12	132 ± 14*	100 ± 7	108 ± 9*
LAD diastolic coronary resistance (mmHg cm^{-1} sec^{-1})	1.6 ± 0.1	1.2 ± 0.1	4 ± 0.2	2.7 ± 0.1*
LCX diastolic coronary resistance (mmHg ml^{-1} min^{-1})	0.64±0.02	0.56±0.02*	0.76±0.03	0.62±0.02*

Table 3. Changes in diastolic coronary blood flow in the anterior descending (LAD) and circumflex (LCX) branches of the left coronary artery before and after the intracoronary administration of nicorandil and levcromakalim.

After 5 min of isosorbide-2-mononitrate infusion diastolic coronary vascular resistance decreased slightly but significantly (from 0.64 ± 0.06 to 0.60 ± 0.05 mmHg $ml^{-1}min^{-1}$; $P < 0.05$), and especially just prior to coronary artery occlusion (e.g. 0.57 ± 0.04 mmHg $ml^{-1}min^{-1}$, $P < 0.05$ at 20 min). This decrease in resistance was due both to an increase in coronary blood flow (of 12 % from the control (diastolic) flow of 139 ± 11 ml min^{-1}) and to a reduction in coronary perfusion pressure.

Administration of nicorandil and isosorbide-2-mononitrate was without effects on epicardial ST-segment or the degree of inhomogeneity of electrical activation. However levcromakalim infusion resulted in a consistent and significant increase in epicardial ST-segment. This elevation of epicardial ST-segment started almost immediatly after the commencement of the levcromakalim infusion (Figure 4).

3.1.2. Haemodynamic changes after coronary artery occlusion in dogs treated with nicorandil, levcromakalim and isosorbide-2-mononitrate

The haemodynamic changes resulted from coronary artery occlusion are summarised in Table 4. In control dogs coronary artery occlusion resulted in significant reductions in arterial blood pressure, positive and negative $LVdP/dt_{max}$ and a marked increase in LVEDP. The heart rate was unchanged. Similar changes were observed following coronary artery occlusion in those dogs in which nicorandil, levcromakalim or isosorbide-2-mononitrate was infused before the occlusion, except that the increase in LVEDP was less pronounced in the nicorandil and

in the isosorbide-2-mononitrate-treated groups than in the control dogs or in the dogs treated with levocromakalim, probably indicating a nitrate-like effect on capacitance vessels.

Table 4.	control		nicorandil	levcromakalim	isosorbide-2-mononitrate
	pre-occl.	post-occl.	post-occl.	post-occl.	post-occl.
Arterial blood pressure					
systolic (mmHg)	126±5	109±5*	97±9*	96±9	104±5*
diastolic (mmHg)	85±4	72±4*	62±5*	61±4	72±3*
mean (mmHg)	99±5	94±4*	74±6*	73±5	83±4*
LVSP (mmHg)	138±7	117±7*	114±9*	112±4	100±5*
LVEDP (mmHg)	5.6±0.4	22.2±1.9*	17±1.3*	28±3*	10±1*
LVdP/dtmax (+ve; mmHg/s)	3027±355	1809±319*	2358±254*	2232±249*	2358±222
LVdP/dtmax (-ve; mmHg/s)	2269±271	1698±266*	1816±332	1512±164*	2547±264
Heart rate (beats/min)	146±5	147±5	167±13	136±10	132±6

*$P < 0.05$

Table 4. Haemodynamic changes following coronary artery occlusion in control dogs and in dogs treated with nicorandil, levcromakalim, isosorbide-2-mononitrate and controls

3.1.3. Severity of myocardial ischaemia following nicorandil, levcromakalim and isosorbide-2-mononitrate administration

The severity of myocardial ischaemia was assessed by changes in epicardial ST-segment and by the degree of inhomogeneity of electrical activation in control dogs and in dogs given nicorandil, levcromakalim and isosorbide-2-mononitrate. These results are illustrated in Figures 4 and 5. In control dogs coronary artery occlusion resulted in significant ST-segment elevation (Figure 4) recorded from epicardial electrodes, and a marked increase in the degree of inhomogeneity of electrical activation (Figure 5), both measured within the area supplied by the occluded vessel. Both nicorandil and isosorbide-2-mononitrate markedly reduced the epicardial ST-segment elevation (Figure 4).

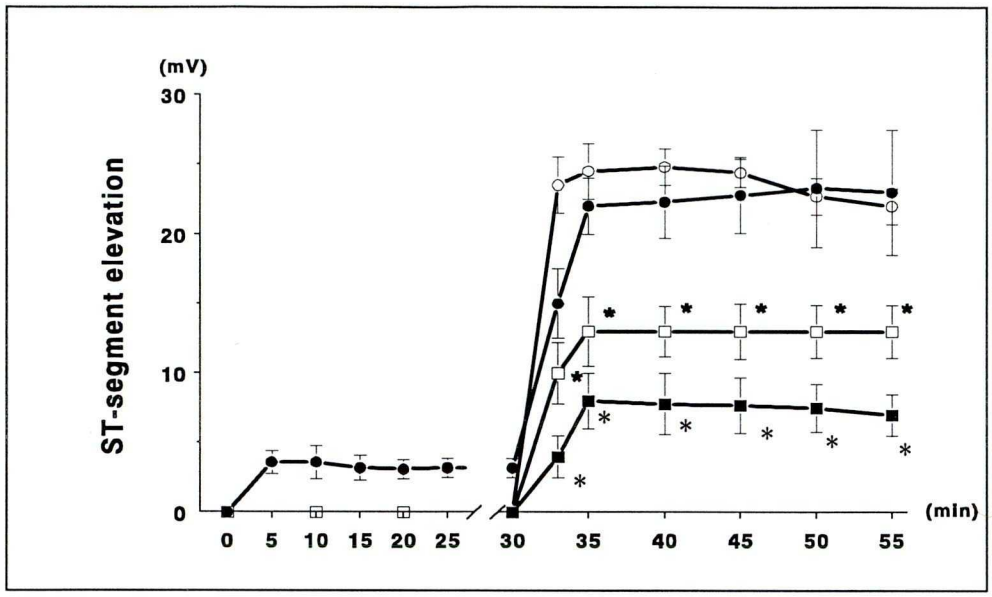

Fig. 4. Changes in ST-segment (mV), obtained from epicardial electrodes during the infusion of nicorandil (open squares), levcromakalim (filled circles) isosorbide-2-mononitrate (filled squares) and of saline control dogs (open circles) during a 25 min coronary artery occlusion.

Similarly, the degree of inhomogeneity of electrical activation was significantly reduced in those dogs in which nicorandil or isosorbide-2-mononitrate was infused (Figure 5). In contrast, the severity of myocardial ischaemia resulting from coronary artery occlusion was not substantially modified by the prior administration of levcromakalim (Figure 4 and 5).

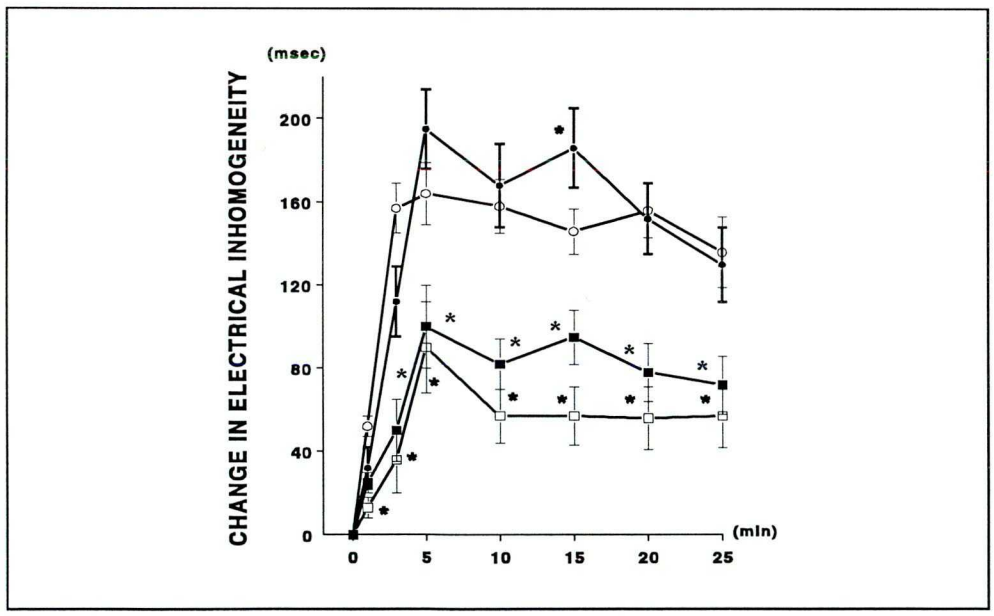

Fig. 5. Changes in the degree of inhomogeneity of electrical activation (ms) during a 25 min coronary artery occlusion in saline control dogs (open circles dogs) and in dogs given nicorandil (open squares), levcromakalim (filled circles) or isosorbide-2-mononitrate (filled squares).

3.1.4. Effects of nicorandil, levcromakalim and isosorbide-2-mononitrate on the incidence and severity of ischaemia-reperfusion induced ventricular arrhythmias

Usually in dogs, subjected to a 25 min occlusion of the LAD marked ventricular ectopic activity results. There are a great number of ventricular premature beats (VPBs 490 ± 74) and a high incidence of VT (80 % of the animals exhibited VT with a mean of 5.9 ± 1.7 episodes per dog) and VF (47 %). All the dogs that survived the 25 min occlusion period fibrillated within 1-2 min of reperfusion. Thus, in the control group no dog survived the combined ischemia- reperfusion insult. In contrast, the severity of ventricular arrhythmias was markedly reduced in those dogs in which nicorandil or isosorbide-2-mononitrate was infused intracoronarily. Thus, compared to the controls, the number of VPBs was markedly suppressed in the presence of nicorandil (163± 87) or isosorbide-2-mononitrate (62 ± 35, $P < 0.05$). Similarly there were fewer episodes of VT over the 25 min occlusion period (1.6 ± 0.6, 0.7 ± 0.4, respectively $P < 0.05$), and lower incidences of ventricular tachycardia (25 %) and ventricular fibrillation (17%) in those dogs which were treated with isosorbide-2-mononitrate. In dogs treated with nicorandil the incidence of ventricular fibrillation was also lower (33 %). Survival from the combined ischaemia-reperfusion insult was significantly increased from 0 % in the controls to 42 % and 50 % in the nicorandil and isosorbide-2-mononitrate treated dogs, respectively ($P < 0.05$).

In contrast, levcromakalim does not reduce the severity of ventricular arrhythmias resulted from a 25 min occlusion of the LAD. The number of VPBs was the same as in the controls, and 56 % of the dogs fibrillated during the occlusion period. In this group 67 % of the dogs exhibited VT and the number of episodes of ventricular tachycardia was 15 ± 7. This increase in the number of VT episodes resulted from that fact that in 1 out of these 9 dogs the number of VT-episodes was more than 60. Although in this group 2 dogs out of nine survived reperfusion, this was not significantly different from the controls.

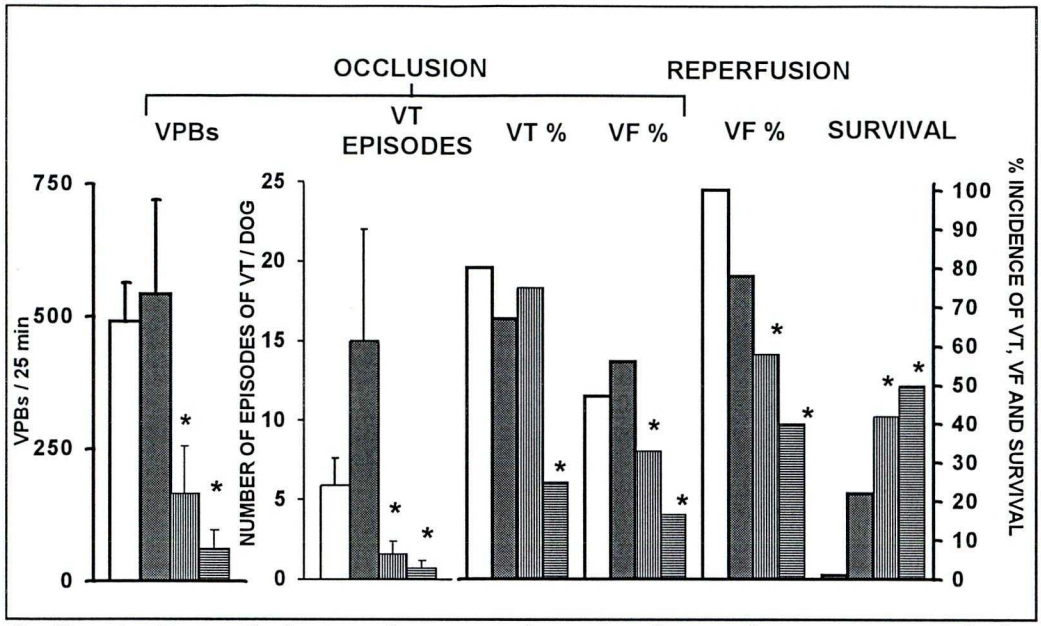

Figure 6. Ventricular arrhythmias during occlusion and reperfusion in control dogs (open histograms), in dogs administered levcromakalim (cross-hatched histograms), nicorandil (vertically shaded histograms) and isosorbide-2-mononitrate (horizontally shaded histograms). VPBs are the total number of ventricular premature beats during occlusion period, VT = ventricular tachycardia, VF = ventricular fibrillation. Survival from the combined ischaemia-reperfusion insult is also illustrated. * $P < 0.05$ compared to the controls.

3.2. Evaluation of the delayed cardioprotective effect of MLA

3.2.1. Effects of MLA on haemodynamic parameters

The administration of MLA had little effect on any haemodynamic parameter measured, under anaesthesia, 24 h later. There were no significant differences between MLA-treated and control dogs in arterial blood pressure, heart rate, LVEDP and + $LVdP/dt_{max}$. These results are summarised in Table 5.

Table 5.	vehicle control	MLA 10 μg/kg	MLA 100 μg/kg
Arterial blood pressure			
systolic (mmHg)	121±8	129±7	114±7
diastolic (mmHg)	81±7	94±6	78±5
mean (mmHg)	94±5	106±7	90±5
LVEDP (mmHg)	5±1.2	5.8±0.5	7.3±0.9
LVdP/dtmax (+ve; mmHg/s)	2753±353	2182±188	3135±196
Heart rate (beats/min)	163±8	154±4	139±2

Table 5. Haemodynamic changes resulting from coronary artery occlusion 24 h after MLA administration

There was no significant difference between the groups in the haemodynamic effects of coronary artery occlusion, which were similar to those previously described in detail (Végh et al. 1992). In the high dose MLA group there were slight decreases in mean arterial pressure (90 ± 5 to 86 ± 4 mm Hg; $P < 0.05$), and in left ventricular dP/dt_{max} (from 3135 ± 196 to 2923 ± 293 mm Hg s^{-1} (positive) and from 3042 ± 141 to 2792 ± 222 mm Hg s^{-1} (negative) and increases in heart rate (from 152 ± 8 to 164 ± 8 beats min^{-1}) and in LVEDP (from 5.3 ± 1.1 to 18.8 ± 1.6 mm Hg; $P < 0.01$).

3.2.2. Effects of aminoguanidine and meclofenamate administration on haemodynamic parameters

Aminoguanidine, given in a dose of 50 mg kg^{-1}, 30 min prior to coronary artery occlusion in five dogs treated with 100 μg kg^{-1} MLA 24 h previously, significantly increased arterial blood pressure (from 119 ± 11 to 135 ± 12 mm Hg systolic, from 86 ± 8 to 96 ± 9 mm Hg diastolic, and from 97 ± 9 to 109 ± 10 mm Hg mean; $P < 0.05$) and positive dP/dt_{max} (from 3467 ± 248 to 4055 ± 246 mm Hg s^{-1}; $P < 0.05$) without substanciallly influencing the other haemodynamic parameters. Similarly, in dogs treated with MLA (100 μg kg^{-1}, 24 h before the occlusion), but given aminoguanidine together with meclofenamate prior to occlusion, the only significant changes were increases in arterial blood pressure (from 133 ± 6 to 156 ± 6 mm Hg systolic, from 82 ± 5 to 121 ± 7 mm Hg diastolic and from 107 ± 4 to 133 ± 7 mm Hg mean; $P < 0.05$) and in positive dP/dt_{max} (from 2673 ± 288 to 3305 ± 280 mm Hg s^{-1}; $P < 0.05$). Occlusion of the left anterior descending coronary artery resulted in similar haemodynamic changes in all these groups.

3.2.3. Effects of MLA on ventricular arrhythmias and on the indices of ischaemia severity

As described previously, coronary artery occlusion in the control dogs results in severe ventricular arrhythmias. The distribution and time course of the various type of arrhythmias in each of the dogs are shown in Figure 7. Eight of the sixteen control dogs fibrillated during the occlusion period between 15 and 25 min, and nearly all the dogs had several episodes of VT. In the control group only one of the sixteen dogs survived the ischaemia-reperfusion insult.

The summarised effects of coronary artery occlusion on arrhythmia severity are shown in Figure 7.

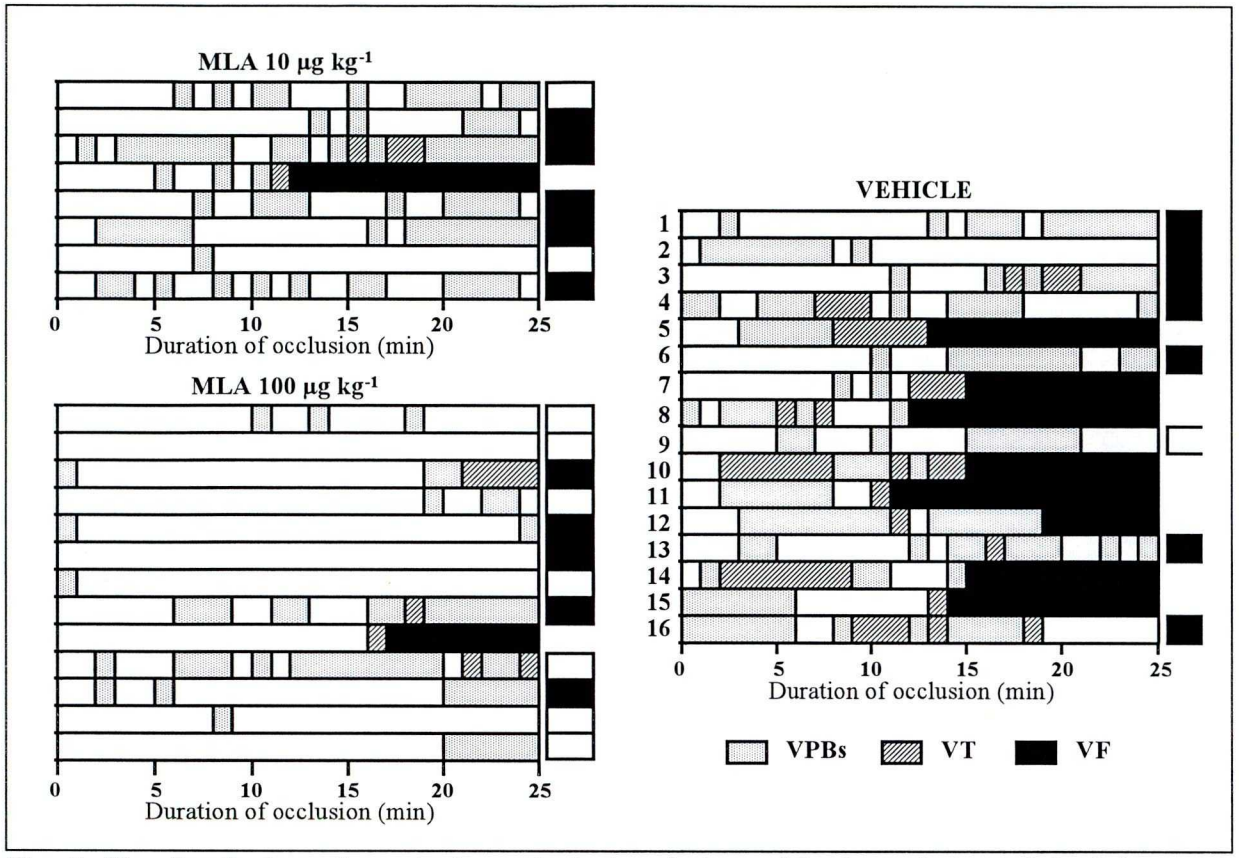

Fig. 7. The distribution of ventricular arrhythmias during a 25 min occlusion of the left anterior descending coronary artery (LAD) in anaesthetised dogs treated with MLA (10 and 100 μg/kg, 24 h prior to occlusion) or the appropriate vehicle controls. The figure shows the occurence of ventricular arrhythmias presenting as premature (ectopic) beats (VPBs: stippled bars), ventricular tachycardia (VT: cross hatched bars) and ventricular fibrillation (VF: black bars).

Prior treatment with MLA, at either dose level, markedly reduced the ischaemic changes (Figures 10 and 11) and the severity of ventricular arrhythmias that occurred both during ischaemia and reperfusion (Figures 7 and 8). There was a marked reduction in the number of VPBs, which occurred during the occlusion period (from 315 ± 84 cp to 89 ± 60 and 53 ± 23 in dogs treated with 10 and 100 μg kg^{-1}, respectively; $P < 0.05$), in the occurrence of VT (from 75 % to 25 %) and in the number of the episodes of VT (from 13.4 ± 4.9 episodes per dog to less than 2 episodes in dogs given MLA). There was also a significant reduction in the incidence of VF during occlusion with the higher dose of MLA (from 50 % to 7 %). Five dogs from the thirteen given the higher dose of MLA, had less than six ectopic beats during the whole of the occlusion period and two of them had no ectopic beats whatsoever (Figure 7). VF during reperfusion was also significantly reduced by the 100 μg kg^{-1} MLA and survival from the combined ischaemia-reperfusion insult was increased.

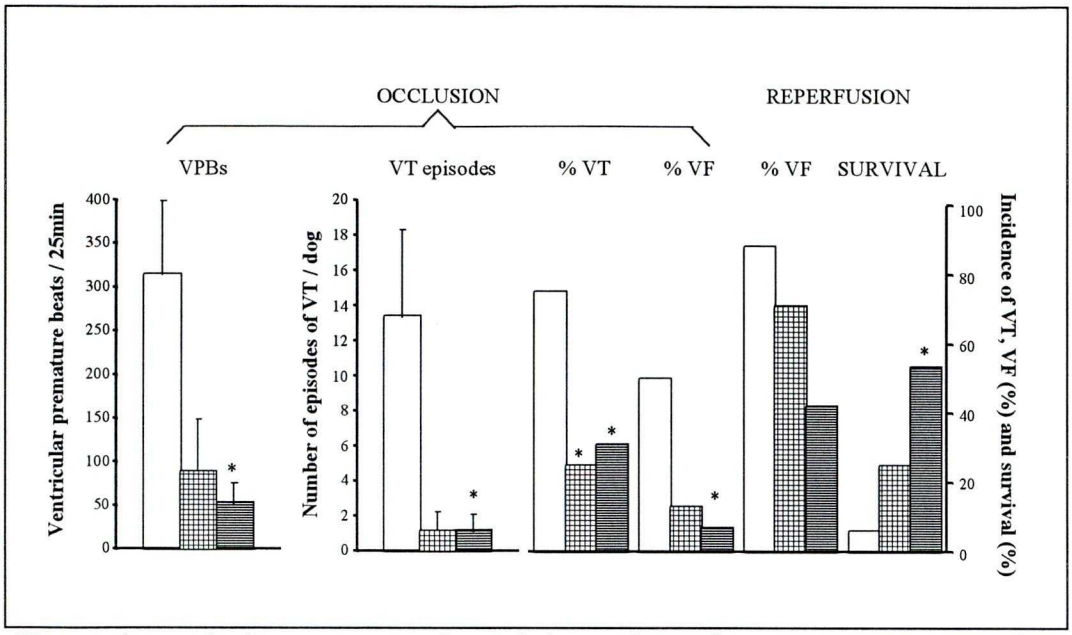

*Fig. 8. Ventricular arrhythmias in anaesthetised dogs subjected to coronary artery occlusion after treatment with MLA 10 μg kg^{-1} (cross shaded histograms), 100 μg kg^{-1} (vertically shaded histograms) or with the appropiate vehicle (open histograms). * $P < 0.05$ compared to the vehicle controls.*

Inhibition of iNOS activity with aminoguanidine only attenuated the protective effects of MLA. However, after dual blockade of both the iNOS activity and cyclooxygenase enzymes with aminoguanidine and meclofenamate, the MLA-induced delayed protection against arrhythmias (Figure 9) was completely abolished.

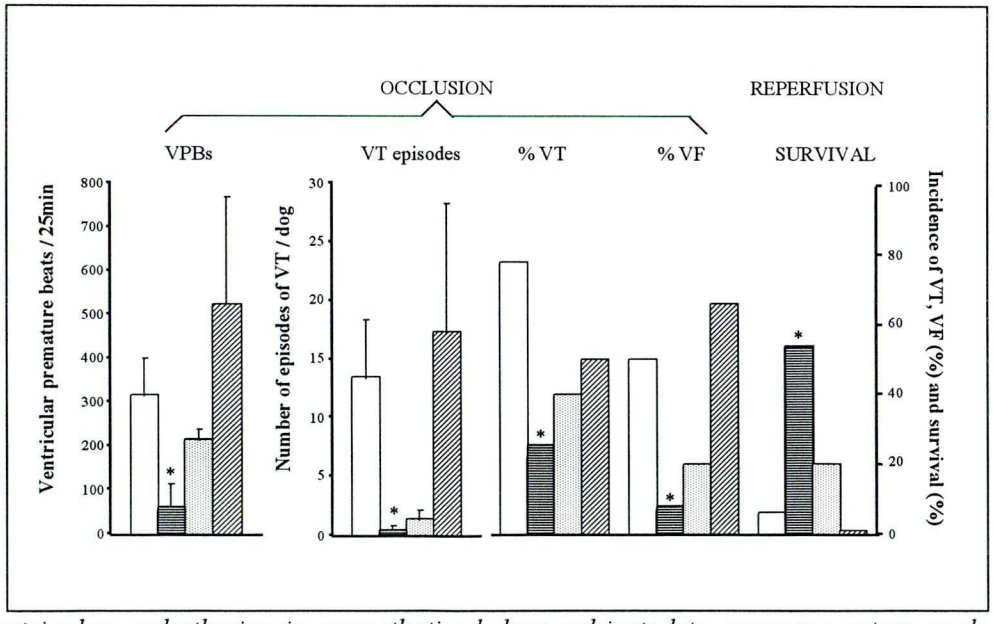

*Fig. 9. Ventricular arrhythmias in anaesthetised dogs subjected to coronary artery occlusion after treatment with MLA 100 μg kg^{-1} (vertically shaded histograms), and when given either aminoguanidine (stippled histograms) or aminoguanidine and meclofenamate (hatched histograms), as well as the appropriate vehicle (open histograms). * $P < 0.05$ compared to the vehicle controls.*

Treatment with MLA also markedly reduced the indices of ischaemia severity. Thus, in the presence of MLA, the epicardial ST-segment elevation (Figure 10a) and changes in the degree of inhomogeneity of electrical activation (Figure 11a) was markedly less than in the untreated controls. Aminoguanidine alone, or in combination with meclofenamate, attenuated or abolished the protective effects of MLA. This is illustrated in Figures 10b, 11b. There was no difference between the area at risk in any of the groups.

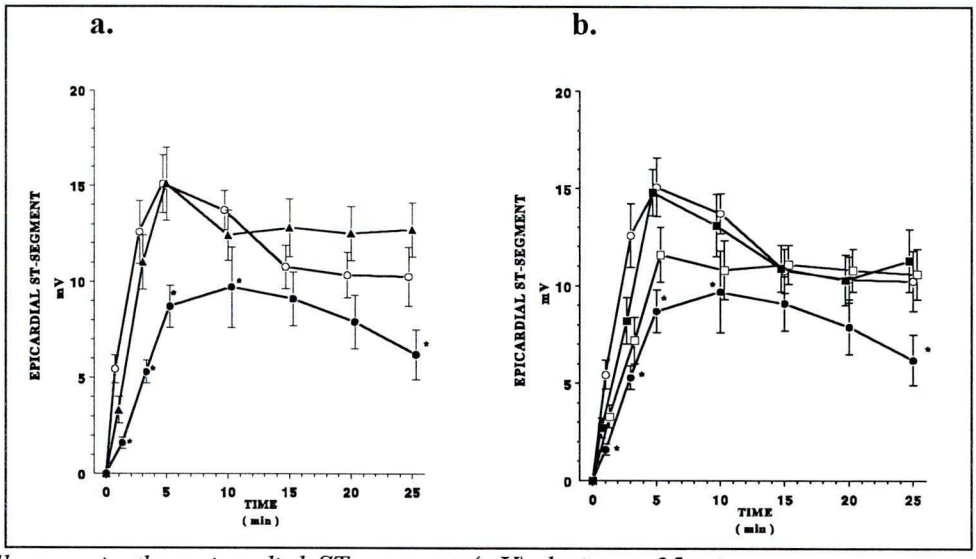

*Fig. 10. Changes in the epicardial ST-segment (mV) during a 25 min coronary artery occlusion in dogs given 24 h previously, MLA 100 μg kg^{-1} (filled circle), 10 μg kg^{-1} (filled triangles) or 100 μg kg^{-1} MLA and then either aminoguanidine (open squares) or aminoguanidine and meclofenamate (filled squares), as well as the appropriate vehicle control (open circles). * P < 0.05 compared to the vehicle controls.*

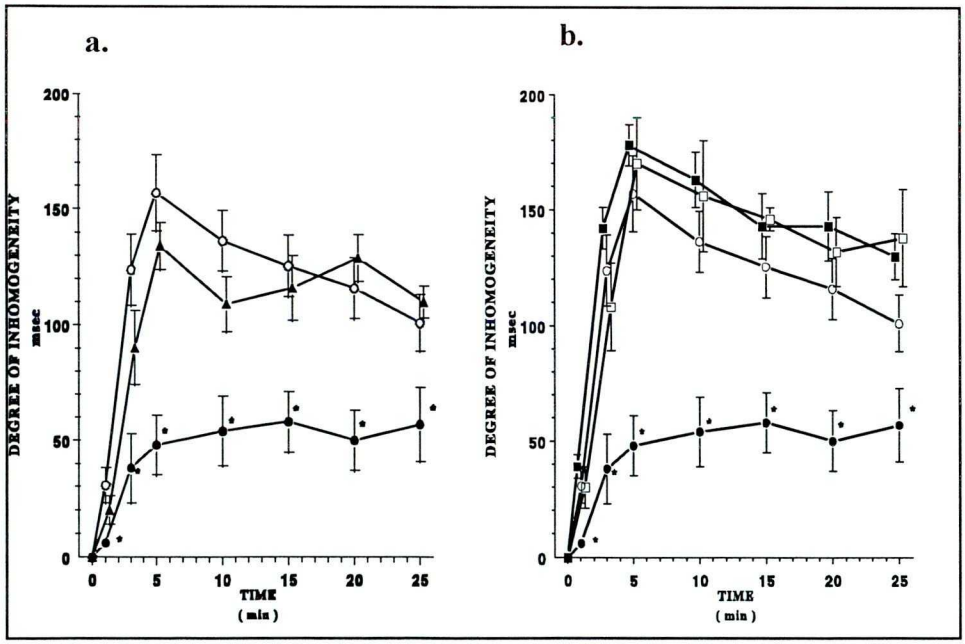

*Fig. 11. Changes in the degree of inhomogeneity of electrical activation (ms) during a 25 min coronary artery occlusion in dogs given 24 h previously, MLA 100 μg kg^{-1} (filled circle), 10 μg kg^{-1} (filled triangles) or 100 μg kg^{-1} MLA and then either aminoguanidine (open squares) or aminoguanidine and meclofenamate (filled squares), as well as the appropriate vehicle control (open circles). * P < 0.05 compared to the vehicle controls.*

3.3. Studies in rat isolated hearts

3.3.1. Effects of MLA on ventricular functional parameters and arrhythmias in rat isolated, Langendorff perfused hearts

The ventricular functional parameters, such as LVDP, $+dP/dt_{max}$ and $-dP/dt_{max}$ were not significantly different between groups at the end of the equilibration period before ischaemia (Table 6).

Table 6.	LVDP (mmHg)	$+dP/dt_{max}$(mmHg/s)	$-dP/dt_{max}$(mmHg/s)
I. vehicle control	66.04±5.24	2026±239	1391±142
II. MLA 0.5 $mg^{-1}kg$/8h	78.27±4.82	1932±284	1417±94
III. MLA 0.5 $mg^{-1}kg$/24h	77.00±7.78	2040±303	1419±194
IV. MLA 2.5 $mg^{-1}kg$/8h	61.90±4.97	1893±215	1441±152
V. MLA 2.5 $mg^{-1}kg$/24h	66.28±9.07	2250±274	1543±196
VI. vehicle control + AG*/24h	74.93±2.63	2624±165	1758±98
VII. MLA 2.5 $mg^{-1}kg$ + AG*/24h	83.13±4.02	2595±176	1867±173
VIII. vehicle control + L-NIL/24h	72.11±9.46	2154±185	1572±169
IX. MLA 2.5 $mg^{-1}kg$ + L-NIL/24h	77.94±5.44	2061±129	1649±187
X. vehicle control + AG*	80.27±5.47	2278±238	1719±218
XI. MLA 2.5$mg^{-1}kg$/24h +AG*perf	77.01±1.60	1908±125	1537±137

*AG=aminoguanidine

Table 6. Ventricular functional parameters before ischaemia (preischaemic values)

The end-reperfusion values were compared with those obtained at the end of equilibration period (30 min). The recoveries of LVDP, $+dP/dt_{max}$ and $-dP/dt_{max}$ after ischaemia-reperfusion in the different experimental groups (expressed as a percentage of the values obtained at the end of the equilibration period) are illustrated in Figure 12 A, B and C respectively, and summarised in Table 7.

The hearts from the control (vehicle) group did not recover to their initial values after an 30 min no-flow ischaemia. MLA at the dose of 0.5 mg kg^{-1} after 8 or 24 h, did not significantly modify the recovery of ventricular contractile function. Eight hours after pretreatment with 2.5 mg kg^{-1} MLA, these parameters presented a slight, albeit not significant recovery. However, 24 h after treatment with the same dose of MLA, recovery of LVDP, $+dP/dt_{max}$, -

dP/dt_{max} values were significantly improved. During reperfusion, 87.5 % of the vehicle control hearts developed VF (Figure 12D). In groups pretreated with 0.5 mg kg^{-1} MLA (8 or 24 h) or with 2.5 mg kg^{-1} MLA (8 h), the incidence of VF was not significantly different from the value obtained in controls. However, it was significantly reduced in the group treated with 2.5 mg kg^{-1} MLA 24 h previously.

The influence of aminoguanidine (2 x 300 mg kg^{-1}) and L-NIL (10 mg kg^{-1}) was studied in animals treated with 2.5 mg kg^{-1} MLA 24 h previously. Both inhibitors prevented the beneficial effects of MLA on LVDP, $+dP/dt_{max}$, $-dP/dt_{max}$ and VF, the parameters reaching values which were not significantly different from those obtained in corresponding controls (Figure 12 and Table 7). However, in hearts removed from MLA treated animals (2.5 mg kg^{-1} for 24 h) and perfused with aminoguanidine (150 μM) 30 min before the onset of ischaemia, the delayed cardioprotection was not attenuated, as indicated by the lack of significant recovery of LVDP, ± dP/dt_{max} and the low incidence of VF (Figure 12 and Table 7).

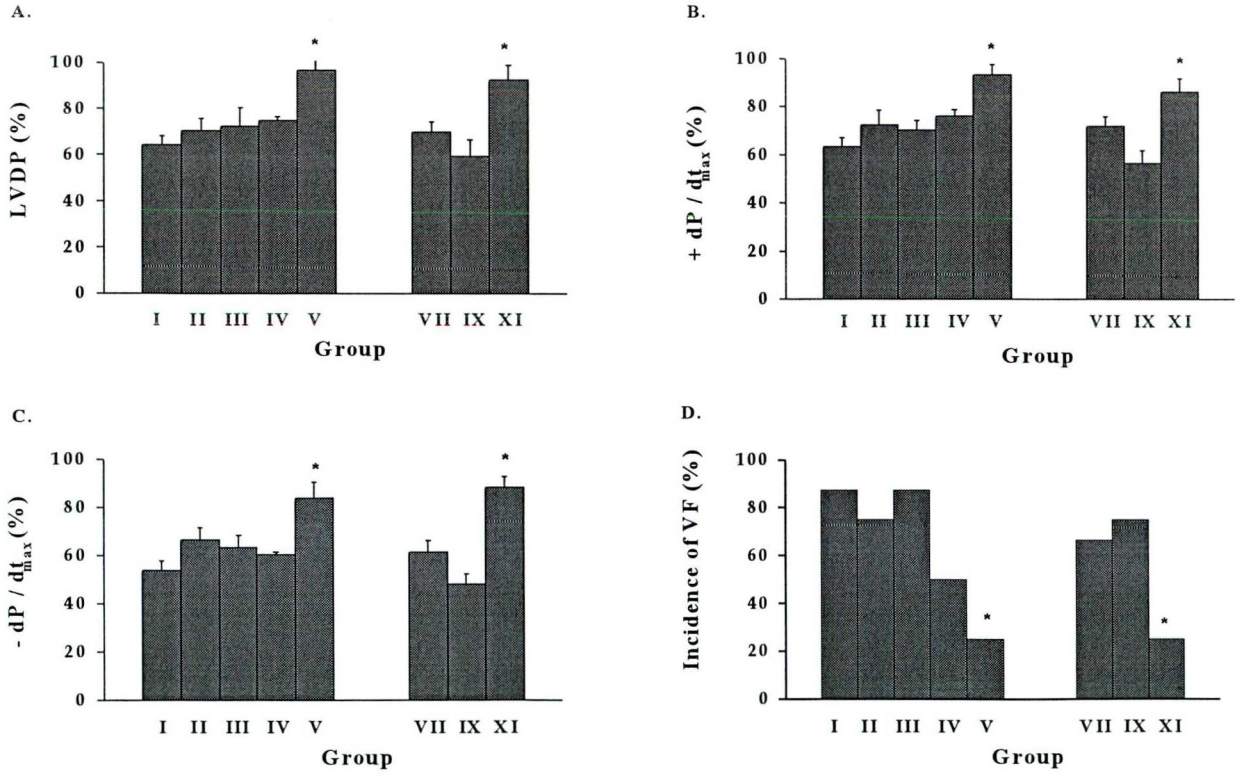

*Fig. 12. Postischaemic recovery of the functional ventricular parameters LVDP (A), $+dP/dt_{max}$ (B), $-dP/dt_{max}$ (C) and incidence of VF during reperfusion (D) in the different experimental groups (see Methods for the description of the groups). * $P < 0.05$ vs control.*

Table 7.	LVDP (%)	$+dP/dt_{max}$ (%)	$-dP/dt_{max}$ (%)	VF (%)
I. vehicle control	64.29±3.76	63.41±3.66	53.73±4.08	87.5
VI. vehicle control + AG/24h	65.63±1.60	67.53±2.80	56.43±0.43	83.3
VIII. vehicle control + L-NIL/24h	58.13±1.23	56.32±2.88	50.87±7.03	75.0
X. vehicle control + AG perf.	56.15±3.16	56.07±5.24	45.29±8.09	83.3

Table 7. Recovery of ventricular functional parameters and the incidence of VF in different control groups

3.4. Effects of MLA treatment on cardiac NO production

In control, vehicle treated rat hearts no EPR spectra characteristic for NO adducts were found (Figure 13B). In groups pretreated with 0.5 mg kg^{-1} MLA for 8 or 24 h, the NO production in the heart was also below detection limit. However, hearts taken from rats pretreated with 2.5 mg kg^{-1} MLA, exhibited a prominent triplet EPR signal (g = 2.035) attributed to a NO-Fe-DETC complex and indicating NO production. The signal was observed in those hearts which were treated with MLA only 8 h previously (Fig. 13C), but not in those given MLA 24 h previously (Fig. 13D) (at 8 h, NO production was 577 ± 73 pmol g^{-1} wet tissue h^{-1}, n = 3). The characteristic EPR signal was not detected in the hearts of animals pretreated for 8 h with MLA and aminoguanidine (Fig. 13E) or with MLA and L-NIL (not shown).

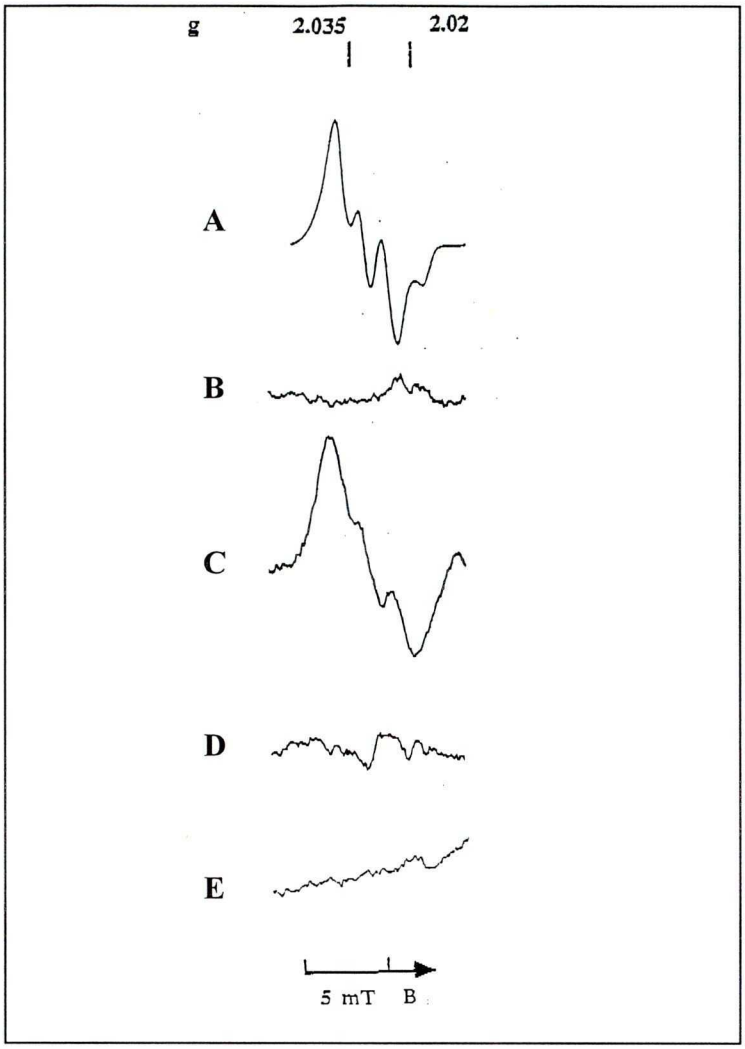

Figure 13. In vivo NO spin trapping by Fe-DETC complex. (A) EPR spectra of rat thoracic aortae treated with sodium diethyldithiocarbamate (DETC, 5 mM) and $FeSO_4$ (50 μM) and incubated at 37°C for 30 min under control conditions (Figure reproduced from Andriambeloson et al. 1997, Br.J.Pharmacol. 120:1053-1058). In our experiments rats were pretreated with vehicle (B), 2.5 mg kg^{-1} MLA 8h (C), 2.5 mg kg^{-1} MLA 24 h (D) or 2.5 mg kg^{-1} MLA plus 300 mg kg^{-1} aminoguanidine 8h previously (E). EPR spectra were recorded at 77K. Then spectra of control heart and Cu-DETC complex were substracted. The values of the spectroscopic splitting factor (g) and the magnetic field scale are shown at the top and at the bottom, respectively.

3.5. Effects of MLA pretreatment on reactivity of rat aortic rings

At the cardioprotective dose of 2.5 mg kg^{-1} (8 or 24 h), MLA did not significantly influence the contractile effect of NA in aortic rings with or without functional endothelium (Figure 14). The NOS inhibitor L-NAME (300 μM) did not significantly modify the sensitivity or the maximal effect of NA in endothelium-denuded rings removed from vehicle controls or MLA pretreated animals, whereas in aortic rings with endothelium from both control and MLA-

treated rats, it produced a significant shift of the concentration-response curve of NA to the left (3.4 to 5.4 fold decrease of the EC_{50} values of NA). L-arginine (1 mM) administration, failed to induce a significant relaxation (0.4 ± 0.4, 0.6 ± 0.4, 5.0 ± 2.8 % relaxation in endothelium-denuded rings removed from control rats, or rats treated with MLA for 8 or 24 h, respectively, and 0.8 ± 0.8, 4.5 ± 4.1, 2.2 ± 1.3 % in rings with endothelium from control rats, or those treated with MLA for 8 or 24 h, respectively).

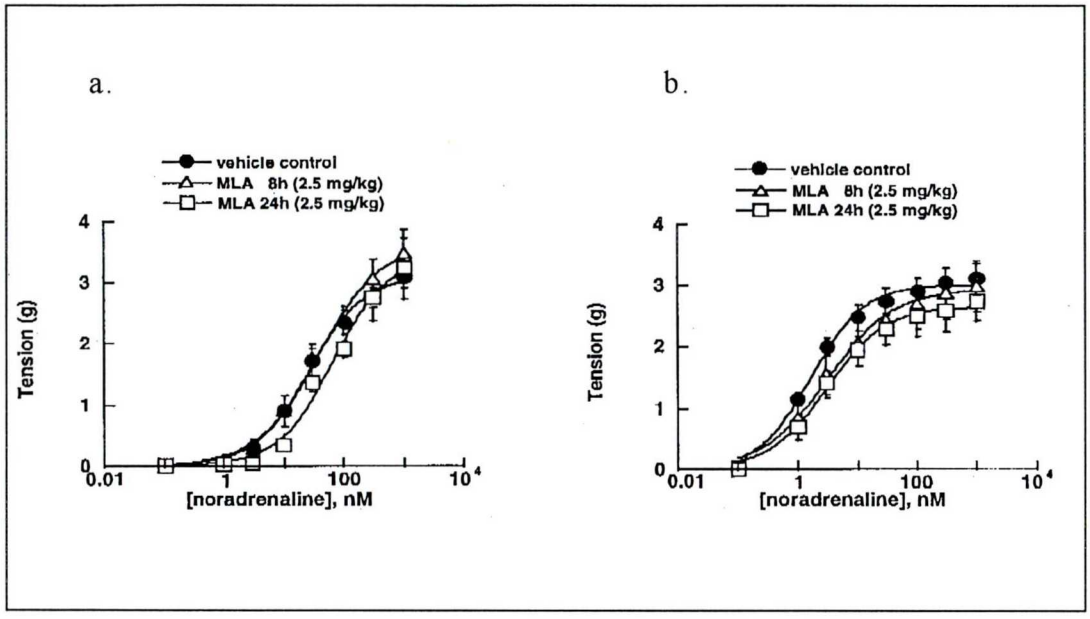

Figure 14. Concentration-response curve of noradrenaline in aortic rings with (a) or without (b) functional endothelium, prepared from vehicle controls and from rats treated 8 or 24 h previously with 2.5 mg kg^{-1} MLA. Results are expressed as mean ± s.e.mean of five experiments.

3.4. Effects of ischaemia-reperfusion on isolated hearts from PARP (+/+) and PARP (-/-) mice

The measured parameters contractile force, heart rate and coronary flow were not significantly different in PARP (+/+) and PARP (-/-) groups at the end of equilibration period before ischaemia (Table 7.).

Table 7	PARP +/+	PARP -/-
Contractile force (g)	2.68±0.13	2.95±0.17
Coronary flow (ml min^{-1})	4.27±0.75	3.56±.17
Heart rate (beats min^{-1})	391±14	389±28

Table 7. Preischaemic values of contractile force, coronary flow and heart rate in PARP +/+, PARP -/- mice.

The end-reperfusion values were compared with those obtained at the end of equilibration period. The recovery of contractile force, coronary flow and heart rate after ischaemia-reperfusion was expressed as a percentage of the values obtained at the end of the equilibration period After 30 min reperfusion both groups exhibited significantly reduced contractile function, with no difference between PARP (+/+) and PARP (-/-) hearts. The heart rate and coronary flow was not affected by ischaemia-reperfusion either in PARP (+/+), or in PARP (-/-) mice. The recovery after 30 min reperfusion of the contractile function, heart rate and coronary flow of the hearts isolated from PARP (+/+) and PARP (-/-) mice are shown in Figure 15.

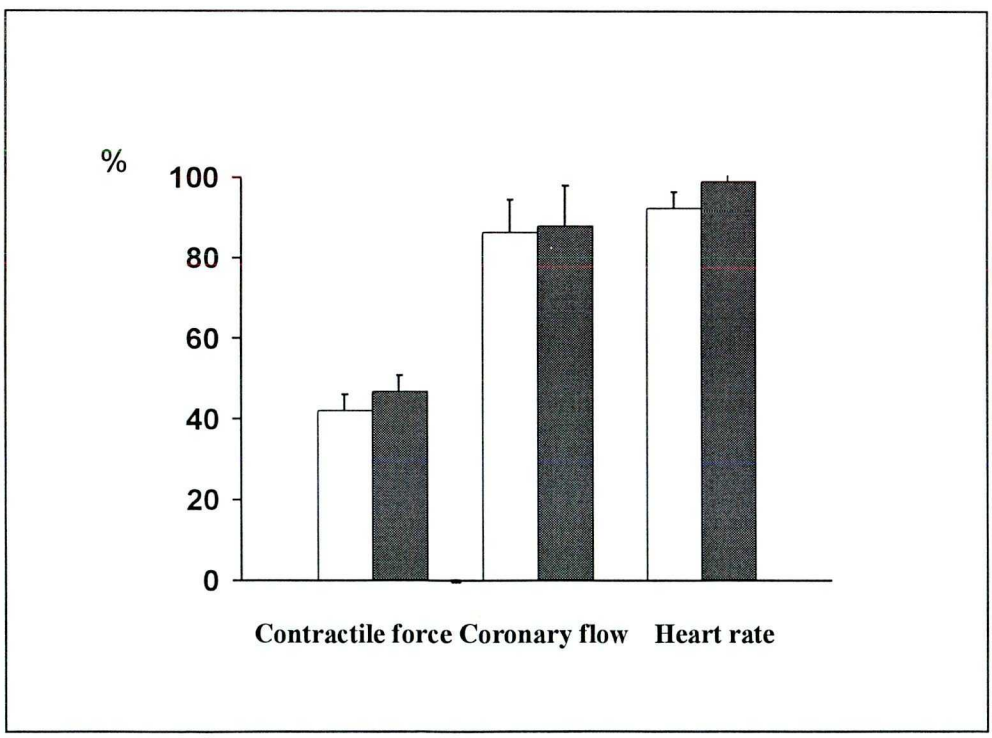

Figure 15. Recovery of contractile force, coronary flow and heart rate after 30 min reperfusion in PARP +/+ (open histograms) and PARP -/- (cross-hatched histograms) mice.

4. Discussion

4.1. New findings

I. We showed that nicorandil and isosorbide-2-mononitrate (described as NO donors), but not cromakalim (a K_{ATP} channel opener) administered intracoronarily, before and throughout coronary occlusion prevent ischaemia-induced ventricular arrhythmias and reduce the incidence of VF following reperfusion in anesthestized dogs.

II. We have described for the first time a protective effect of MLA against ischaemia/reperfusion-induced ventricular arrhythmias in dogs. This cardioprotection was only slightly attenuated by the prior administration of an iNOS inhibitor (aminoguanidine), but markedly reduced by addition of a COX inhibitor (meclofenamate).

III. In rats, we demonstrated that pretreatment with MLA increases NO levels in the heart and that the increased NO formation preceeded the myocardial protection and had returned to undetectable levels by the time the protective effects were evident. iNOS inhibitors abolished both the MLA-induced NO elevation and the cardioprotection. We described that with MLA pretreatment increased NO production in hearts, yet was without side effects on vascular contractility.

IV. Finally, studies performed with hearts from PARP -/- mice, indicate that PARP did not play an essential role in the impairment of cardiac contractility induced by ischaemia/reperfusion.

4.2.The role of NO in the cardioprotective effects of nicorandil and isosorbide mononitrate

In anaesthetised dogs administration of nicorandil (a NO donor and K_{ATP} opener) or isosorbide mononitrate (a NO donor), prior to ischaemia and throughout the occlusion period markedly reduced the severity of ventricular arrhythmias which occurred during a coronary artery occlusion and particularly, following reperfusion of the ischaemic myocardium. In this study the dose of the drugs and the administration route (local, intracoronary infusion) were chosen to avoid the confounding effect of systemic hypotension on ischaemia-induced arrhythmias. After 20 min infusion only slight reductions in arterial blood pressure occurred; LVdP/dt and heart rate were not significantly affected. A regional increase of coronary blood

flow could also contribute to the beneficial effect of NO donors (98). In our studies, intracoronary administration of levcromakalim (a pure K_{ATP} channel opener) increased coronary blood flow to a similar extent to that obtained with nicorandil or isosorbide mononitrate. However, levcromakalim failed to influence either the severity of ischaemic changes following coronary artery occlusion or ischaemia-induced ventricular arrhythmias. These results would argue against this mild degree of coronary dilatation being a major factor responsible for the marked arrhythmia suppression in this anaesthetised dog model. Furthermore, these results also indicated that opening of K_{ATP} channels before an ischaemic episode did not result in protection against ischaemia-reperfusion-induced arrhythmias in this model. Although there is much evidence for the cardioprotective effects of K^+ channel openers, as demonstrated by their ability to reduce myocardial necrosis (99) and to enhance recovery of cardiac contractility following ischaemia and reperfusion (100), the antiarrhythmic effect of opening of these channels is still controversial. Taken together, our results suggest that the suppression of ventricular arrhythmias with nicorandil and also with isosorbide mononitrate is due to their ability to donate NO. Both of these agents undergo metabolic conversion (by gluthatione S-transferases or cytochrome P450 activity) to donate NO. To further strengthen our conclusion, investigations with agent that donates the NO radical spontaneously, without metabolic conversion (for instance NONOates) should be performed. Another point which needs to be clarified, concerning the cardioprotective effects of NO donors, is the respective contribution of NO elevation before the onset of ischaemia and during ischaemia itself.

The present studies now provide evidence that, in the same canine model as that used for our previous classical preconditioning studies, the local intracoronary infusion of NO donors mimic the protective effects of ischaemic preconditioning. Although it is difficult to strictly compare these two protective manipulations, they produced the same characteristics of myocardial protection: a reduction in epicardial ST-segment elevation, a decrease in the inhomogeneity of electrical activation within the ischaemic area and a markedly reduced severity of ventricular arrhythmias. This supports the hypothesis that an endogenous production of NO by NOS may be involved in the protective effect of ischaemic preconditioning induced by short coronary occlusion. Our explanation for NO-induced protection might be the elevation of myocardial cGMP levels. It has been previously reported in the same model that methylene blue abolished the protective effect of preconditionning

induced by short coronary occlusion (55). This might indicate that nitric oxide by activating soluble guanylyl cyclase plays a role in the protective effect of ischaemic preconditioning. However, methylene blue cannot be considered as a specific inhibitor of the catalytic activity of soluble guanylyl cyclase. It is rather considered to prevent the activation of guanylyl-cyclase by NO, and therefore may inhibit other NO-induced mechanisms as well. In a conscious dog model of ischaemia and increased sympathetic activity, stable analogues of cGMP reduce arrhythmia severity (101). The involvement of cGMP in NO-induced protective effects, as well the subsequent mechanisms warrants further investigations.

4.3. The role of NO in the delayed cardioprotection induced by MLA

MLA is a non-toxic derivative of LPS which induces delayed cardioprotection in various species (73). In the present study we demonstrated, for the first time, that MLA induces protection against ischaemia and reperfusion-induced ventricular arrhythmias in dogs. We also demonstrated a protective effect against ischaemia reperfusion–induced contractile dysfunction and ventricular fibrillation in isolated hearts removed from MLA-pretreated rats. .

The induction of iNOS has been proposed to be involved in the delayed cardioprotection induced by several procedures (e.g. administration of endotoxin, short coronary occlusions, cardiac pacing). Recently, a role of iNOS has been proposed in the delayed cardioprotective effects of MLA in rats and rabbits (77, 76). In anaesthetised dogs, administration of the iNOS inhibitor aminoguanidine, prior to the ischaemic period, only slightly attenuated the protection against ventricular arrhythmias induced by ischaemia and reperfusion in dogs pretreated 24 h previously with MLA. However, when in addition to aminoguanidine sodium meclofenamate (an inhibitor of the cyclooxygenase pathway) was given, the antiarrhythmic protection afforded by MLA was completely abolished. These results indicate that in dogs endogenous substances other than NO, such as prostanoids (more likely prostacyclin) derived from cyclooxygenase activation are also involved in the antiarrhythmic effect of MLA.

In hearts removed from rats treated 24 h before with MLA aminoguanidine, given prior to ischaemia did not attenuate protection. However, when aminoguanidine was administered at the same time as MLA, 24 h before global ischaemia was induced in isolated hearts, the cardioprotective effects of MLA were abolished. Although the ability of aminoguanidine to

inhibit iNOS is well documented (102, 103) the contribution of other mechanisms of action cannot be excluded. For instance, it was reported that aminoguanidine possesses direct scavenging properties against free radicals and $ONOO^-$ (104) and inhibits the activity of various enzymes (105). Therefore, in a separate series of experiments we have studied the effect of L-NIL, a reasonable more selective inhibitor of iNOS, structurally different from aminoguanidine (106). We have demonstrated that pretreatment of rats with L-NIL also inhibited the cardioprotective effects of MLA. Thus, inhibition of iNOS activity is the most probable mechanism by which these drugs prevent MLA-induced protection. Although the results obtained in this study show that activation of iNOS seems to be a pathway involved in the protective effects of MLA in dogs and rats, some difference was found between these two models. Whereas in anaesthetised dogs, administration of aminoguanidine just before the ischaemia slightly attenuated the protective effect of MLA, in rat isolated hearts it did not modify this protection.

In rats, NO level within cardiac tissue was directly evaluated by Fe-DETC spin trapping followed by EPR spectroscopy. The advantage of this technique is to detect NO as a radical within the tissue (97). An increased level of NO was detected by EPR in cardiac tissue 8 h after MLA pretreatment, but not 24 h after MLA treatment. This time course of NO production after MLA is closely related to that of iNOS mRNA expression, reported by others (77) and is consistent with the view that the NO detected by the EPR method is mainly derived from MLA-induced iNOS activity. This was further supported by the ability of both aminoguanidine and L-NIL to prevent MLA-induced NO elevation in heart. Thus, there was a marked difference in the time course of MLA-induced cardioprotection and NO production; 8 h after MLA, NO production was increased in the heart whereas no functional protection was evident. By contrast, 24 h after MLA, there was no EPR detectable NO in cardiac tissue whereas a cardioprotective effect was observed. This temporal dissociation between the effects of MLA on cardiac NO production and cardioprotection, and the ability of iNOS inhibition to abolish the MLA-induced NO elevation and cardioprotection, suggests that NO acts as a trigger of protective mechanisms in hearts from MLA-treated rats.

The ability of NO to trigger and/or to mediate the late phase of preconditioning induced by short coronary occlusion was recently investigated in more detail by Bolli and collegues. They proposed that the initial signals elicited by a brief ischaemic stress (NO, ROS, PKC and

tyrosine kinases) are transduced into iNOS gene expression via an NF-kB-dependent mechanism (68). From our results it appears that, once iNOS induced in the heart, it may subsequently trigger some adaptative mechanisms, perhaps similar to those mentioned above, which are then responsible for the delayed protection.

There are several potential mechanisms triggered by iNOS-derived NO which may contribute to protection against ischaemia-reperfusion damage. It is well etablished that the severe consequences of the ischaemia-reperfusion are ultimately due to a burst of reactive oxidative species that occurs upon readmission of oxygen (21, 14). Therefore induction of antioxidant enzymes like catalase, superoxide dismutase or glutathione peroxidase, may exert cardioprotective effects. For example, it has been demonstrated that iNOS derived NO upregulates Cu/Zn SOD expression (107) and that NO can also trigger the expression of genes coding heat shock proteins (HSP) (108). NO may also upregulate cyclooxygenase-2 or heme-oxygenase-1, which in a cell specific way may attenuate apoptotic cell death. NO triggered protective principles may further arise from processes that are mediated by elevated levels of cGMP, thiol modification, for instance caspase inactivation (109). Interestingly, MLA induced cardioprotection is associated with increased catalase (44) and Mn SOD activity (50), and with the induction of HSP 72 (51). The contribution of these adaptative mechanisms to iNOS-induced cardioprotective effects requires further investigation.

The results obtained in rats show that, although MLA increased NO production in the heart, the pre-ischaemic ventricular parameters were not affected. This distinguishes MLA from its parent molecule LPS which, at cardioprotective doses, was reported to decrease cardiac contraction by a NO-dependent mechanism (110). Another difference between MLA and LPS is their effect on aortic reactivity. In aortic rings removed from rats treated 8 or 24 h before with MLA, the contractile response to NA was not significantly affected either in the absence or presence of L-NAME. Further, the NOS substrate L-arginine failed to induce a significant relaxing effect. These data do not provide evidence for NO-mediated aortic hyporeactivity in the aortae removed from MLA-treated rats. Moreover in rings with intact endothelium, MLA did not affect the potentiating effect of L-NAME on vascular response to NA, indicating no modification of the basal influence of endothelium-derived NO. The lack of effect of MLA on aortic contractility and cardiac function in the absence of ischaemia is consistent with the

observation that in a similar model, MLA did not modify blood pressure or heart rate (61). By contrast, it was reported that a cardioprotective dose of LPS produced NO-mediated hyporeactivity to contractile agonists in aortic rings, as well as a decrease of eNOS activity (110). The reasons for these differential effects of MLA and LPS, especially in blood vessels, remains to be investigated.

4.4. Role of PARP in ischaemia reperfusion–induced injuries

As described above, reperfusion injury can be attributed to reactive oxidative species that are generated following reperfusion of the ischaemic myocardium (21, 14). The cytotoxic effect of these species, especially peroxynitrite and hydroxyl radicals, has been mainly attributed to activation of the nuclear enzyme PARP. This idea derives from experiments showing that inhibitors of PARP activity can reduce the cytotoxic effect of peroxynitrite generated during reperfusion. However, PARP inhibitors represent a class of drugs about which little is known concerning their pharmacological specificity. The use of transgenic mice lacking for the PARP gene represent an alternative approach to investigate the role of PARP. The results that we obtained with PARP(-/-) mice do not support the idea that PARP activation plays an obligatory role in the contractile dysfunction that occur during reperfusion of mice isolated ischaemic hearts. In contrast, recent data obtained by other groups show that in PARP(-/-) mice the biochemical and functional consequences of cardiac ischaemia-reperfusion are significantly reduced. For instance, tissue necrosis and serum creatine phosphokinase were significantly reduced in PARP(-/-) mice compared with their wild-type PARP (+/+) littermates (89). In isolated working hearts from mice lacking PARP and previously subjected to hypoxia, no contractile dysfunction occured during reoxygenation (111). At the present time there is no obvious explanation for these different results. In addition to differences related to various experimental protocols and models (ischaemia versus hypoxia, isolated-perfused hearts versus *in vivo* studies), the recent description of several PARP isoforms (PARP 1,2,3,4,5) may result in the generation of various transgenic models between different laboratories.

The main objective of this thesis was to examine the role of NO in ischaemia and reperfusion-induced myocardial injury. This was conducted using different models: *in vivo* anaesthetised

dogs and isolated, Langendorff-perfused rat and mouse hearts. The two latter models allow to differentiate phenomena which take place within cardiac tissue from those which may affect circulating cells and cardiac load.

The results obtained in the present work are consistent with the hypothesis that NO may behave as an endogenous myocardial protective substance against ischaemia and/or reperfusion injuries. This hypothesis is supported by the fact that cardioprotection could be obtained by exogenous NO delivery (from pharmacological agents which are able to donate NO) and by increased formation of endogenous NO (by agents which induces iNOS expression).

Ischaemia-reperfusion injury is an important phenomenon during cardiac surgery and the pharmacological manipulation of cardioplegic solutions has emerged as a powerful clinical tool in the preservation of myocardial integrity. Recently, two studies have demonstrated the cardioprotective role of NO during cardiac surgery by blood supplementation with L-arginine (112) and a cystein containing NO donor (SPM-5185) (113). Both of these agents reduced infarct size and improved post-ischaemic contractile performance. If NO can precondition the myocardium then nitrates may also have a protective role before routine cardiac surgery. From the emerging evidence for the role of NO in cardioprotection from experimental and clinical results perhaps the emphasis should now be shifted to extensively re-exploring a role for nitrates, as well as other NO-donating agents, before ischaemia has occurred. Large, properly designed trials are required to answer this question. What is likely to be of paramount importance is the timing of therapy in relation to ischaemia, dose regime and the concept of nitrate tolerance. Future therapeutic strategies are likely to involve the development of more stable NO donors, the adjunctive use of free radical scavengers and the pharmacological use of agents downstream of NO.

The conditions in which NO exerts either protective or deleterious effects in tissues are not well defined. The excessive production of NO which occurs after iNOS induction has been generally considered as deleterious. The present work, together with some recent informations, indicate that NO produced by iNOS may play a protective role. NO levels are probably not the only determining factor for the role of NO, but also the redox state of the tissue and the balance between NO and $O_2^{\cdot}$. From the data obtained in the present work, it appears that NO

produced by iNOS can trigger some adaptative mechanisms against oxidative stress. These mechanisms are likely to be involved in detoxifying pathway, for instance the conversion of peroxynitrite to another form, perhaps a nitrosothiol or NO itself. Modulating the biological activity of NO by modifying the redox state in tissues, particularly O_2^- production, is a very exciting field which offers many possibilities. However, the cell sources of O_2^- are not well known at the present time.

The recent discovery of complex biochemical pathway controlling the biological activity of NO in the cardiovascular system offers a great number of novel approaches for pharmacological modulation. However, there are a number of unsolved questions on the mechanisms directing the actions of NO towards beneficial rather than deleterious consequences. Thus a greater scientific understanding of the basis of NO-induced cytoprotection against ischaemic injury may help to answer these important questions.

5. RESUME

L'infarctus du myocarde est une des causes principales de morbidité et de mortalité cardiovasculaires dans notre société. Il est la conséquence d'une occlusion prolongée d'une artère coronaire. La reperfusion du myocarde, utile lorsqu'elle est suffisamment précoce pour limiter l'étendue de la nécrose, voire l'empêcher, peut aussi avoir des conséquences délétères (dysfonctions contractiles, survenue de fibrillations ventriculaires fatales). En dehors d'une prévention primaire de la maladie coronarienne, une stratégie thérapeutique consiste à limiter les dommages liés à un épisode d'ischémie/reperfusion. Des progrès significatifs dans ce domaine ont été réalisés en cardiologie expérimentale ces dernières années: la possibilité, par différentes manoeuvres, de rendre le coeur „résistant" à l'ischémie/reperfusion (préconditionnement myocardique) et l'identification de mécanismes susceptibles d'être impliqués dans les dommages induits par la reperfusion.

Le préconditionnement peut être induit par des épisodes d'ischémie transitoire ou par la stimulation du coeur à haute fréquence; il se caractérise par une première phase précoce de protection (se développant immédiatement après le stimulus et se maintenant quelques heures) et une seconde, retardée (se manifestant 24 h après le stimulus initial et persistant quelques jours). Divers agents pharmacologiques, capables d'induire la NO synthase inductible (iNOS), tels le lipopolysaccharide et son dérivé synthétique le „monophosphoryl lipid A" (MLA) sont capables de reproduire les effects du préconditionnement. Néanmoins, le NO pourrait aussi être impliqué dans les dommages de reperfusion, via la génération de peroxynitrites (formés à partir de NO et des anions superoxydes lors de la réadmission de l'oxygène). Les effets cytotoxiques des peroxynitrites produits dans ces conditions ont été récemment attribués, sur la base d'arguments pharmacologiques, à l'activation de la poly (ADP-ribose) polymérase (PARP).

OBJECTIFS DU TRAVAIL DE THESE

L'objectif principal de ce travail est de préciser les conditions dans lesquelles le NO peut exercer un effet protecteur vis-à-vis des dommages provoqués par l'ischémie/reperfusion myocardique. Plus spécifiquement, ce travail a pour objet: (1)- de déterminer si un apport de NO, à partir d'agents utilisés en thérapeutique comme anti-angoreux, peut exercer un effet protecteur similaire à celui induit par la première phase de préconditionnement; (2)- d'analyser l'implication et le rôle du NO produit par la iNOS dans un modèle de préconditionnement retardé; (3)- d'examiner l'implication de la PARP dans les dommages engendrés par un épisode d'ischémie/reperfusion dans le coeur de souris chez lesquelles le géne de la PARP a été éradiqué.

Ce travail a été mené sur différents modèles:

- *in vivo* chez le Chien, où l'ischémie est provoquée par l'occlusion de l'artère coronaire gauche; l'effet cardioprotecteur a été évalué principalement par le nombre et la nature des arrythmies ventriculaires;
- sur coeur isolés/perfusés (modèle de Langendorff) de Rat et de Souris: les coeurs sont soumis à une ischémie globale et l'effet cardioprotecteur a été évalué sur la survenue des fibrillations ventriculaires et/ou la récupération de la fonction contractile ventriculaire

L'approche expérimentale est essentiellement pharmacologique. Dans certains cas, la production de NO dans le tissu cardiaque a été évaluée par résonance paramagnétique électronique.

RESULTATS

Effet du nicorandil, du levcromakalim et du mononitrate d'isosorbide sur les arrythmies ventriculaires induites par la ligature d'une artère coronaire chez le Chien

Chez le Chien anesthesié, l'occlusion de l'artère coronaire gauche pendant une période de 25 min provoque des extrasystoles et des épisodes de tachycardie ventriculaire pouvant aller jusqu'à la fibrillation ventriculaire. Aucun des animaux ne survit après reperfusion. Dans une première série d'expériences, l'influence du nicorandil, un agent connu comme „donneur de NO" mais également comme activateur de canaux potassiques, a été étudiée sur les arrythmies cardiaques provoquées par l'ischémie et la reperfusion. Afin de s'affranchir d'un effet systémique, le nicorandil a été infusé par voie intracoronaire (20 min avant d'occlusion et pendant l'occlusion). Dans ces conditions, le nicorandil diminue de manière significative les troubles du rythme et augmente la survie des animaux. Afin d'évaluer la contribution du NO ou des canaux potassiques dans l'effet cardioprotecteur, l'effet du nicorandil a été comparé à celui du levcromakalim (activateur de canaux potassiques) et du mononitrate d'isosorbide (dérivé nitré „donneur de NO"). L'administration intracoronaire de levcromakalin ne modifie pas significativement les arrythmies ventriculaires. Par contre, le mononitrate d'isosorbide administré dans ces mêmes conditions, diminue de manière significative les arrythmies ventriculaires induites par l'ischémie.

Ces résultats montrent que des agents connus pour leurs propriétés anti-angoreuses sont capables d'exercer un effet protecteur vis à vis des arrythmies provoquées par un épisode ischémique, et que leur effet protecteur semble être lié à leur propriété de libérer NO.

Rôle de iNOS dans l'effet cardioprotecteur à long terme induit par le MLA

L'effet cardioprotecteur du MLA a été étudié chez le Chien (modèle décrit précédement) et chez le Rat (modèle d'ischémie globale sur des coeurs isolés/perfusés). Le prétraitement des animaux 24 h avant l'épisode ischémique induit un effet protecteur marqué. Chez le Chien, le MLA diminue significativement le nombre des extrasystoles, la survenue de tachycardie ventriculaire, la survenue des fibrillations ventriculaires et améliore la survie. Chez le Rat, il améliore la récupération de la fonction contractile ventriculaire et réduit la survenue des fibrillations ventriculaires lors de la reperfusion. Par contre, aucun effet protecteur n'est observé 8h après administration de MLA. L'effet cardioprotecteur du MLA est aboli chez des animaux (chiens et rats) prétraités 24 h auparavant par le MLA et l'aminoguanidine ou par le MLA et la L-N^6-(1-Iminoethyl)-lysine (L-NIL). L'aminoguanidine et la L-NIL sont deux composés, structuralement différents, tous les deux décrits comme inhibiteurs sélectifs de iNOS.

Le rôle de NO produit par iNOS dans l'effet cardioprotecteur du MLA a été analysé de manière plus détaillée chez le Rat. Pour cela, la production de NO dans le tissu cardiaque a été évaluée par résonance paramagnétique électronique, et la relation temporelle entre la production de NO et l'effet cardioprotecteur a été étudiée. Vingt-quatre heures après l'administration de MLA (c'est à dire lorsqu'un effet cardioprotecteur est manifeste), aucune élévation de NO n'est détectable dans le tissu cardiaque. Par contre, une élévation de NO est observée sur des cœurs prélevés chez des animaux traités 8 h auparavant par le MLA (c'est à dire à un temps où aucun effet cardioprotecteur n'a pu être mis en évidence). Cette élévation de NO n'est pas observée chez des animaux prétraités par le MLA et l 'aminoguanidine ou par le MLA et la L-NIL.

Ces résultats montrent l'implication de la iNOS dans l'effet cardioprotecteur retardé du MLA chez le Rat et le Chien. La dissociation temporelle observée chez le Rat entre l'effet protecteur et l'élévation de NO dans le tissu cardiaque (l'élévation de NO précédant de plusieurs heures l'effet protecteur) suggère que, dans ce modèle, la cardioprotection n'est pas liée à la production immédiate de NO par la iNOS, mais plutôt aux conséquences à plus long terme de cette élévation de NO.

Rôle de la PARP dans les dommages engendrés par un épisode d'ischémie/reperfusion

Ce travail a été abordé sur des souris transgéniques, 'knock-out» pour le gène de la PARP. Pour cela, nous avons mis au point un modèle de coeur de isolé/perfusé de Souris, afin de mettre en évidence les modifications de la fonction contractile ventriculaire lors de la reperfusion du myocarde ischémié. Un épisode d'ischémie globale, suivi de reperfusion induit une diminution marquée de la force de contraction ventriculaire chez le souris PARP +/+. Un

effet comparable a également été observé chez les souris PARP -/-. Ces résultats ne sont pas en faveur d'une implication obligatoire de la PARP dans les dysfonctions contractiles provoquées par un épisode d'ischémie/reperfusion sur coeur isolé de souris.

CONCLUSION

Les résultats obtenus confortent l'hypothèse qu'une élévation de NO dans le tissu cardiaque avant un épisode d'ischémie, protège le coeur vis-à-vis des dommages de reperfusion. Un tel effet protecteur peut être obtenu par des agents pharmacologiques „donneurs de NO" , ou à plus long terme par induction de la iNOS dans le tissu cardiaque. Dans ce dernier cas, la cardioprotection est probablement due à la capacité du NO à initier des mécanismes d'adaptation dans le tissu cardiaque. La PARP ne semble pas nécessairement impliquée dans l'altération de la contractilité cardiaque produite par un épisode d'ischémie-reperfusion chez la Souris.

6. References

1. Jennings R.B., Ganote C.E., Reimer K.A. Ischaemic tissue injury *Am. J. Pathol.* 1975; 81:179-198
2. Jennings R.B., Hawkins H.K., Lowe J.E., Hill M.L., Klotman S., Reimer K.A. Relation between high energy phosphate and lethal injury in myocardial ischaemia in the dog. *Am. J. Pathol.* 1978; 92: 187-214
3. Jennings R.B., Reimer K.A., Steenbergen C.Jr., Schaper J. Total ischaemia III: Effect of inhibition of anaerobic glycolysis. *J. Mol. Cell. Cardiol.* 1989; 21 (Suppl 1): 37-54
4. Reimer K.A., Jennings R.B., Hill M.L. Total ischaemia in dog hearts, in vivo 2. High energy phosphate depletion and associated defects in energy metabolism, cell volume regulation, and sarcolemmal integrity. *Circ. Res.* 1981; 49: 901-911
5. Opie L.H. Reperfusion injury and its pharmacologic modification. *Circulation* 1989; 80: 1049-1062
6. Hearse D.J. Reperfusion-induced injury: a possible role for oxidant stress and its manipulation. *Cardiovasc. Drugs Ther.* 1991; 5 (Suppl 2): 225-235
7. Hearse D. J. Reperfusion of the ischemic myocardium. *J. Mol. Cell. Cardiol.* 1977; 9(8): 605-16
8. Jennings R.B., Reimer K.A., Hill M.L., Mayer S.E. Total ischemia in dog hearts, in vitro. 1. Comparison of high energy phosphate production, utilization, and depletion, and of adenine nucleotide catabolism in total ischemia in vitro vs. severe ischemia in vivo. *Circ. Res.* 1981; 49(4): 892-900
9. Corr P.B. & Witkowski F.X. Potential electrophysiologic mechanisms responsible for dysrhythmias associated with reperfusion of ischemic myocardium. *Circulation* 1983; 68:I16-24
10. Kelly R.A., Balligand J-L., Smith T.W. Nitric oxide and cardiac function *Circ. Res.* 1996; 7: 363-380
11. Moncada S., Palmer R.M., Higgs E.A. Nitric oxide: physiology, pathophysiology, and pharmacology *Pharmacol Rev* 1991; 43(2):109-42
12. Lohmann S.M., Fischmeister R., Walter U. Signal transduction by cGMP in heart. *Basic Res Cardiol* 1991; 86(6):503-14
13. Liu Z, Rudd MA, Freedman JE, Loscalzo J S. Transnitrosation reactions are involved in the metabolic fate and biological actions of nitric oxide. *J Pharmacol Exp Ther* 1998; 284(2): 526-34
14. Yasmin W., Strynadka K.D., Schulz R. Generation of peroxynitrite contributes to ischaemia-reperfusion injury in isolated rat hearts. *Cardiovasc. Res.* 1997; 33: 422-432
15. Curtis M.J. & Pabla R. Nitric oxide supplementation or synthesis block-which is the better approach to treatment of heart disease? *Trends Pharmacol Sci* 1997; 18(7): 239-44
16. Lefer A.M. Attenuation of myocardial ischaemia-reperfusion injury with nitric oxide replacement therapy. *Ann. Thorac. Surg.* 1995; 60: 847-851
17. Woditsch I. & Schrör K. Prostacyclin rather than endogenous nitric oxide is a tissue protective factor in myocardial ischaemia *Am. J. Physiol.* 1992; H1390-H1396
18. Pabla R. & Curtis M.J. Effect of endogenous nitric oxide on cardiac systolic and diastolic function during ischaemia and reperfusion in the rat isolated perfused heart *J. Mol. Cell. Cardiol.* 1996; 28: 2111-2121
19. Matheis G., Sherman M.P., Buckberg G.D., Haybron D., Young H.H., Ignarro L.J. Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury *Am. J. Physiol.* 1992; 262: H616-H620
20. Schulz R. & Wambolt R. Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury *Cardiovasc. Res.* 1995; 30:432-439
21. Wang P. & Zweier, J.L. Measurement of nitric oxide and peroxynitrite generation in the postischaemic heart: evidence for peroxynitrite-mediated reperfusion injury. *J. Biol. Chem.* 1996; 271: 29223-29230
22. Murry C.E., Jennings R.B., Reimer K.A. Preconditioning with ischaemia: a delay of the lethal cell injury in ischaemic myocardium. *Circulation* 1986; 74: 1124-1136
23. Liu Y. & Downey J.M. Ischaemic preconditioning protects against infarction in rat heart. *Am. J. Physiol.* 1992; 263: H1107-H1112
24. Schott R.J., Rohmann S., Braun E.R., Schapper W. Ischaemic preconditioning reduces infarct size in swine myocardium. *Circ. Res.* 1990; 66: 1133-1142
25. Végh A, Szekeres L., Parratt J.R. Protective effects of preconditioning of the ischaemic myocardium involve cyclo-oxygenase products. *Cardiovasc. Res.* 1990; 24:1020-1023
26. Végh A., Szekeres L., Parratt J.R. Preconditioning of the ischamic myocardium; Involvement of the L-arginine nitric oxide pathway. *Br. J. Pharmacol* 1992; 107: 648-652
27. Li Y.W, Whittaker P., Kloner R.A. The transient nature of the effect of ischaemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am. Heart. J.* 1992; 123: 346-353
28. Shiki K., Hearse D.J. Preconditioning of ischaemic myocardium: reperfusion-induced arrhythmias. *Am. J. Physiol.* 1987; 253: H1470-H1476
29. Hagar J.M., Hale S.L., Kloner R.A., Effect of preconditioning ischaemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. *Circ. Res.* 1991; 68: 61-68

30. Cave A.C. Preconditioning induced protection against post-ischaemic contractile dysfunction: Characteristics and mechanisms *J. Mol. Cell. Cardiol.* 1995; 27: 969-979
31. Shaper W. Molecular mechanisms in "stunned" myocardium. *Cardiovasc. Drugs Ther.* 1991; 5: 925-932
32. Asimakis G.K., Innersmcbride K., Conti V.R. Attenuation of postischaemic dysfunction by ischaemic preconditioning is not mediated by adenosine in the isolated rat heart. *Cardiovasc. Res.* 1993; 27: 1522-1530
33. Kaplan L.J., Bellows, C.F., Blum H., Mitchell M., Whitman G.J.R. Ischaemic preconditioning preserves end-ischaemic ATP, enhancing functional recovery and coronary flow during reperfusion. *J. Surg. Res.* 1994; 57:179-184
34. Richard V., Blanc T., Kaeffer N., Tron C., Thuillez C. Myocardial and coronary endothelial protective effects of acetylcholine after myocardial ischaemia and reperfusion in rats: role of nitric oxide. *Br J Pharmacol* 1995; 115(8):1532-8
35. Yellon D.M., Alkhulaifi A.M., Pugsley W.B. Preconditioning the human myocardium. *Lancet* 1993; 342: 276-277
36. Végh A., Szekeres L., Parratt J.R. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc. Res.* 1991; 25:1051-1053
37. Ovize M., Przyklenk K., Kloner R.A. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992; 71(5):1165-73
38. Iwamoto T., Bai X.J., Downey H.F. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc. Res.* 1993; 27: 2071-2076
39. Szekeres L. On the mechanism and possible therapeutic application of delayed cardiac adaptation to stress. *Can J Cardiol* 1996; 12(2):177-85
40. Downey J.M., Liu G.S., Thornton J.D. Adenosine and the anti-infarct effects of preconditioning *Cardiovasc. Res.* 1993; 27(1): 3-8
41. Linz W., Wiemer G., Scholkens B.A. Role of kinins in the pathophysiology of myocardial ischemia. In vitro and in vivo studies *Diabetes* 1996; 45 Suppl 1: S51-8
42. Hattori Y., Szabo C., Gross S., Thiemermann C., Vane J.R. Lipid A and the lipid A analogue anti-tumour compound ONO-4007 induce nitric oxide synthase in vitro and in vivo. *Eur J Pharmacol* 1995; 291(2):83-9
43. Brown, J.M., Grosso, M.A., Terada, L.S., Whitman, G.J.R., Banerjee, A., White, C.W., Harken, A.H., Repine, J.E. Endotoxin pretreatment increases endogenous myocardial catalase activity and decreases ischaemia-reperfusion injury of isolated rat hearts. *Proc. Natl. Acad. Sci.* 1989; 86: 2516-2520
44. Nelson, D.W., Brown, J.M., Banerjee, A., Bensard, D.D., Rogers, K.B., Locke-Winter, C.R., Anderson, B.O., Harken, A.H. Pretreatment with a nontoxic derivative of endotoxin induces functional protection against cardiac ischemia/reperfusion injury. *Surgery* 1991; 110: 365-9
45. Kuzuya T., Hoshida S., Yamashita N., Fuji H., Oe H., Hori M., Kamada T., Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia *Circ. Res.* 1993; 72: 1293-1299
46. Marber M.S., Latchman D.S., Walker J.M., Yellon D.M.Cardiac stress protein elevation 24 hours after brief ischaemia or heat stress is associated with resistance to myocardial infarction *Circulation* 1993; 88:1264-1272
47. Végh A., Papp J. Gy., Kaszala K., Parratt J.R. Cardiac pacing in anaesthetised dogs preconditions the heart against arrhytmias when ischaemia is induced 24h later. *J.Physiol. (Lond)* 1994; 480: 89
48. Liu G.S., Thornton J., Van Winkle D.M., Stanley A.W., Olsson R.A., Downey J.M. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart *Circulation* 1991; 84: 350-356
49. Parratt J.R., Végh A., Zeitlin I.J., Ahmad M., Oldroyd K., Kaszala K., Papp J.Gy. Bradykinin and endothelial-cardiac myocyte interactions in ischemic preconditioning *Am. J. Cardiol.* 1997; 80(3A):124A-131A
50. Yamashita N., Hoshida S., Taniguchi N., Kuzuya T., Hori M. A "second window of protection" occurs 24 h after ischemic preconditioning in the rat heart. *Mol Cell Cardiol* 1998; 30(6):1181-9
51. Nayeem, M.A., Elliott, G.T., Shah, M.R., Hastillo-Hess, S.L., Kukreja, R.C. Monophosphoryl lipid A protects adult rat cardiomyocytes with specific induction of the 72-KD heat shock protein *J. Mol. Cell. Cardiol.* 1997; 29: 2303-2310
52. Grover G.J., Sleph P.G., Dzwonczyk S. Role of myocardial ATP-sensitive potassium channels in mediating preconditioning in the dog heart and their possible interaction with adenosine A1-receptors *Circulation* 1992; 86(4):1310-6
53. Gross G.J., Auchampach J.A. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992; 70(2): 223-33
54. Mei D.A., Elliott G.T., Gross G.J. KATP channels mediate late preconditioning against infarction produced by monophosphoryl lipid A. *Am J Physiol* 1996; 271(6 Pt 2): H2723-9

55. Végh A., Papp J. Gy., Szekeres L., Parratt J.R. The local intracoronary administration of methylene blue prevents the pronounced antiarrhythmic effect of ischaemic preconditioning *Br. J. Pharmacol* 1992; 107:910-911
56. Kaszala K. Protection against ischaemia and reperfusion-induced ventricular arrhythmias resulting from brief periods of cardiac pacing. Annals of Albert Szent-Györgyi Medical University
57. Parratt J.R. Endogenous protective substances. Cardiovasc. Res. 1993, 27: 693-702
58. Bilinska M., Maczewski M., Beresewicz A. Donors of nitric oxide mimic effects of ischaemic preconditioning on reperfusion induced arrhythmias in isolated rat heart Mol. Cell. Biochem. 1996; 160-161:265-271
59. Thiemermann C., Wu C.C., Szabo C., Perretti M., Vane J.R. Role of tumour necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. *Br J Pharmacol* 1993;110(1):177-82
60. Liu S., Adcock I.M., Old R.W., Barnes P.J., Evans TW Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem Biophys Res Commun* 1993; 196(3):1208-13
61. Song W., Furman B.L., Parratt J.R. Attenuation by dexamethasone of endotoxin protection against ischaemia-induced ventricular arrhythmias. *Br J Pharmacol* 1994; 113(4):1083-4
62. Kis A., Végh A., Papp J.Gy., Parratt J.R. Pacing–induced delayed protection against arrhythmias is attenuated by aminoguanidine, an inhibitor of nitric oxyde synthase *Br. J. Pharmacol* 1999; 127:1545-1550
63. Kis A., Végh A., Papp J.Gy., Parratt J.R. Repeated cardiac pacing extends the time during which canine hearts are protected against ischaemia-induced arrhythmias: role of nitric oxide. *J. Mol. Cell. Cardiol.* 1999; 31:1229-1241
64. Takano H., Manchikalapudi S., Tang X.L., Qiu Y., Rizvi A., Jadoon A.K., Zhang Q., Bolli R. Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 1998; 98(5):441-9
65. Imagawa J., Yellon D.M., Baxter G.F. Pharmacological evidence that inducible nitric oxide synthase is a mediator of delayed preconditioning. *Br J Pharmacol* 1999;126(3):701-8
66. Bolli R., Manchikalapudi S., Tang X.-L., Takano H., Qiu Y., Guo Y., Zhang Q., Jadoon A.K. The protective effects of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxyde synthase. *Circulation* 1997; 81: 1094-1107
67. Bolli R., Dawn B., Tang X.L., Qiu Y., Ping P., Xuan Y.T., Jones W.K., Takano H., Guo Y., Zhang J. The nitric oxide hypothesis of late preconditioning *Basic Res Cardiol* 1998; 93(5): 325-38
68. Xuan Y.-T., Tang X.-L., Banerjee S., Takano H., Li R.C.X., Han H., Qiu Y., Li J.J., Bolli R. Nuclear factor-kB an essential role in the late phase of ischaemic preconditioning in conscious rabbits. *Circ. Res.* 1999; 1095-1109
69. Ribi, E. Beneficial modification of the endotoxin molecule. *J. Biol. Response Modif.* 1984; 3: 1-9
70. Yao, Z., Auchampach, J.A., Pieper, G.M., Gross, G.J. Cardioprotective effects of monophosphoryl lipid A, a novel endotoxin analog, in the dog. *Cardiovasc. Res.* 1993; 27: 832-838
71. Yao, Z., Rasmussen, J.L., Hirt, J.L., Mei, D.A., Pieper, G.M., Gross, G.J. Effects of monophosphoryl lipid A on myocardial ischaemia/reperfusion injury in dogs. *J. Cardiovasc. Pharmacol.* 1993; 22: 653-663
72. Végh A., György K., Rastegar M.A., Papp J.Gy., Parratt J.R. Delayed protection against ventricular arrhythmias by monophosphoryl lipid A in a canine model of ischaemia and reperfusion. Eur. J. Pharmacol. 1999; 382: 81-90
73. Elliott, G.T. Monophosphoryl lipid A induces delayed preconditioning against cardiac ischaemia-reperfusion injury *J. Mol. Cell. Cardiol.* 1998; 30: 3-17
74. Baxter, G.F., Goodwin, R.W., Wright, M.J., Kerac, M., Heads, R.J., Yellon, D.M. Myocardial protection after monophosphoryl lipid A: studies of delayed anti-ischaemic properties in rabbit heart. *Br. J. Pharmacol.* 1996; 117: 1685-1692
75. Song, W., Furman, B.L., Parratt, J.R. Monophosphoryl lipid A reduces both arrhythmia severity and infarct size in a rat model of ischaemia. *Eur. J. Pharmacol.* 1998; 345: 285-287
76. Zhao, L, Weber, P.A., Smith, J.R., Comerford, M.L., Elliott, G.T. Role of inducible nitric oxide synthase in pharmacological «preconditioning» with monophosphoryl lipid A. *J. Mol. Cell. Cardiol.* 1997; 29: 1567-1576
77. Tosaki, A., Maulik, N., Elliott, G.T., Blasig, I.E., Engelman, R.M., Das, D.K. Preconditioning of rat heart with monophosphoryl lipid A: a role for nitric oxide. *J. Pharmacol. Exp. Ther.* 1998; 285: 1274-1279
78. Xi L., Jarrett N.C., Hess M.L., Kukreja R.C. Essential role of inducible nitric oxide synthase in monophosphoryl lipid A-induced late cardioprotection: evidence from pharmacological inhibition and gene knockout mice. *Circulation* 1999; 99: 2157-2163
79. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P. Superoxide generation by endothelial nitric oxide synthase. the influence of cofactors. Proc. Natl. Acad. Sci. 1998, 95: 9220-9225

80. Kukreja, R.C., Hess, M.L. The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. *Cardiovasc. Res.* 1992; 6: 641-655
81. Zweier, J.L., Flaherty, J.T., Weisfeldt, M.L. Direct measurement of free radical generation following reperfusion of ischaemic myocardium. *Proc. Natl. Acad. Sci.* 1987; 84: 1404-1407.
82. Henry, T.D., Archer, S.L., Nelson, D., Weir, E.K., From, A.H. Enhanced chemiluminescence as a measure of oxygen-derived free radical generation during ischaemia and reperfusion. *Circ. Res.* 1990; 67: 1453-1461
83. Bolli, R., Patel, B.S., Jeroudi, M.O., Lai, E.K., McCay, P.B. Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alphaphenyl-N-tert-butyl nitrone. *J. Clin. Invest.* 1988; 82: 476-485
84. Ambrosio, G., Becker, L.C., Hutchins, G.M., Weisman, H., Weisfeldt, M.L. Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury. *Circulation* 1986; 74: 1424-1433
85. Jolly, S.R., Kane, W.J., Bailie, M.B., Abrams, G.D., Lucchesi, B.R. Canine myocardial reperfusion injury. Its reduction by combined administration of superoxide dismutase and catalase. *Circ. Res.* 1984; 54: 277-285
86. Schraufstatter, I.U., Hyslop, P.A., Hinshaw, D.B., Spragg, R.G., Sklar, L.A., Cochrane, C.G. Hydrogen peroxide induced injury and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc. Natl. Acad. Sci. U.S.A.*, 1986; 83: 4908-4912
87. Le Page, C., Sanceau, J., Drapier, J.C., Wietzerbin, J. Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NFkB activation. Biochem. Biophys. Res. Commun. 1998, 243: 451-457
88. Thiemermann C., Bowes J., Myint F., Vane J.R. Inhibition of the activity of poly(ADP-ribose) synthetase reduces ischaemia-reperfusion injury in the heart and skeletal muscle. *Proc. Natl. Acad. Sci. USA* 1997; 94: 679-683
89. Zingarelli B., Cuzzocrea S., Zsengeller Z., Salzman A.L., Szabo C. Protection against myocardial ischaemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthetase. *Cardiovasc. Res.* 1997; 36: 205-215
90. Docherty J.C., Kuzio B., Silvester J., Bowes J., Thiemermann C. An inhibitor of poly(ADP-ribose) synthetase activity reduces contractile dysfunction and preserves high energy phosphate levels during reperfusion of the ischaemic rat heart. *Br. J. Pharmacol.* 1999; 127: 1518-1524
91. Végh A, Komori S., Szekeres L., Parratt J.R. Antiarrhythmic effects of preconditioning in anaesthetized dogs and rats. *Cardiovasc. Res.* 1992; 26: 487-495
92. Walker M.J.A., Curtis M.J., Hearse D.J., Campbell R.W.F., Janse M.J., Yellon D.M., Cobbe S.M., Coker S.J., Harness J.B., Harron D.W.G., Higgins A.J., Julian D.G., Lab M.J., Manning A.S., Northover B.J., Parratt J.R., Riemersma R.A., Riva E., Russell D.C., Sheridan D.J., Winslow E., Woodward B., The Lambeth Conventions: guidelines for the study of arrhytmias in ischaemia, infarction and reperfusion. *Cardiovasc. Res.* 1988; 22: 447-455
93. Végh A., Szekeres L., Parratt J.R. Protective effects of preconditioning of the ischaemic myocardium involve cyclo-oxygenase products. *Cardiovasc. Res.* 1990; 24: 1020-1023
94. Misko T.P., Moore W.M., Kasten T.P., Nickols G.A., Corbett J.A., Tilton R.G., McDaniel M.L., Williamson J.R., Currie M.G. Selective inhibition of inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.* 1993; 233: 119-125
95. Hasan K., Heesen B-J., Corbett J.A., McDaniel M.L., Chang K., Allison W., Wolffenbuttel B.H.R., Williamson J.R., Tilton R.G. Inhibition of nitric oxide formation by guanidines. *Eur. J. Pharmacol.* 1993; 249: 101-106
96. Vanin A.F., Mordvintcev P.I., Kleschyov A.L. Appearance of nitrogen oxide in animal tissues *in vivo*. Stud. Biophys. 1984; 102: 135-143
97. Mülsch, A., Mordvintcev, P., Bassenge, E., Lung, F., Clement, B., Busse, R. In vivo spin trapping of glycerol trinitrate-derived nitric oxide in rabbit blood vessels and organs *Circulation.* 1995; 92: 1876-1882
98. Stockman M.B., Verrier R.L., Lown B. Effect of nitroglycerin on vulnerability to ventricular fibrillation during myocardial ischaemia and reperfusion. *Am. J. Cardiol.* 1979; 43: 233-238
99. Gross G.J. The cardioprotective effects of nicorandil. *Rev. Contemp.* Pharmacother. 1993; 4: 99
100. Grover G.J. Protective effects of ATP sensitive potassium channel openers in models of myocardial ischaemia. *Cardiovasc. Res.* 1994; 28: 778
101. Billman G.E. Role of ATP sensitive potassium channel in extracellular potassium accumulation and cardiac arrhythmias during myocardial ischaemia. *Cardiovasc. Res.* 1994; 28: 762
102. Connor, J.R., Manning, P.T., Settle, S.L., Moore, W.M., Jerome, G.M., Webber, R.K., Tjoeng, F.S., Currie, M.G. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase *Eur. J. Pharmacol.* 1995; 273, 15-24
103. Griffiths, M.J.D., Messent, M., Macallister, R.J., Evans, T.W. Aminoguanidine selectively inhibits inducible nitric oxide synthase *Br. J. Pharmacol.* 1993; 11, 963-968

104.Yildiz G., Demiryurek A.T., Sahin-Erdemli I., Kanzik I. Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. *Br J Pharmacol* 1998; 124(5): 905-10
105.Southan, G.J. & Szabo, C. Selective pharmacological inhibition of distinct nitric oxide synthases with variable isoforms selectivity. *Biochem. Pharmacol.* 1996; 51: 383-394
106.Moore, W.M., Webber, R.K., Jerome, G.M., Tjoeng, F.S., Misko, T.P., Currie, M.G. L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J. Med. Chem.* 1994; 37: 3886-3888.
107.Frank S., Zacharowski K., Wray G.M., Thiemermann C., Pfeilschifter J. Identification of copper/zinc superoxide dismutase as a novel nitric oxide-regulated gene in rat glomerular mesangial cells and kidneys of endotoxemic rats. *FASEB J* 1999; 13(8): 869-82
108.Malyshev, I.Y., Malugin, A.V., Manukhina, E.B., Larionov, N.P., Malenyuk, E.B., Malysheva, E.V., Mikoyan, V.D., Vanin, A.F. Is HSP70 involved in nitric oxide-induced protection of the heart ? *Physiol. Res.* 1996; 45, 267-272
109.Brune B., von Knethen A., Sandau K.B. Nitric oxide and its role in apoptosis. *Eur J Pharmacol* 1998; 351(3): 261-72
110.Yang, B.C., Chen, L.Y., Saldeen, T.G.P., Mehta, J.L. Reperfusion injury in the endotoxin-treated rat heart: reevaluation of the role of nitric oxide. *Br. J. Pharmacol.* 1997; 120: 305-311
111.Grupp I.L., Jackson T.M., Hake P., Grupp G., Szabo C. Protection against hypoxia-rexygenation in the absence of poly(ADP-ribose) synthetase in isolated working hearts. *J. Mol. Cell. Cardiol.* 1999; 31: 297-303
112.Sato H., Zhao Z.Q., McGee D.S. Supplemental L-arginine during cardioplegic arrest and reperfusion avoids regional postischaemic injury. *J. Thorac. Cardiovasc. Surg.* 1995; 110: 302-314
113.Nakanishi K, Zhoa Z.Q., Vinten-Johansen J. Blood cardioplegia enhanced with the nitric oxide donor SPM-5185 counteracts postischaemic endothelial and ventricular dysfunction. *J. Thorac. Cardiovasc. Surg.* 1995; 109:1146-1154

6. Acknowledgements

I would like to thank Professor Julius Gy. Papp for providing me with the facilities to carry out my studies in the Department of Pharmacology and Pharmacotherapy, Albert Szent-Györgyi Medical University and also for his support in the development of my thesis.

I would like to express my respect and gratefulness to my friend and teacher, Professor Ágnes Végh for her encouragement and her guidance with excellent scientific knowledge in my studies.

I would like to thank Professor Jean-Claude Stoclet for giving me the opportunity to realise a „co-tutelle" thesis and his excellent guidance in my experiments.

I must also thank Professor James R. Parratt, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow for his great help and his kind support over the past years.

I wish to acknowledge the fruitful collaboration and the nice moments shared with my friends Bernard Muller and Andrei L. Kleschyov within the Strasbourg Department.

I also thank all of my collegues from Szeged - Erika Tóth, Gábor Girst, Ágnes Krajevszki, Mária Kosztka and from Strasbourg - Christophe Untereiner and Denis Wagner for their assistance in carrying out my experiments.

A special thank-you goes to my parents and my brother for their help, patience and understanding during the past years and finally to my husband without who I wouldn't have been able to finish my PhD studies.