Protection against ischaemia and reperfusion-induced ventricular arrhythmias: role of bradykinin

PhD Thesis

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SUMMARY

There is a great deal of evidence that in the canine ischaemic preconditioning induced by one or more brief episodes of coronary artery occlusion provides marked protection against ventricular arrhythmias which result from a more prolonged ischaemic episode. The aim of the present study was three fold; (*i*), to analyse the time-course of the antiarrhythmic protection induced by a single brief 5 (min) preconditioning occlusion, (*ii*), to examine whether the antiarrhytmic protection achieved by ischaemic preconditioning can be enhanced or prolonged by the coadministration of the angiotensin converting enzyme (ACE) inhibitor enalaprilate or by the combined ACE and neutral endopeptidase (NEP) inhibitor Z13752A; both of which are known to increase endogenous bradykinin levels, and (*iii*), to investigate the possible antiarrhythmic effect of atrial natriuretic peptide (ANP) in our canine model of ischaemia and reperfusion.

We have demonstrated that one brief (5 min) period of occlusion of the left anterior descending coronary artery (LAD) resulted in marked protection against ventricular arrhythmias, induced by prolonged (25 min) occlusion of the same artery. This protection started to fade 15 min after ischaemic preconditioning and almost completely disappeared if the time interval between the preconditioning occlusion and the prolonged occlusion was extended to 60 min. Neither enalaprilate nor Z13752A were able to prolong or enhance this antiarrhythmic effect of ischaemic preconditioning although both drugs, given alone prior to prolonged ischaemia significantly reduced the severity of ventricular arrhythmias during occlusion and increased survival of the dogs (50% and 67%, respectively) from the combined ischaemia and reperfusion insult. Thus, when these drugs were infused in preconditioning dogs with 15 and 60 min reperfusion, the severity of arrhythmias was again increased and survival decreased, particularly in preconditioned dogs treated with enalaprilate. We have also demonstrated that ANP markedly reduced the incidence and severity of venricular arrhythmias resulted from ischaemia and reperfusion.

We conclude from these results that (*i*) one single episode of coronary artery occlusion can protect the myocardium against severe ventricular arrhythmias and this protection lasts less than 1h., (*ii*) increasing bradykinin levels by drugs in preconditioned dogs does not provide additional protection to preconditioning, (*iii*) ANP protects the myocardium against ischaemia and reperfusion-induced ventricular arrhythmias.

CONTENTS

LIST OF PUBLICATIONS

Full papers

1. **Rastegar M.A.,** Marchini F., Morazzoni G., Végh Á., Papp J.Gy., Parratt J.R. The effects of Z13752A, a combined ACE/NEP inhibitor, on responses to coronary artery occlusion; a primary protective role for bradykinin. *Br. J. Pharmacol* 2000; **129:** 671-680.
2. **Rastegar M.A.,** Végh. Á., Papp J.Gy., Parratt J.R. Atrial natriuretic peptide reduces the severe consequences of coronary artery occlusion in anaesthetised dogs. *Cardiovasc Drugs Ther* 2000. (in press)
3. Végh Á., 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.
4. György K., Végh Á., **Rastegar M.A.,** Papp J.Gy., Parratt J.R. Isosorbide-2-mononitrate reduces the consequences of myocardial ischaemia, including arrhythmia severity: implications of preconditioning. *Cardiovasc Drugs Ther* 2000. (in press)

Abstracts

1. **Rastegar M.A.,** Végh Á., Papp J.Gy., Parratt J.R. Can the already preconditioned myocardium be additionally protected by preconditioning at a distance? *J Mol Cell Cardiol* 1997; **29**: A121.
2. György K., Végh Á., **Rastegar M.A.,** Papp J.Gy., Parratt J.R. 2-isosorbide mononitrate reduces the consequences of ischaemia in anaesthetised dogs. *J Mol Cell Cardiol* 1998; **30**: A82.
3. **Rastegar M.A.,** Végh Á., Papp J.Gy., Parratt J.R., Semeraro C., Marchini F. Does inhibition of bradykinin catabolism modify the severity of arrhythmias in myocardial ischaemia. *J Mol Cell Cardiol* 1998; **30**: A6.
4. **Rastegar M.A.,** Végh Á., Papp Gy., Parratt J.R. The antiarrhythmic effects of ANP in a canine model of ischaemia-reperfusion. *Szote IV. Ph.D. Előadói Napok.*, Szeged, 1998; **38**p.
5. Végh Á., **Rastegar M.A.,** Papp J.Gy., Parratt J.R. The antiarrhythmic effects of ANP in a canine model of ischaemia-reperfusion. *J Mol Cell Cardiol* 1998; **30**: A7.
6. **Rastegar M.A.,** Végh Á., Papp J.Gy., Parratt J.R., Semeraro C., Marchini F. Does inhibition of bradykinin catabolism reduces the severity of ventricular arrhythmias resulting from ischaemia-reperfusion in anaesthetised dogs. *The 6th Joint Meeting of the Italian, Hungarian and Polish Pharmacological Societies.*, Pisa, 1998; 45p.

7. Végh Á., **Rastegar M.A.,** Papp J.Gy., Parratt J.R. Atrial natriuretic peptide infusion reduces the severity of ventricular arrhythmias in a canine model of ischaemia-reperfusion. *6th Joint Meeting of the Italian, Hungarian and Polish Pharmacological Societies.*, Pisa, 1998; 45p.
8. **Rastegar M.A.,** Végh Á., Papp J.Gy., Parratt J.R. Does ACE or NEP inhibition provide additional protection to ischaemic preconditioning? *British Society for Cardiovascular Research* 1999; London, Ap 4.
9. **Rastegar M.A.,** Papp J.Gy., Marchini F., Pradella L., Parratt J.R., Végh Á. Involvement of bradykinin in the antiarrhythmic effect of Z13752A, a novel neutral endopeptidase inhibitor. *Szote V. Ph.D. Előadói Napok.*, Szeged, 1999; 36.
10. **Rastegar M.A.,** Végh Á., Papp J.Gy., Marchini F., Pradella L., Parratt J.R. The role of bradykinin in the antiarrhythmic effects of the neutral endopeptidase inhibitor, Z13752A. *J Mol Cell Cardiol* 1999; **31**: A64.
11. **Rastegar M.A.,** Papp J.Gy., Marchini F., Pradella L., Parratt J.R., Végh Á. Involvement of bradykinin in the antiarrhythmic effect of Z13752A, a novel neutral endopeptidase inhibitor. *Fundam Clin Pharm* 1999; **13** (suppl. 1): 224s.
12. **Rastegar M.A.,** Papp J.Gy., Parratt J.R., Végh Á. Atrial natriuretic peptide (ANP) reduces the severity of ventricular arrhythmias resulting from coronary artery occlusion and reperfusion in anaesthetised dogs. *Cardiol Hung* 1999; Suppl. **2**: 29.
13. **Rastegar M.A.,** Papp J.Gy., Parratt J.R., Végh Á. Does the increased bradykinin level following ACE and NEP inhibition provide additional protection to ischaemic preconditioning. *8th Alpe Adria Cardiology Meeting*, Portoroz, Slovenia 2000; 55p.

1. INTRODUCTION

Interruption of the nutritional blood flow to the heart muscle results in a state of ischaemia. The heart muscle cells begin to die approximately 20 min after the onset of ischaemia and 3h later the whole ischaemic myocardium virtually becomes infarcted. Myocardial ischaemia is often accompanied by serious ventricular tachyarrhythmias, which are still the most common cause of sudden cardiac death in the developed countries. This is due, at least in part, to the lack of the success of the reperfusion therapy, if the time interval between the onset of the acute ischaemic attack and the initiation of treatment increases. In the absence of early reperfusion, more myocardium could be saved if the development of ischaemic injury is slowed or delayed.

1.1. Cardioprotection by brief periods of ischaemia; the phenomenon of ischaemic preconditioning

In 1986, Murry and colleagues (1) showed in anaesthetised dogs that myocardial infarction, that resulted from a 40 min coronary artery occlusion and reperfusion of the left circumflex coronary artery, was markedly reduced if the same coronary artery had been occluded for four 5 min periods just prior to the prolonged ischaemia. In these dogs myocardial ATP levels were also significantly preserved. This phenomenon was described and termed as "ischaemic preconditioning" (1).

We now know that ischaemic preconditioning not only offers an extremely powerful protection against ischaemic damage (2,3), but it also reduces the severity of ischaemia (4-7) and reperfusion-induced (6,8,9) ventricular arrhythmias, decreases the postischaemic contractile dysfunction (10), enhances the recovery of myocardial function during reperfusion (11-13), blunts the loss in vasodilator reserve (14-16) and improves metabolic disturbances that associated with myocardial ischaemia (17). These protective effects of preconditioning can occur in all species studied so far, such as in dogs (1,5), rats (8,18), rabbits (19,20), pigs (3,21) and also in humans (22,23).

There is now good evidence that preconditioning can be induced by other stimuli than short coronary artery occlusions. Thus, rapid cardiac pacing (24), partial occlusion of the coronary artery (25), and alteration in myocardial oxygen supply-demand balance (26) can serve as preconditioning stimuli. Administration of various substances, such as prostacycline (27), adenosine (28), angiotensin converting enzyme (ACE) inhibitor (29), interferon χ (30), bacterial endotoxin (31), monophosphoryl lipid A (32,33) and nitric oxide donors (34,35) may also protect the myocardium. Application of these drugs can be considered as "pharmacological preconditioning".

1.2. The antiarrhythmic effects of ischaemic preconditioning

There are good reasons for believing that the antiarrhythmic effect of ischaemic preconditioning is an even more important manifestation of this adaptive phenomenon than that is the reduction in myocardial ischaemic damage (35) since the occurrence of severe ventricular

arrhythmias and sudden cardiac death, resulting from ventricular fibrilation, are threatening consequences of the ischaemia and reperfusion injury. Shiki and Hearse (8) were the first to demonstrate the ability of ischaemic preconditioning to protect against reperfusion induced arrhythmias in rats. Then, Komori and his colleagues (4) showed in anaesthetized rats, that one brief period (3 min) of ischaemia reduced the severity of arrhythmias during a subsequent more prolonged (30 min) period of coronary artery occlusion. Using an anaesthetized dog model, Végh and colleagues (5,6) showed that two brief (5 min) periods of occlusion of the left anterior desending coronary artery, resulted in marked protection against those ventricular arrhythmias which occurred during a 25 min period of occlusion of that same artery. The most striking feature of this protection was the absence of ventricular fibrillation in preconditioned dogs during occlusion; in contrast to 40% in the controls. Furthermore, all the control dogs died following reperfusion whereas 50% of dogs subjected to preconditioning survived the combined ischaemia reperfusion insult. The antiarrhythmic effects of ischaemic preconditioning, thus demonstrated in rats and dogs, were *marked* (absence of ventricular fibrillation), *real* (if the duration of the occlusion was extended to 60 min the occurrence of arrhythmias was not shifted to a later time of the occlusion), but *transient*, i.e. the protection was lost if the time interval between the preconditioning stimulus and the prolonged occlusion was extended to 30 or 60 min (35-37).

1.3. The time-dependent characteristics of ischaemic preconditioning

Among the several unanswered questions, relating to the antiarrhythmic effects of preconditioning, one is that which concerns with the number and the duration of the preconditioning stimulus required for optimum protection. For example, in rat isolated perfused hearts the protection against reperfusion-induced ventricular arrhythmias can be enhanced by increasing the number of cycles of the preconditioning occlusion (37). In contrast, a single period of preconditioning occlusion for 5 min appears to be sufficient to provide maximal protection against myocardial necrosis in dogs and rabbits (38-40) and no further benefit is derived from additional cycles. Similarly, Végh and colleagues showed in anaesthetised dogs that preconditioning, induced either by one or two 5 min periods of occlusion, results in almost similar protection against ischaemia and reperfusion induced ventricular arrhythmias (41). They have also demonstrated that the optimum duration of the preconditioning occlusion against arrhythmias in rats is 3 min; 1 min period of preconditioning occlusion was ineffective, whereas the 5 min occlusion period resulted in high incidence of ventricular arrhythmias following reperfusion (6). Furthermore, Li and colleagues (39) showed in dogs that one 5 min period of preconditioning occlusion reduced the incidence of ventricular fibrillation but when the number of the preconditioning occlusion had been increased to twelve, the mortality was markedly increased during the subsequent prolonged occlusion. There is evidence that repeated, brief periods of coronary artery occlusion lead to progressive deterioration of cardiac myocyte function especially at the level of the mitochondria (42). In the rabbit, Miura and colleagues (40) showed that a single 5 min preconditioning occlusion was more effective to limit infarct size than the 2 min period of coronary artery occlusion. However, if this 2 min occulusion was repeated

twice (2x2 min) the protection was similar to that with a single 5 min preconditioning occlusion. Although the protection, resulting from preconditioning is very prounounced, it is transient. The power of the protection may depend on the strengh of the ischaemic stimulus. This seems to be determined by the number and the duration of the preconditioning occlusion (36,37) and also by the time interval that elapse between the preconditioning stimulus and the prolonged ischaemic insult (6,36). For example, if the time interval between the preconditioning stimulus and the prolonged occlusion is increased to 1 h, the protection is markedly attenuated or even abolished (6,43,44). However, the protection reappears 24 h later and then it may last for at least 72 h (45-47). This second phase of the protection is termed as delayed, or "second window of protection" (48).

1.4. The possible mechanisms of ischaemic preconditioning; involvement of endogenous myocardial protective substances

Although the precise mechanism of ischaemic preconditioning is still uncertain, most of the investigators accept the hypothesis that endogenous myocardial substances, released either from ischaemic cardiac myocytes or endothelial cells or both, are involved (49-51). These mediators, which are released during the early phase of myocardial ischaemia, might be either protective (adenosine, nitric oxide, prostanoids, bradykinin) or potentially detrimental (endothelin, noradrenaline, potassium, etc; 50). It is also likely that more than one protective mediator is released during ischaemia to compensate the harmful consequences of the ischaemic injury. These endogenous mediators, acting at different receptors, might induce protection in different ways or, perhaps, there is a final common pathway. The role of adenosine, as originally described by Downey and colleagues (19), with the subsequent activation of protein kinase C (52), in mediating the limitation of infarct size associated with ischaemic preconditioning, is well described in most species. However, it seems likely that adenosine plays no substantial role in the antiarrhythmic effect of ischaemic preconditioning either in rats (53,54) or dogs (55), despite the fact that in these species adenosine is an endogenous antiarrhythmic substance (56). The first evidence, that other endogenous protective substances, such as bradykinin, nitric oxide and prostanoids, are also involved in the cardioproptective effects of ischaemic preconditioning, comes from studies performed in anaesthetised dogs (35,57,58). According to this hypothesis, as illustrated in figure 1, during the early stages of ischaemia (i.e. preconditioning), bradykinin is released from endothelial cells. This activates endothelial bradykinin B_2 receptors and induces the formation and the release of nitric oxide (NO) and prostacyclin from endothelial cells. Nitric oxide then diffuses to cardiac myocytes, stimulates soluble guanylate cyclase and elevates cyclic GMP levels. This would reduce myocardial contractility and energy demand perhaps by stimulating the cGMP-dependent phosphodiesterase enzyme. Cyclic GMP could also inhibit the voltage-dependent L-type calcium channels (59) and reduce the influx of calcium which is a key player in the generation of arrhythmias and also in myocardial cell death.

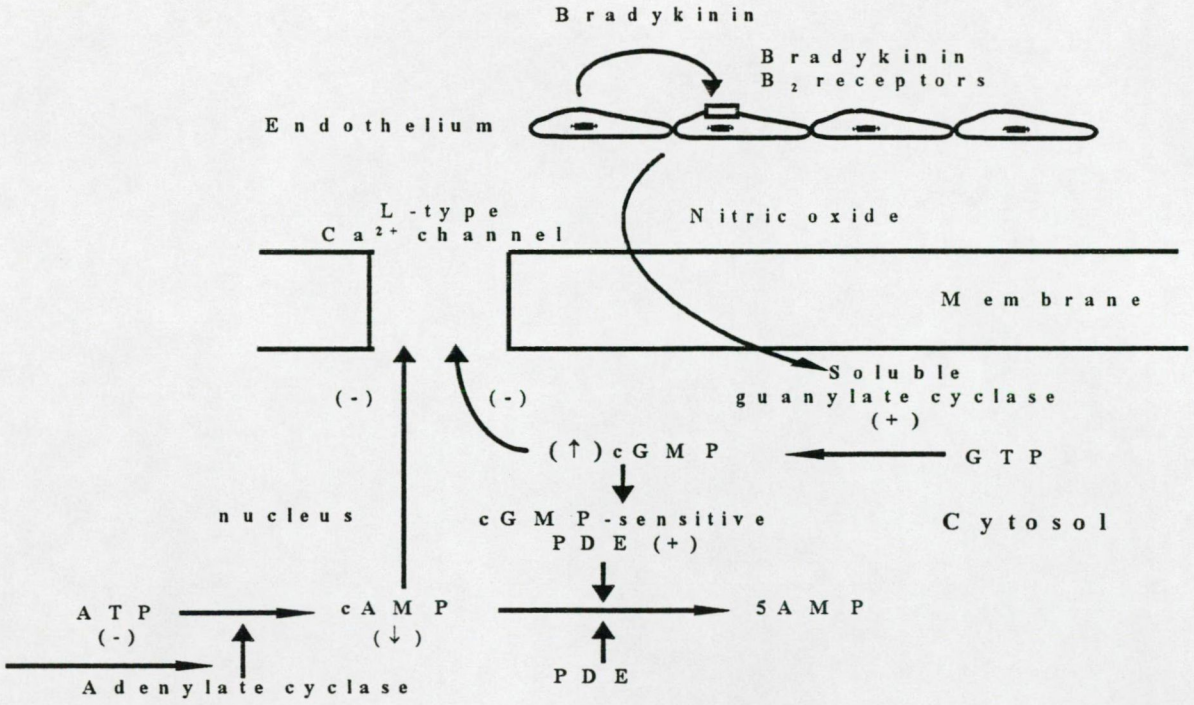


Figure 1. Possible mechanisms involved in the antiarrhythmic effects of preconditioning (adapted, by permission, from ref. 35).

1.4.1. The role of bradykinin in the antiarrhythmic effects of ischaemic preconditioning

The first evidence that bradykinin might be involved in the cardioprotective effects of preconditioning comes first from studies of Végh and colleagues (60,61). They showed in anaesthetised dogs that local administration of bradykinin markedly reduced the severity of ventricular arrhythmias resulted from a 25 min occlusion of the left anterior descending coronary artery (60). Furthermore, the protective effect of preconditioning against arrhythmias was abolished by icatibant, a selective antagonist of bradykinin at bradykinin B_2 receptors (61). They have also demonstrated that the antiarrhythmic effect of bradykinin is largely mediated by NO, since the protection was markedly attenuated in the presence of L-NAME (62). Similarly, Hecker and colleagues reported that activation of bradykinin B_2 receptors is responsible for the release of prostacycline and the generation of nitric oxide by bradykinin (63).

There is evidence that bradykinin levels can be elevated by other means than ischaemic preconditioning. It is well documented that ACE plays an important role role in bradykinin metabolism and that inhibition of ACE elevates bradykinin levels (64-66). The cardioprotective effects of ACE inhibition in myocardial ischaemia are well documented both in experimental animals (67,68) and humans (69,70) and there is a clear role for bradykinin in mediating these protective effects (64,67,71-73). The other enzyme which is also involved in bradykinin metabolism is the neutral endopeptidase (NEP) which is a membrane bound metallopeptidase present in endothelial cells (74) and in cardiomyocytes (75). NEP has a high affinity for a variety of vasoactive peptides including substance P, bradykinin, atrial natriuretic peptide (ANP) and endothelin (74). In the heart this is a particularly important enzyme responsible for kinin degradation (75-77). If we accept the hypothesis that bradykinin is an important mediator of

ischaemic preconditioning and that drugs which inhibit both ACE and NEP enzymes act by elevating bradykinin levels, then we might expect that these drugs can mimic the protective effects of preconditioning and they would be important in the therapy of ischaemic heart disease. Such a combined ACE/NEP inhibitor is the Z13752A (N-[(2S)-3-mercapto-2-phenylmethylpropionyl]-4-(2-thiazolyl)-L-phenylalanine) compound. Z13752A is a newly developed ACE/NEP inhibitor with an IC_{50} of 3.2 nM on ACE and of 1.8 on NEP (78). Z13752A has been found to potently inhibit plasma and tissue ACE, as well as tissue NEP activity in various *in vitro* and *in vivo* experiments. Z13752A resulted in a long lasting antihypertensive effect in both SHR and DOCA-salt hypertensive rats after intravenous or oral administration (79,80).

1.4.2. The role of nitric oxide in ischaemic preconditioning

Végh and colleagues porposed for the first time that nitric oxide is involved in the antiarrhythmic effects of ischaemic preconditioning. In anaesthetised dogs inhibition of the L-arginine-nitric oxide pathway by L-NAME (N^G-nitro-arginine methyl ester) markedly attenuated the antiarrhythmic effects of preconditioning (81). Similarly, the local, intracoronary infusion of methylene blue (an inhibitior of both L-arginine-NO synthesis and guanylyl cyclase enzyme) completely abolished the protective effect of preconditioning against arrhythmias (82). Thus, it was concluded that NO, acting through the guanylyl-cyclase-cGMP system, is one of the main mediators of the antiarrhythmic effects of classical preconditioning (81).

The proposal, that elevation of cGMP in the myocardium might reduce the susceptibility to ventricular arrhythmias, raised first by Opie (83). This was confirmed by studies of Billman (84), using a canine model with healed myocardial infarction. He showed that both carbacol and 8-bromo-cyclic GMP reduced the incidence of ventricular fibrillation, induced by a brief coronary artery occlusion and exercise. Elevation of cGMP can influence arrhythmogenesis in different ways (Figure 1). These might include depression of myocardial contractility by nitric oxide (85), inhibition of calcium influx through the L-type calcium channels (86) and stimulation of cGMP dependent phosphodiesterase and subsequent reduction in myocardial cAMP levels.

1.4.3. The cardioprotective effects of atrial natriuretic peptide

ANP is another endogenous myocardial substance which elevates myocardial cGMP. This peptide is secreted into the circulation mainly from the atrium (87) in response to a variety of stimuli, such as atrial stretch (88), acut hypoxia (89), and cardiac pacing (90). ANP has a wide range of potent biological effects, including coronary vasodilation (91,92), natriuresis and inhibition of the renin-angiotensin-aldosterone system (93). Marmuo and colleagues reported that ANP aguments induction of iNOS in the rat vascular smooth muscle cells (94). Similarly, Yamamoto and colleagues showed that ANP potentiates the cytokine-stimulated NO synthesis in cardiac myocytes and this is mediated partially via activation of a cGMP-dependent protein kinase (95). In a canine model, Takata and colleagues (96) found that administration of ANP

elevates cGMP and prevents reperfusion arrhythmias. However, there is no information whether ANP reduces ischaemia-induced ventricular arrhythmias.

1.5. Aim of the study

1. To analyse the time-course of the antiarrhythmic protection induced by a single brief (5 min) preconditioning occlusion in anaesthetised dogs.
2. To examine the effect of the ACE inhibitor enalaprilate and the combined ACE/NEP inhibitor Z13752A against ischaemia and reperfusion-induced ventricular arrhythmias in anaesthetised dogs.
3. To explore whether protection, resulted from ischaemic preconditioning, can be enhanced or prolonged by elevating bradykinin levels. For this purpose enalaprilate and Z13752A were administrated in preconditioned dogs. Both drugs supposed to increase bradykinin levels.
4. To examine whether ANP, given intravenously in anaesthetised dogs, protects the myocardium against ischaemia and reperfusion-induced ventricular arrhythmias.

2. METHODS

2.1. Experimental animals

Adult mongrel dogs of either sex, weighing in excess of 17 kg and allowed to access to food and water *ad libitum a day before* until starting the experiments, were used. All animals received humane treatment according to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication number NO 85-23, 1985) and local institutional policy.

2.2. Surgical interventions

Dogs were anaesthetised intravenously with a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively) and ventilated with room air using a Harvard ventilator at a rate of 15 strokes min^{-1}. The stroke volume was adjusted to maintain pH and blood gases within normal limits: pH 7.40 ± 0.04; PaO_2 85 ± 1.3 mm Hg, $PaCo_2$ 30 ± 1.1 mmHg. Body temperature was monitored from the oesophagus and maintained at 37 ± 0.6 °C by means of a heating pad.

Thoracotomy was performed at the fifth intercostal space and the heart was suspended in a pericardial cradle. The anterior branch of the left coronary artery (LAD) was dissected free about 2 cm from its origin, just above the first marginal branch, and a silk thread was loosely placed around it (Figure 2). Myocardial ischaemia was induced by passing the thread through a small plastic tube and then pulling the suture while pressing the tube against the surface of the myocardium. Reperfusion was initiated by releasing the ligature and removing the plastic tube. In some experiments, proximal to the occlusion site, a Doppler flow probe was placed around the coronary artery by which changes in flow velocity (cm s^{-1}) were evaluated. The circumflex branch (LCX) of the left coronary artery was also prepared. An electromagnetic flow probe was placed around this coronary artery, attached to a Statham SP 2202 flowmeter.

Polyvinyl catheters were inserted into the right femoral artery for monitoring arterial blood pressure (systolic: SABP and diastolic DABP), into the left ventricle via the left carotid artery to measure the left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures and dP/dt_{max}. Catheters introduced into the right femoral vein were used for the administration of anaesthetic and drugs. All these parameters were measured by means of a Statham P23XL pressure transducer and recorder on a Medicor R-81 recorder (Figure 2).

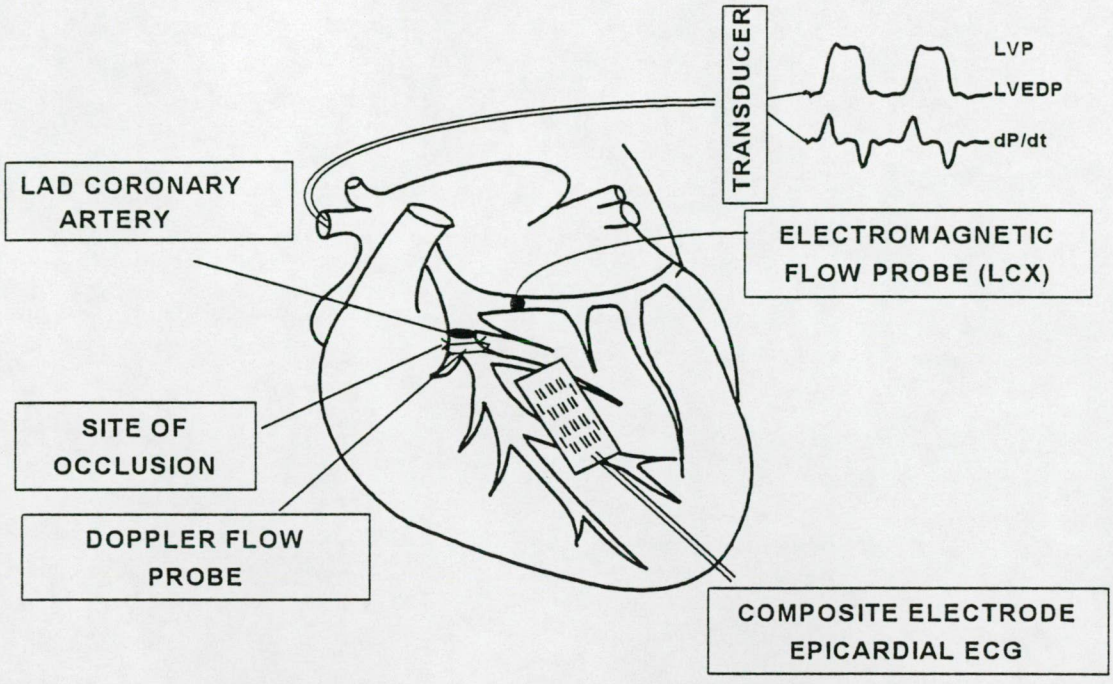


Figure 2. The experimental model in anaesthetised dogs for the measurements of the haemodynamic parameters, epicardial ST-segment changes, the degree of inhomogeneity of electrical activation and for the assessment of ventricular arrhythmias.

2.3. Assessment of the severity of myocardial ischaemia

The severity of myocardial ischaemia following coronary occlusion was assessed by evaluating changes in epicardial ST-segment (mV) and in the degree of inhomogeneity of electrical activation (msec). These were measured by means of a small rubber pad (composite electrode), containing 32 measuring points, ended in a bipolar lead for the measurement of inhomogeneity and four unipolar electrodes by which changes in epicardial ST-segment were evaluated. The composite electrode was sutured on the epicardial surface of the myocardium within the ischaemic zone (Figure 2). Inhomogeneity was determined from the summarised recording of R-waves, collected from these 32 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 (97).

2.4. Evaluation of ventricular arrhythmias

Ventricular arrhythmias, occurring following ischaemia and reperfusion, were analysed according to the "Lambeth Conventions" (98), except that no distinction was made between couplets and salvos, which were included as single ventricular ectopic (premature) beats (VPBs), and that we defined ventricular tachycardia (VT) as a run of four or more ectopic beats at a rate faster than the resting sinus rate. We also evaluated the number of episodes of ventricular

tachycardia (VT) which occurred in each dog, as well as the incidences of VT and ventricular fibrillation (VF) during occlusion. The only arrhythmia that we evaluated during reperfusion was VF. Survival indicates those dogs that were predominantly in sinus rhythm 10 min after reperfusion.

2.5. The measurement of the area at risk

Since the size of the occluded area can modify the severity of ventricular arrhythmias, at the end of the experiments the 'area at risk' was assessed by infusing patent blue V dye into the occluded artery at a pressure equivalent to that of mean arterial pressure. The risk area was then measured and expressed as the precentage of the left ventricular wall including the septum.

2.6. Drugs

Z13752A was a kind gift from Zambon, Milan, Italy. Enalaprilate, ANP, ∝-Chloralose and Urethane were purchased from Sigma, Grenoble, France. Icatibant (Hoe-140) was provided by Hoechst AG., Germany.

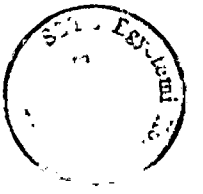
2.7. Statistical evaluation

All data were expressed as means ± s.e.mean and the differences between means were compared by analysis of variance (ANOVA for repeated measures) or the Student's *t*-test as appropriate. A one-way ANOVA was undertaken to determine whether or not there were significant haemodynamic differences between the groups. VPBs were compared by using the Mann-Whitney Rank sum test, and the incidences of arrhythmias were compared using the Fisher Exact test. Diferences between groups were considered significant when $P< 0.05$.

2.8. Experimental protocols

2.8.1. Experimental protocol to examine the time-course of preconditioning induced by a single brief (5 min) preconditioning occlusion

In this study 5 groups of dogs were used. In the control group (group1; n = 16) dogs were infused with saline for 60 min and then subjected to a 25 min occlusion of the LAD. The coronary artery was then opened, to allow for rapid reperfusion. Four groups of dogs were subjected to preconditioning by occluding the LAD for 5 min. At various time afterwards (i.e. 5 min in group2, n = 9; 15 min in group3, n = 8; 30 min in group4, n = 8 and 60 min in group5, n = 12) these dogs were subjected to prolonged occlusion (Figure 3).



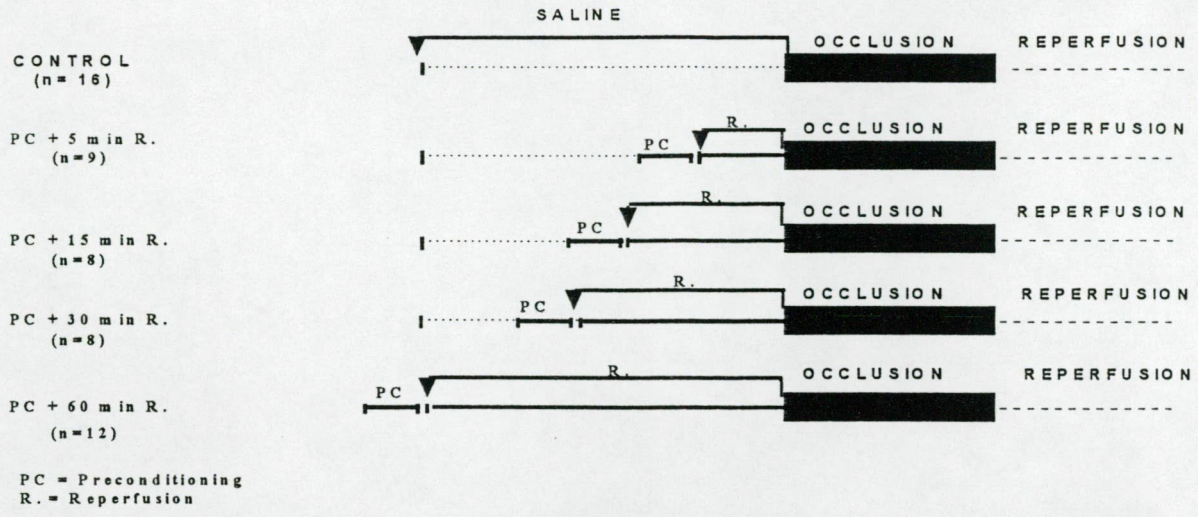


Figure 3. Experimental protocol for evaluating the time-course of classical preconditioning

2.8.2. Experimental protocol for evaluating the antiarrhythmic effects of enalaprilate and Z13752A

In this study we examined the effects of enalaprilate and Z13752A on responses of acute coronary artery occlusion and reperfusion in anaesthetized dogs (Figure 4).

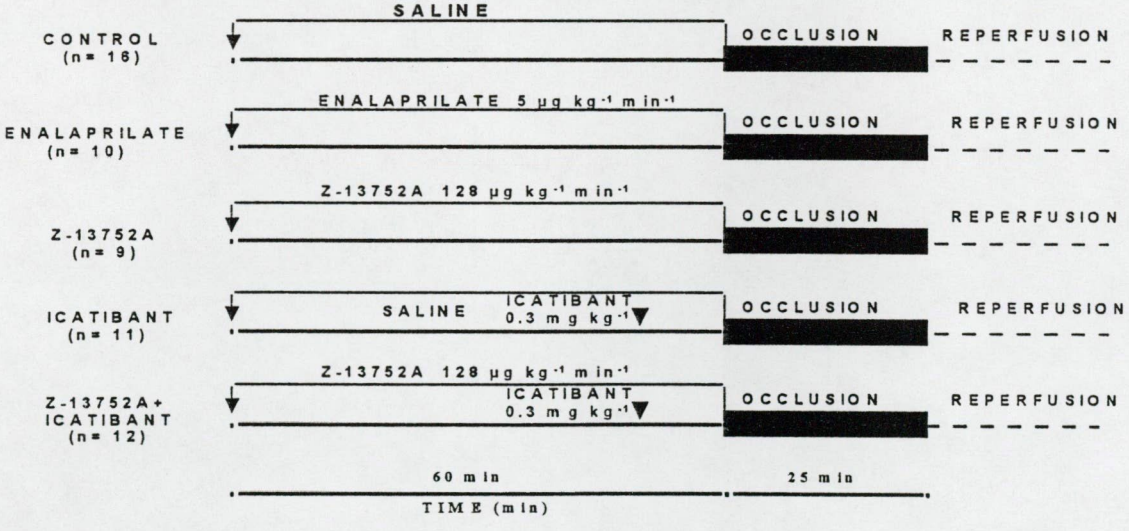


Figure 4. Experimental protocol to examine the effects of enalaprilate, Z13752A and Z13752A in the presence of icatibant.

Ten dogs were infused with enalaprilate, in a dose of 5 µg kg^{-1} min^{-1} and 9 dogs with Z13752A, in a dose of 128 µg kg^{-1} min^{-1} intravenously over a period of 60 min. At the end of the infusion the LAD was occluded for 25 min followed by reperfusion. In a third group of 11 dogs, icatibant, an antagonist of bradykinin at B_2 receptors, was given intravenously in a dose of 0.3 mg kg^{-1}, starting the infusion 10 min prior to coronary artery occlusion. A fourth group of 12 dogs

was also infused with Z13752A, as described above, but 50 min later these dogs were given icatibant. The responses were compared with those of 16 control dogs which were infused with a similar volume of the vehicle for 60 min and then subjected to coronary artery occlusion followed by reperfusion (Figure 4).

2.8.3. Experimental protocol to examine whether protection induced by preconditioning can be enhanced or prolonged by enalaprilate or Z13752A

In this part of the study we examined whether the antiarrhythmic effect of preconditioning can be enhanced or prolonged by the administration of enalaprilate and Z13752A, giving these drugs in preconditioned dogs at a time when the protection resulted from preconditioning was still present or has already faded. For this purpose preconditioned dogs with reperfusion intervals of 15 and 60 min were used (see the protocol in section 2.8.1.). In two groups of dogs, in which the reperfusion interval between the preconditioning occlusion and the prolonged occlusion was 15 min, either enalaprilate (n = 6) or Z13752A (n = 6) was infused in doses as described above (see section 2.8.2.), commencing the infusions 60 min prior to prolonged ischaemia (Figure 5a). Other groups of preconditioning dogs, with a reperfusion interval of 60 min, were also infused with enalaprilate (n = 13) and with Z13752A (n = 12), starting the infusions immediately after the preconditioning occlusion (Figure 5b). The results obtained from these experiments were compared to those groups in which dogs were simply subjected to preconditioning with 15 min and 60 min reperfusion intervals (described in section 2.8.1) and to dogs which were treated with either enalaprilate or Z13752A (described in section 2.8.2.), as well as to the controls.

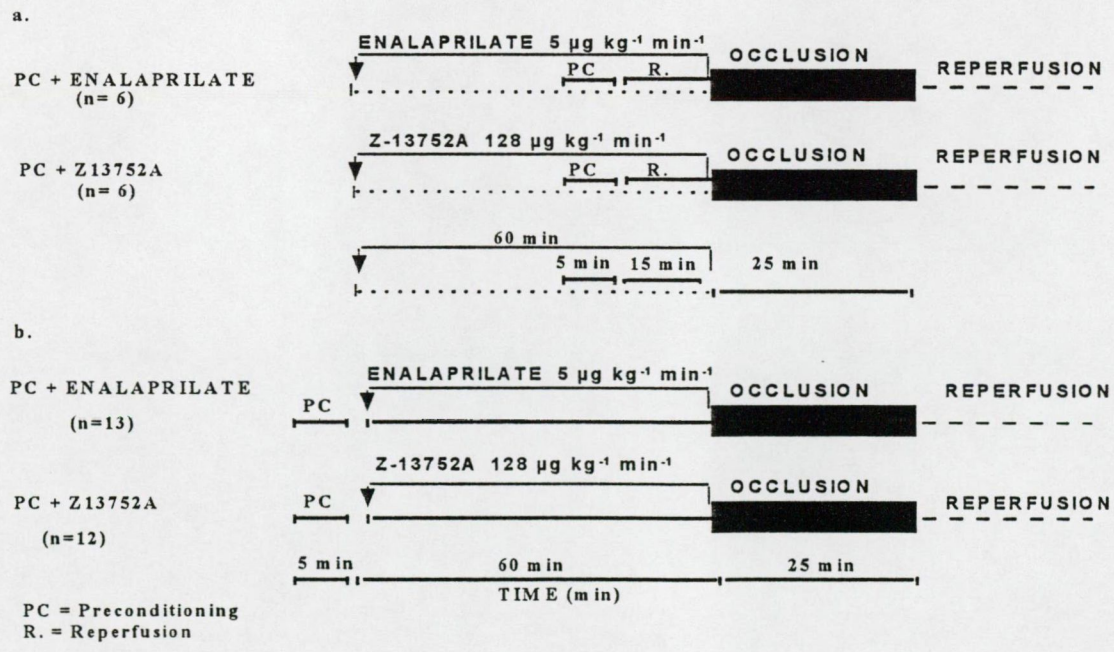


Figure 5. Experimental protocol to explore whether protection induced by preconditioning can be enhanced or prolonged by enalaprilate or Z13752A.

2.8.4. Experimental protocol to examine the antiarrhythmic effect of atrial natriuretic peptide

In these studies 2 groups of dogs, were used. In eleven dogs human synthetic ANP was given intravenously in a bolus injection of 10 μg kg^{-1} followed by infusion of 0.1 μg kg^{-1} min^{-1} over a period of 40 min, commencing 30 min prior to, and 10 min during the 25 min occlusion of the LAD. The responses were compared with those of 14 control dogs which were given a similar volume of saline and then subjected to coronary artery occlusion for 25 min. The artery was then re-opened rapidly to allow reperfusion (Figure 6).

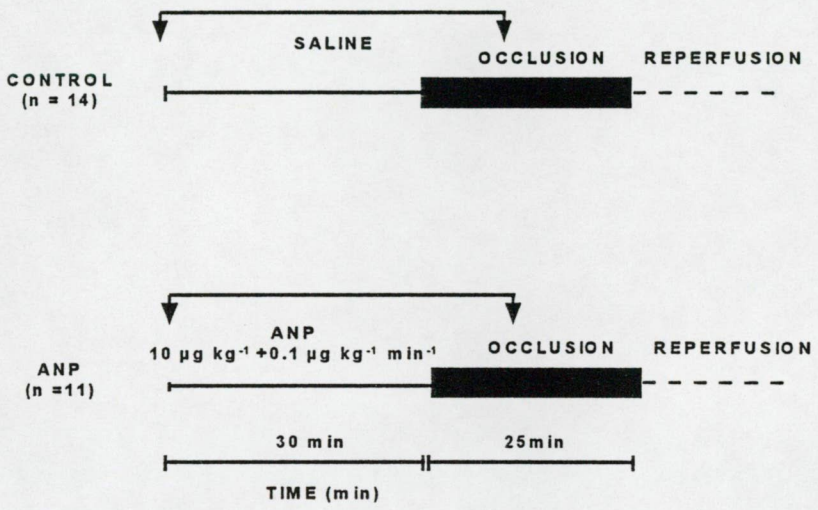


Figure 6. Experimental protocol to evaluate the effects of ANP.

3. RESULTS

3.1. Evaluation of the duration of the antiarrhythmic effects of preconditioning induced by a single 5 min occlusion of the LAD in anaesthetised dogs

3.1.1. Haemodynamic changes following coronary artery occlusion in control and in preconditioned dogs

These are summarised in Table 1. In all groups, occlusion of the LAD resulted in similar reduction in arterial blood pressure. However, reductions in LVSP, positive and negative $LVdP/dt_{max}$ were significantly less pronounced and the increase in LVEDP was less marked in the preconditioned dogs with 5 min, 15 min, 30 min reperfusion intervals than in the controls. These haemodynamic changes in preconditioned dogs with 60 min reperfusion were, however, similar to the controls.

Occlusion of the LAD resulted in marked and prolonged increase in blood flow of the LCX coronary artery as a result of the significant reduction in coronary resistance. This compensatory flow increase was somewhat more pronounced in the preconditioning dogs than in the controls (Table 1).

3.1.2. The severity of myocardial ischaemia during a 25 min occlusion of the LAD in control and in preconditioned dogs

In our experiments the severity of myocardial ischaemia was assessed by two parameters; changes in the epicardial ST-segment and the degree of inhomogeity of electrical activation, both measured within the ischaemic myocardium. In control dogs, coronary artery occlusion resulted in a significant elevation in the epicardial ST-segment (Figure 7a) and a marked increase in the degree of inhomogeneity of electrical activation (Figure 7b). One 5 min preconditioning occlusion markedly reduced these indices of ischaemia severity. Thus, compared to the controls, the epicardial ST-segment elevation was significantly less pronounced in all groups of the preconditioned dogs (Figure 7a). Similarly, the degree of inhomogeniety of electrical activation was less marked in dogs subjected to preconditioning than in the controls, except that group in which the time interval between the preconditioning occlusion and the prolonged ischaemia was extended to 60 min (Figure7b).

Table 1. Haemodynamic changes during a 25 min occlusion of the LAD in control and in preconditioned dogs.

	Control (n = 16)		PC 5 min R (n = 8)		PC 15 min R (n = 9)		PC 30 min R (n = 11)		PC 60 min R (n = 12)	
	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change
Arterial blood pressure										
systolic (mmHg)	125 ± 5	-14 ± 2*	113 ± 4	-12 ± 3*	101 ± 2	-11 ± 3*	116 ± 6	-13 ± 3*	124 ± 5	-14 ± 3*
diastolic (mmHg)	90 ± 4	-13 ± 1*	73 ± 3	-8 ± 1*	65 ± 3	-8 ± 2*	80 ± 3	-7 ± 2*#	87 ± 3	-10 ± 1*
mean (mmHg)	102 ± 4	-13 ± 3*	84 ± 3	-10 ± 1.5*	77 ± 2	-9 ± 2*	93 ± 4	-9± 2*	99 ± 4	-14 ± 5*
LVSP (mmHg)	128 ± 7	-16 ± 3*	120 ± 4	-11 ± 2*#	-106 ± 2	-7 ± 4*#	122 ± 5	-10 ± 2*#	117 ± 5	-14 ± 2*
LVEDP (mmHg)	6.0 ± 0.3	12.7 ± 0.6*	5 ± 0.3	9.1 ± 1.2*#	6.3 ± 0.6	4.8 ± 1.5*#	6.0 ± 0.4	10.0 ± 2.1*#	5.2 ± 0.5	12.1 ± 0.8*
$LVdP/dt_{max}$:										
(+ve: mmHg s^{-1})	2622 ± 216	-644 ± 99*	3136 ± 233	-427 ± 200*#	2784 ± 177	-267 ± 123*#	2834 ± 269	-441 ± 82*#	3466 ± 342	-311 ± 95*
(-ve: mmHg s^{-1})	2914 ± 242	-641 ± 116*	2805 ± 172	-117 ± 51#	2594 ± 174	-194 ± 84#	2784 ± 171	-226 ± 70#	2844 ± 212	-433 ± 76*
Heart rate (beats min^{-1})	155 ± 4	1 ± 1	134 ± 4	3 ± 1	127 ± 5	2 ± 1	141 ± 6	9 ± 2*	148 ± 6	7 ± 2*
Coronary (LCX) diastolic blood flow (ml min^{-1})	82 ± 8	14 ± 3*	164 ± 11	27 ± 4*#	156 ± 31	31 ± 7*#	113 ± 21	35 ± 7*#	156 ± 12	44 ± 5*#
Coronary (LCX) diastolic resistance (mmHg ml^{-1} min^{-1})	1.13 ± 0.12	-0.23 ± 0.05	0.44 ± 0.02	-0.10 ± 0.01*	-0.51 ± 0.1	-0.21 ± 0.04*	0.88 ± 0.15	-0.21 ± 0.05*	0.56 ± 0.03	-0.81 ± 0.65*

**P < 0.05 vs initial (value pre-occlusion); # P < 0.05 vs control.*

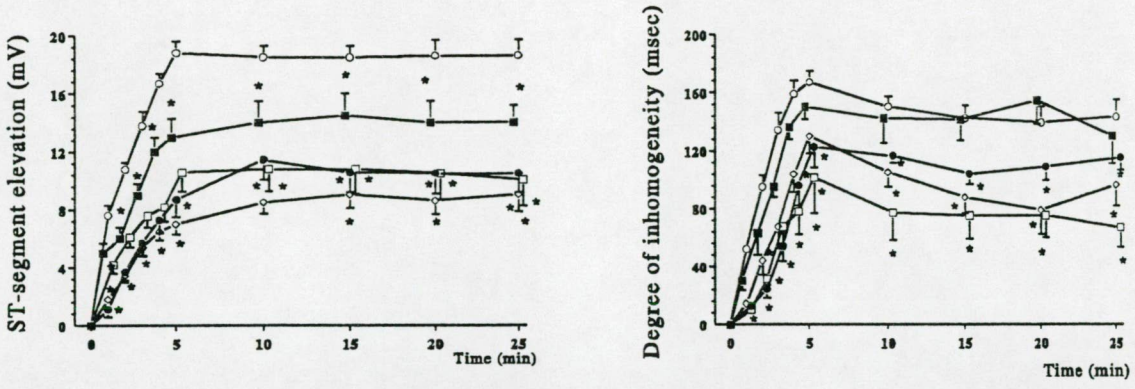


*Figure 7. Changes in epicardial ST-segment elevation (a) and in the degree of inhomogeneity of electrical activation (b) during a 25 min occlusion of the LAD in control dogs (open circles) and in dogs preconditioned 5 min (open squares), 15 min (filled circles), 30 min (open rhombs) and 60 min (filled squares) previously. *P < 0.05 vs controls.*

3.1.3. Time-course of the antiarrhythmic protection resulted from a single 5 min occlusion of the LAD

Figure 8 illustrates the severity of ventricular arrhythmias that occur during a 25 min occlusion and reperfusion of the LAD in control dogs and in dogs subjected to preconditioning at various time intervals previously. Compared to the controls, preconditioning significantly reduced the number of VPBs (353 ± 79 to 83 ± 37, 72 ± 27 and 32 ± 9), the number of episodes of VT (10.7 ± 3.3 to 1.6 ± 0.7, 0.3 ± 0.2 and 0.4 ± 0.2) and the incidence of VT (100% to 62%, 25% and 37%) during coronary artery occlusion, if the time interval between the preconditioning occlusion and the prolonged occlusion was 5, 15 and 30 min, respectively. None of these preconditioned dogs fibrillated during occlusion, in contrast to 44% in the controls (Figure 8). Survival from the combined ischaemia-reperfusion insult, thus significantly increased in the preconditioned dogs (63%, 38% and 38% cp controls 0%). This antiarrhythmic protection was almost completely abolished if the time interval between the preconditioning occlusion and the prolonged occlusion had been extended to 60 min (VPBs: 273 ± 87, VT episodes: 8 ± 4.1, VT incidence: 58%, occlusion VF incidence: 17%, reperfusion VF incidence: 66%, survival: 17%; Figure 8).

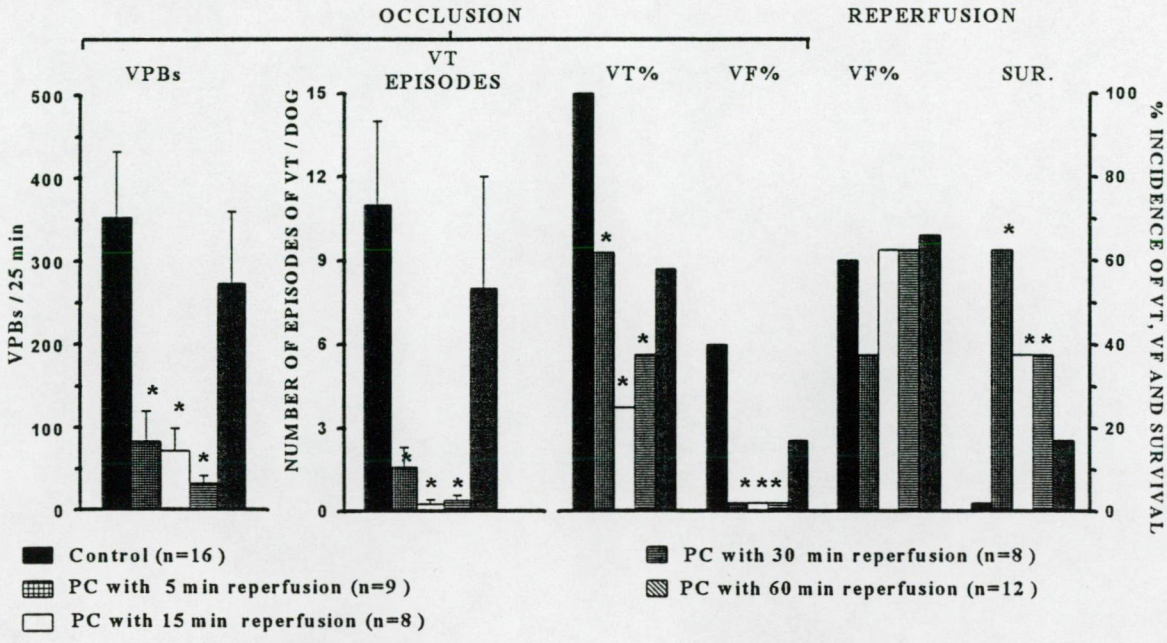


*Figure 8. The incidence and the severity of ventricular arrhythmias resulting from coronary artery occlusion and subsequent reperfusion in anaesthetised control dogs and in dogs preconditioned 5 min, 15 min, 30 min and 60 min previously. *P < 0.05 vs controls.*

3.2. The cardioprotective effects of enalaprilate and Z13752A in anaesthetised dogs. The role of bradykinin

3.2.1. The haemodynamic effects of enalaprilate, Z13752A, icatibant and Z13752A in the presence of icatibant.

These data are summarised in Table 2. Intravenous infusions of Z13752A and enalaprilate resulted in significant reductions in arterial blood pressure, left ventricular systolic pressure and negative $LVdP/dt_{max}$ without substantialy affecting LVEDP. In addition, enalaprilate also significantly reduced heart rate and positive $LVdP/dt_{max}$. Both drugs caused marked increase in coronary blood flow through the circumflex branch of the left coronary artery as a result of a decrease in coronary vascular resistance (Table 2).

Icatibant given alone resulted in no significant haemodynamic changes. There was only a moderate increase in arterial blood pressure and a slight decrease in positive dP/dt_{max}. However, this dose of icatibant completely abolished the haemodynamic effects of Z13752A (Table 2).

Table 2. Haemodynamic changes following intravenous infusions of enalaprilate, Z13752A icatibant and Z13752A in the presence of icatibant.

	Enalaprilate (n = 10)		Z13752A (n = 9)		Icatibant (n = 11)		Z13752A + Icatibant (n = 12)	
	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change
Arterial blood pressure								
systolic (mmHg)	124 ± 3	-29 ± 3*	129 ± 16	-12 ± 4*#	118 ± 4	3 ± 0.8	125 ± 3	7 ± 3*
diastolic (mmHg)	79 ± 2	-27 ± 4*	80 ± 9	-9 ± 3*#	84 ± 3	3 ± 0.9	86 ± 3	5 ± 2*
mean (mmHg)	94 ± 2	-28 ± 3*	97 ± 11	-11 ± 3*#	95 ± 3	4 ± 1*	98 ± 3	5 ± 1*
LVSP (mmHg)	116 ± 3	-25 ± 4*	134 ± 10	-11 ± 4*#	111 ± 4	2 ± 0.7	110 ± 4	3 ± 3
LVEDP (mmHg)	5.0 ± 0.4	0 ± 0	5.6 ± 1.0	0.3 ± 0.7	5 ± 0.2	0.7 ± 0.5	5.0 ± 0.6	0.3 ± 0.2
$LVdP/dt_{max}$:								
(+ve: mmHg s^{-1})	3778 ± 144	-771 ± 113*	3666 ± 305	-133 ± 184#	3137 ± 169	-274 ± 123	4061 ± 206	-336 ± 130*
(-ve: mmHg s^{-1})	3067 ± 137	-599 ± 129*	3242 ± 461	-363 ± 167*	2747 ± 86	-402 ± 207	2807 ± 226	-80 ± 150
Heart rate (beats min^{-1})	150 ± 7	-6 ± 1*	142 ± 7	-4 ± 2	153 ± 7	-4 ± 1*	161 ± 6	-5 ± 2*
Coronary (LCX) diastolic blood flow (ml min^{-1})	111 ± 5	11 ± 3*	113 ± 13	14 ± 5*	83 ± 5	-9 ± 3	102 ± 7	-3 ± 5
Coronary (LCX) diastolic resistance (mmHg ml^{-1} min^{-1})	0.82 ± 0.10	-0.38 ± 0.11*	0.81 ± 0.13	-0.63 ± 0.11*	1.04 ± 0.08	0.15 ± 0.05	0.87 ± 0.05	0.09 ± 0.06

**P < 0.05 vs initial (value pre-drug), #P < 0.05 vs enalaprilate.*

3.2.2. Haemodynamic changes induced by coronary artery occlusion in control dogs, and in dogs given enalaprilate, icatibant, Z13752A and Z13752A in the presence of icatibant

These results are summarised in Table 3. Occlusion of the LAD resulted in almost similar decreases in arterial blood pressure, LVSP and $LVdP/dt_{max}$ in all dogs. However, the occlusion-induced increase in LVEDP was significantly less pronounced in dogs given enalaprilate or Z13752A than in the control dogs or in dogs given icatibant either alone or in the presence of Z13752A (Table 3).

In all groups, occlusion of the LAD led to an immediate increase in blood flow of the other major (circumflex) branch of the left coronary artery. This 'compensatory' flow increase was somewhat more pronounced in dogs treated with icatibant either alone or in the presence of Z13752A (Table 3).

Table 3. Heamodynamic changes following occlusion of the LAD in dogs pretreated with saline, enalaprilate, Z13752A, icatibant and with Z13752A in the presence of icatibant.

	Control (n = 16)		Enalaprilate (n = 10)		Z13752A (n = 9)		Icatibant (n = 11)		Z13752 + Icatibant (n = 12)	
	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change
Arterial blood pressure										
systolic (mmHg)	125 ± 5	-14 ± 2*	95 ± 3	-12 ± 3*	117 ± 3	-13 ± 3*	114 ± 4	-20 ± 5*	131 ± 4	-15 ± 2*
diastolic (mmHg)	90 ± 4	-13 ± 1*	52 ± 3	-5 ± 2*#	71 ± 3	-7 ± 2*#	81 ± 4	-19 ± 4*	92 ± 3	-13 ± 2*
mean (mmHg)	102 ± 4	-13 ± 3*	66 ± 3	-7 ± 2*	86 ± 3	-9 ± 2*	92 ± 4	-19 ± 1*	105 ± 3	-14 ± 2*
LVSP (mmHg)	128 ± 7	-16 ± 3*	91 ± 4	-8 ± 2*#	123 ± 5	-13 ± 4*	108 ± 4	-20 ± 4*	117 ± 3	-15 ± 2*
LVEDP (mmHg)	6.0 ± 0.3	12.7 ± 0.6*	4.8 ± 0.5	8.6 ± 1*#	5.3 ± 0.3	8.3 ± 1.5*#	4.0 ± 0.4	16.0 ± 1.2*#	5.3 ± 0.7	14.1 ± 0.9*
$LVdP/dt_{max}$:										
(+ve: mmHg s^{-1})	2622 ± 216	-644 ± 99*	3149 ± 248	-729 ± 87*	3666 ± 252	-635 ± 264*	3315 ± 274	-624 ± 139*	3275 ± 213	-1134 ± 160*
(-ve: mmHg s^{-1})	2914 ± 242	-641 ± 116*	2620 ± 136	-551 ± 104*	2879 ± 252	-165 ± 131#	2748 ± 278	-551 ± 106*	3126 ± 320	-487 ± 133*
Heart rate (beats min^{-1})	155 ± 4	1 ± 1	144 ± 7	1 ± 3	138 ± 8	3 ± 2	149 ± 7	5 ± 2	157 ± 6	10 ± 3*
Coronary (LCX) diastolic blood flow (ml min^{-1})	82 ± 8	14 ± 3*	101 ± 8	21 ± 4*	123 ± 18	21 ± 3*	82 ± 4	35 ± 8*#	99 ± 9	40 ± 4*#
Coronary (LCX) diastolic resistance (mmHg ml^{-1} min^{-1})	1.13 ± 0.12	-0.23 ± 0.05*	0.54 ± 0.04	-0.15 ± 0.09*	0.70 ± 0.12	-0.21 ± 0.01*	1.04 ± 0.03	-0.30 ± 0.66*	0.98 ± 0.08	-0.41 ± 0.06*

**P < 0.05 vs initial (value pre-occlusion); #P < 0.05 vs control.*

3.2.3. Angiotensin responses before and after enalaprilate and Z13752A treatment

In order to evaluate the inhibitory effects of enalaprilate and Z13752A on ACE, in a separate group of dogs the responses to intravenous bolus injections of angiotensin I (AG I) and angiotensin II (AG II), in doses of 5, 10, 15 and 20 ng kg^{-1} were examined prior to and at the end of the 60 min infusion period of enalaprilate (n = 9; Figure 9b) and Z13752A (n = 11; Figure 9c). All these responses were compared to those obtained from control dogs (n = 6; Figure 9a) in which enalaprilate and Z13752A were replaced by the vehicle. The AG I responses were significantly reduced after any of these two drugs (Figure 9b and 9c). Interestingly, the AG II responses were significantly potentiated at all dose levels after administration of Z13752A (Figure 9c).

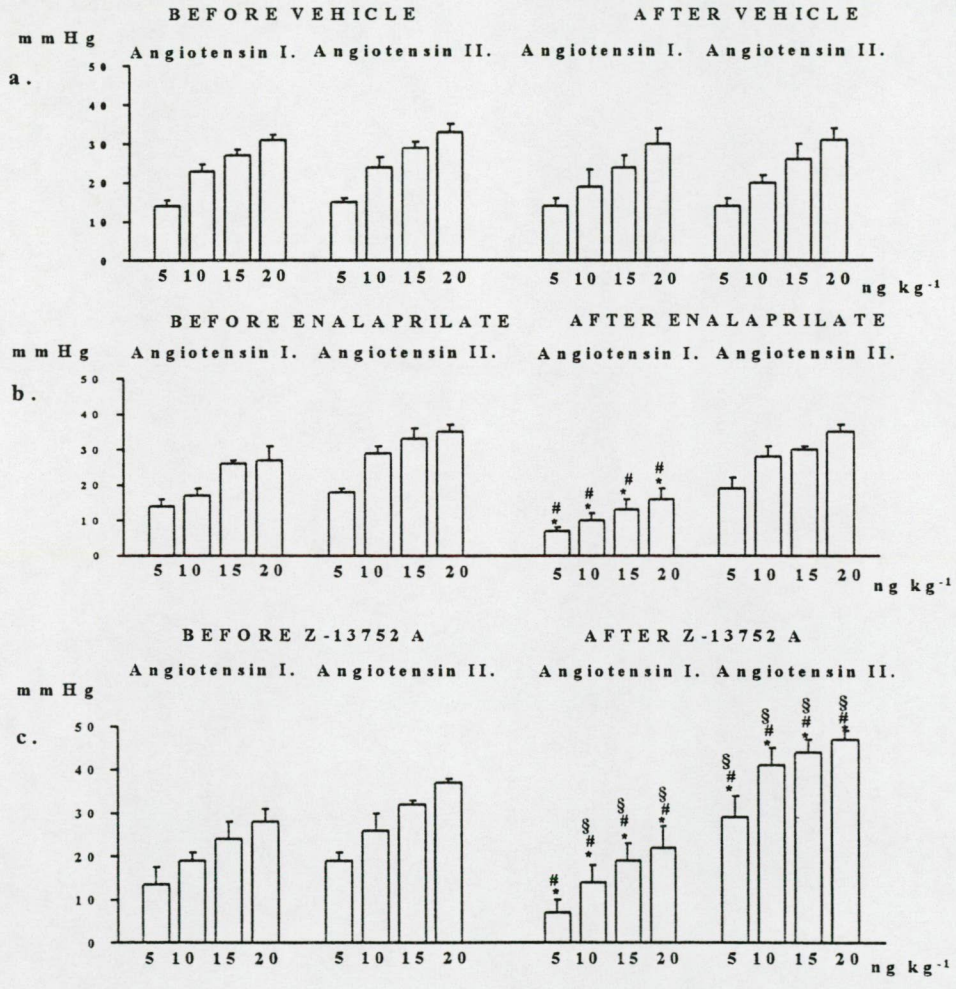


*Figure 9. Changes in arterial blood pressure induced by bolus injections of AGI and AGII given before or after the administration of the vehicle (a), enalaprilate (5 μg kg^{-1} min^{-1}, b) and Z13752A (128 μg kg^{-1} min^{-1}, c). *P < 0.05 vs before drug treatment; #P < 0.05 vs control. §P < 0.05 vs enalaprilate*

3.2.4. Bradykinin responses before and after Z13752A administration

In these experiments we tested whether inhibition of ACE and NEP enzymes with Z13752A would result in enhanced responses to bradykinin. Therefore, in a separate group of dogs intravenous bolus injections of bradykinin were given in doses of 0.1, 0.25, 0.5 and 1 μg kg^{-1} prior to and after the administration of Z13752A (n = 4) and changes in arterial blood pressure were compared to the pre-drug values and to the vehicle treated dogs (n = 4, Figure 10a). Bradykinin responses were significantly potentiated at all dose levels after the administration of Z13752A (Figure 10b).

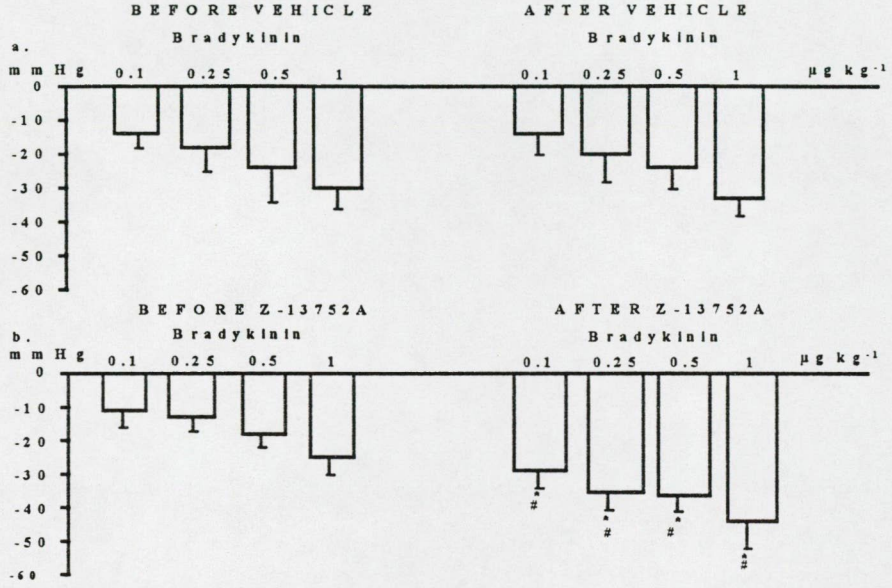


*Figure 10. Changes in arterial blood pressure induced by bolus injections of bradykinin given before and after the administration of the vehicle (a) and of Z13752A (128 μg kg^{-1} min^{-1}, b). *P < 0.05 vs before drug administration; #P < 0.05 vs controls.*

3.2.5. The severity of myocardial ischaemia following coronary artery occlusion in control dogs and in dogs treated with enalaprilate, Z13752A, icatibant and with the combination of Z13752A and icatibant

The epicardial ST-segment and the degree of inhomogeneity of electrical activation (Figures 11a and 11b) were rapidly increased within the first 5 min of the onset of coronary artery occlusion. These changes were significantly less marked in dogs given either enalaprilate or Z13752A (Figures 11a and 11b). Icatibant reversed the protective effects of Z13752A on these indices of ischaemia severity (Figures11a and 11b).

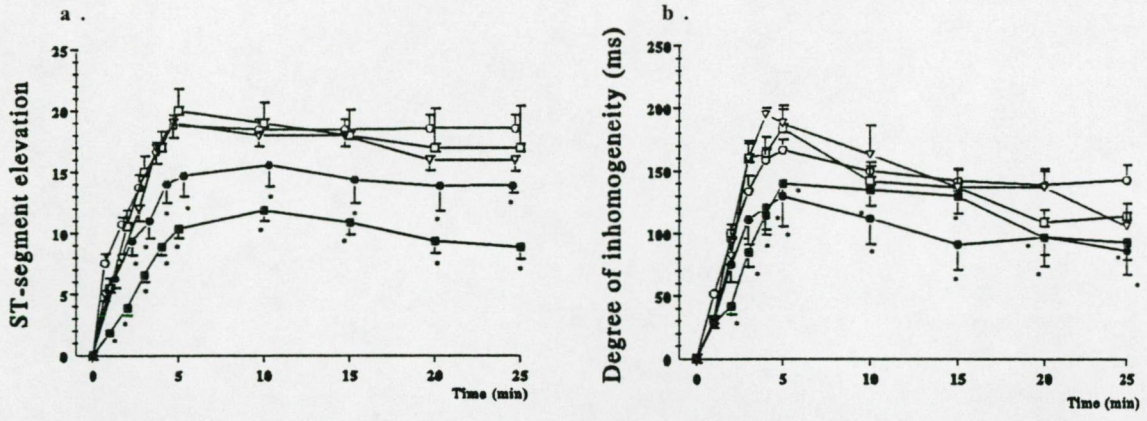


*Figure 11. Changes in epicardial ST-segment (a) and in the degree of inhomogenity of electrical activation (b) during a 25 min occlusion of the LAD in anaesthetised dogs, given saline (open circles), enalaprilate (filled squares), Z13752A (filled circles), icatibant (open triangles) and Z13752A in the presence of icatibant (open squares). *P < 0.05 vs controls.*

3.2.6. The effects of enalaprilate, Z13752A, icatibant and Z13752A together with icatibant on ventricular arrhythmias following coronary artery occlusion and reperfusion

This is illustrated in Figure 12. Both enalaprilate and Z13752A markedly reduced the incidence and the severity of ventricular arrhythmias, resulted from a 25 min occlusion of the LAD. Thus, compared to the controls, in dogs given enalaprilate and Z13752A, the number of VPBs (353 ± 79 to 103 ± 42 and 91 ± 40), the number of episodes of VT (10.7 ± 3.3 to 1.8 ± 1.2 and 0.22 ± 0.15), the incidences of VT (100% to 40% and 22%) and VF (44% to 30% and 0%) during occlusion were markedly reduced and survival was significantly increased (0% to 50% and 67%). This protection was not seen in dogs which were infused with Z13752A and then given icatibant (Figure 12). Thus, at this time, there was a high number of VPBs (632 ± 300, $P<0.05$ vs Z13752A alone) and all dogs exhibited a large number of episode of VT (22.5 ± 13.8, $P<0.05$ vs Z13752A alone). Furtheremore, 58% of the Z13752A treated dogs fibrillated during the occlusion period in the presence of icatibant and no dog survived the combined occlusion-reperfusion insult. The severity of ventricular arrhythmias after icatibant alone was not significantly different from those seen in the controls (VPBs: 524 ± 166, VT episodes: 25 ± 10, VT: 73%, VF during occlusion: 46% and survival: 9%; not shown in the figure).

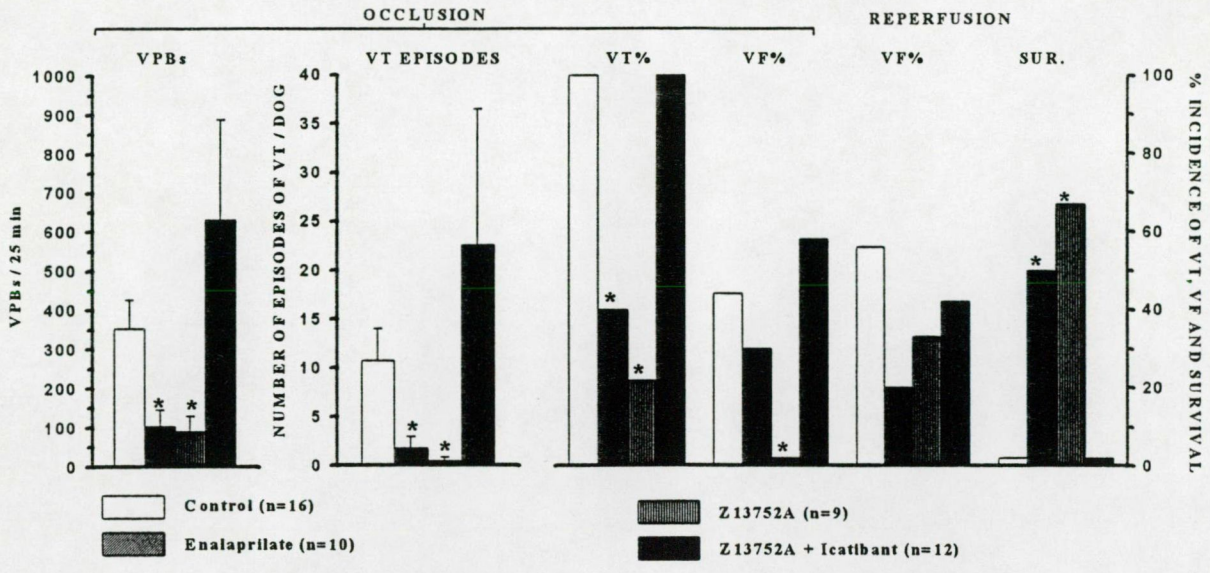


*Figure 12. The effects of enalaprilate, Z13752A alone and with icatibant in comparison with controls on ventricular arrhythmias resulting from coronary artery occlusion and subsequent reperfusion. *P < 0.05 vs controls.*

3.3. Evaluation of the cardioprotective effects of preconditioning in combination with drug treatment

This study aimed to explore whether elevation of bradykinin levels following administration of enalaprilate or Z13752A can provide additional protection to ischaemic preconditioning induced by a single 5 min occlusion of the LAD, 15 min and 60 min previously.

3.3.1. Haemodynamic changes during coronary artery occlusion in preconditioned dogs in the presence of enalaprilate and Z13752A treatment

These results are shown in Table 4. In all dogs, coronary artery occlusion resulted in significant decreases in arterial blood pressure, LVSP and coronary diastolic resistance. At the same time LVEDP and blood flow of the LCX coronary artery were increased. These changes were almost identical in all groups of dogs, except those preconditioned dogs with a 60 min reperfusion interval, in which Z13752A was given. In these dogs the increase in LVEDP following coronary artery occlusion was less marked than in the controls. Similarly, compared to the controls in preconditioned dogs, treated with either enalaprilate or Z13752A, a significantly less marked reduction could be observed in positive and negative dP/dt_{max} during occlusion of the LAD (Table 4).

Table 4. Haemodynamic changes during coronary artery occlusion in dogs subjected to preconditioning 15 and 60 min previously, either before or after the administration of enalaprilate and Z13752A.

	Control (n = 16)		enalaprilate + PC 15 min R (n = 6)		PC 60 min R + enalaprilate (n = 13)		Z13752A + PC 15 min R (n = 6)		PC 60 min R + Z13752A (n = 12)	
	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change	Initial value	max. change
Arterial blood pressure										
systolic (mmHg)	125 ± 5	-14 ± 2*	90 ± 5	-10 ± 3*	87 ± 5	-8 ± 1*	100 ± 16	-12 ± 3*	100 ± 4	-10 ± 2*
diastolic (mmHg)	90 ± 4	-13 ± 1*	53 ± 4	-8 ± 2*	49 ± 4	-5 ± 2*	62 ± 9	-8.5 ± 2*	62 ± 3	-8 ± 1*
mean (mmHg)	102 ± 4	-13 ± 3*	66± 4	-9 ± 2*	61 ± 4	-6 ± 2*#	75 ± 11	-9 ± 2*	74 ± 3	-9 ± 1*
LVSP (mmHg)	128 ± 7	-16 ± 3*	93 ± 4	-8 ± 3*	85 ± 4	-10 ± 3*	94 ± 10	-11 ± 3*	88 ± 3	-5 ± 0.8*
LVEDP (mmHg)	6.0 ± 0.3	12.7 ± 0.6*	4.3 ± 0.4	11.2 ± 1.7*	3.3 ± 0.3	14.5 ± 0.4*	4.1 ± 1.0	10.1 ± 1.4*	4.1 ± 0.2	8.5 ± 1.1*#
$LVdP/dt_{max}$:										
(+ve: mmHg s^{-1})	2622 ± 216	-644 ± 99*	1376 ± 262	-196 ± 81#	1450 ± 140	-250 ± 101#	1505 ± 305	-143 ± 79#	2981 ± 293	-247 ± 94#
(-ve: mmHg s^{-1})	2914 ± 242	-641 ± 116*	1191 ± 189	-177 ± 40#	1169 ± 227	-75 ± 31#	1906 ± 461	-302 ± 95#	2560 ± 182	-181 ± 88#
Heart rate (beats min^{-1})	155 ± 4	1 ± 1	131 ± 6	5 ± 2	141 ± 7.5	3 ± 1	133 ± 7	5 ± 2	124 ± 4	2 ± 1
Coronary (LCX) diastolic blood flow (ml min^{-1})	82 ± 8	14 ± 3*	95 ± 9	19 ± 4*	90 ± 6	23 ± 7*	123 ± 15	15 ± 3*	97 ± 8	17 ± 4*
Coronary (LCX) diastolic resistance (mmHg ml^{-1} min^{-1})	1.13 ± 0.12	-0.23 ± 0.05*	0.60 ± 0.09	-0.19 ± 0.04*	0.58 ± 0.08	-0.26 ± 0.09*	0.59 ± 0.07	-0.14 ± 0.03*	-0.69 ± 0.07	-0.14 ± 0.02*

**P < 0.05 vs initial (value pre-occlusion); #P < 0.05 vs controls.*

3.3.2. The effects of enalaprilate and Z13752A on the severity of myocardial ischaemia in preconditioned dogs

The epicardial ST-segment elevation and the degree of inhomogeneity, following coronary artery occlusion, were markedly reduced in preconditioned dogs with 15 min reperfusion and also in dogs treated with enalaprilate (Figures 13a and b). These changes were abolished when the preconditioned dogs were treated with enalaprilate (Figures 13a and b).

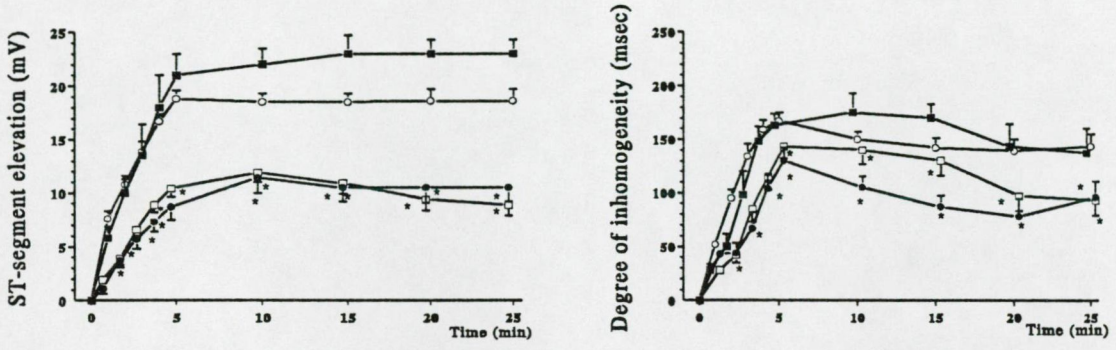


*Figure 13. Changes in epicardial ST-segment elevation (a) and in the degree of inhomogeneity (b) during a 25 min occlusion of the LAD, in control dogs (open circles), in dogs given enalaprilate (open squares) and in preconditioned dogs with 15 min reperfusion, either in the absence (filled circles) or in the presence (filled squares) of enalaprilate. *P < 0.05 vs controls.*

In dogs preconditioned 60 min prior to occlusion the epicardial ST-segment was slightly decreased whereas the degree of inhomogeneity was similar to that in the controls (Figure 14a and b). Enalaprilate, given to these preconditioned dogs did not modify ischaemia severity.

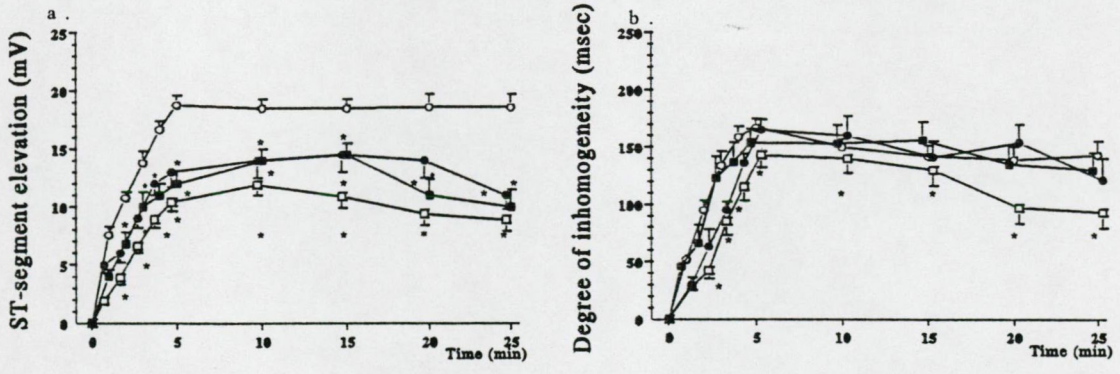


Figure 14. Changes in epicardial ST-segment elevation (a) and in the degree of inhomogeneity (b) during a 25 min occlusion of the LAD in control dogs (open circles), in dogs given enalaprilate (open squares) and in preconditioned dogs with 60 min reperfusion,

*either in the absence (filled circles) or in the presence (filled squares) of enalaprilate. *P < 0.05 vs controls.*

Similarly, when Z13752A was infused in dogs preconditioned 15 min prior to prolonged occlusion, the marked protection, resulted either from preconditioning or Z13752A was completely abolished. Thus, changes in epicardial ST-segment (Figure 15a) and in the degree of inhomogeneity of electrical activation (Figure 15b) following coronary artery occlusion were as marked as in the controls.

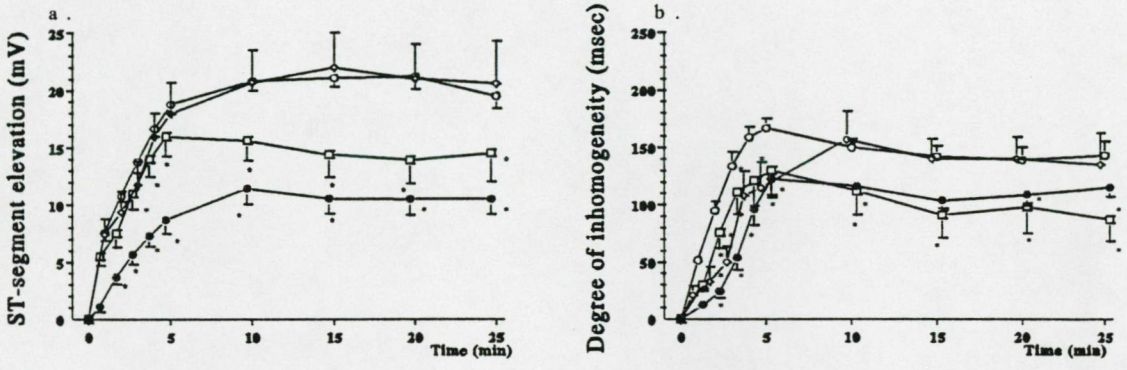


*Figure 15. Changes in the epicardial ST-segment (a) and in the degree of inhomogenity of electrical activation (b) during a 25 min occlusion of the LAD in control dogs (open circles), in dogs treated with Z13752A (open squares) and in preconditioned dogs with 15 min reperfusion, either in the absence (filled circles) or presence (open rhombs) of Z13752A. *P < 0.05 vs control.*

However, when Z13752A was given in dogs subjected to preconditioning 60 min prior to the 25 min occlusion of the LAD, the reduction in both the epicardial ST-segment (Figure 16a) and the degree of inhomogeneity (Figures 16), resulted from Z13752A treatment was largely preserved.

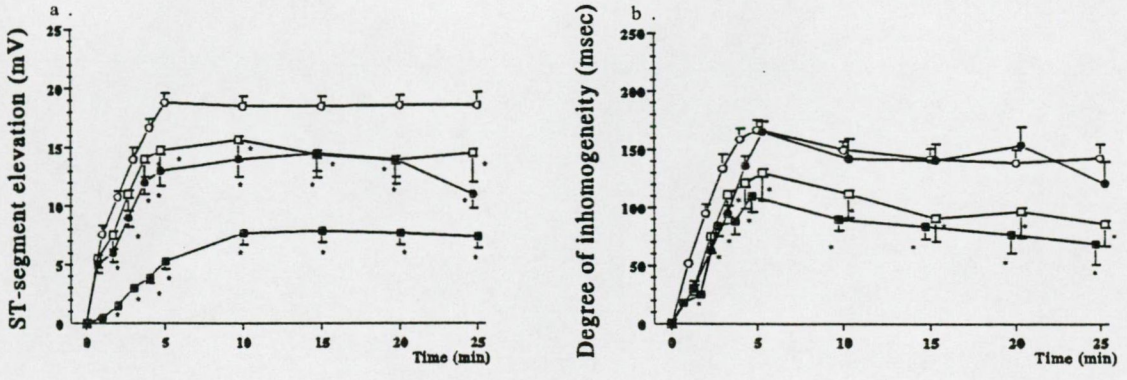


Figure 16. Changes in epicardial ST-segment (a) and in the degree of inhomogenity of

*electrical activation (b) during a 25 min occlusion of the LAD in control dogs (open circles), in dogs treated with Z13752A (open squares) and in preconditioned dogs with 60 min reperfusion, either in the absence (filled circles) or presence (filled squares) of Z13752A. *P < 0.05 vs control.*

3.3.3. The effects of enalaprilate and Z13752A on the severity of ventricular arrhythmias in preconditioned dogs

When enalaprilate was infused in dogs subjected to preconditioning either15 min (Figure 17) or 60 min (Figure 18) prior to occlusion of the LAD, no additional protection occurred against arrhythmias. There was even an attenuation in the antiarrhythmic protection, resulted either from drug treatment or preconditioning. For example, when enalaprilate was administered in dogs preconditioned 15 min prior to prolonged occlusion, the number of VPBs (204 ± 94), the number episodes of VT (2.0 ± 1.6) during occlusion were somewhat higher than either in dogs given enalaprilate alone or in dogs subjected to preconditioning 15 min prior to prolonged ischaemia without drug treatment (Figure 17). Although no dog fibrillated during the occlusion period, all the dogs died following reperfusion. Thus, similar to the controls, none of these preconditioned dogs treated with enalaprilate survived the combined ischaemia/reperfusion insult.

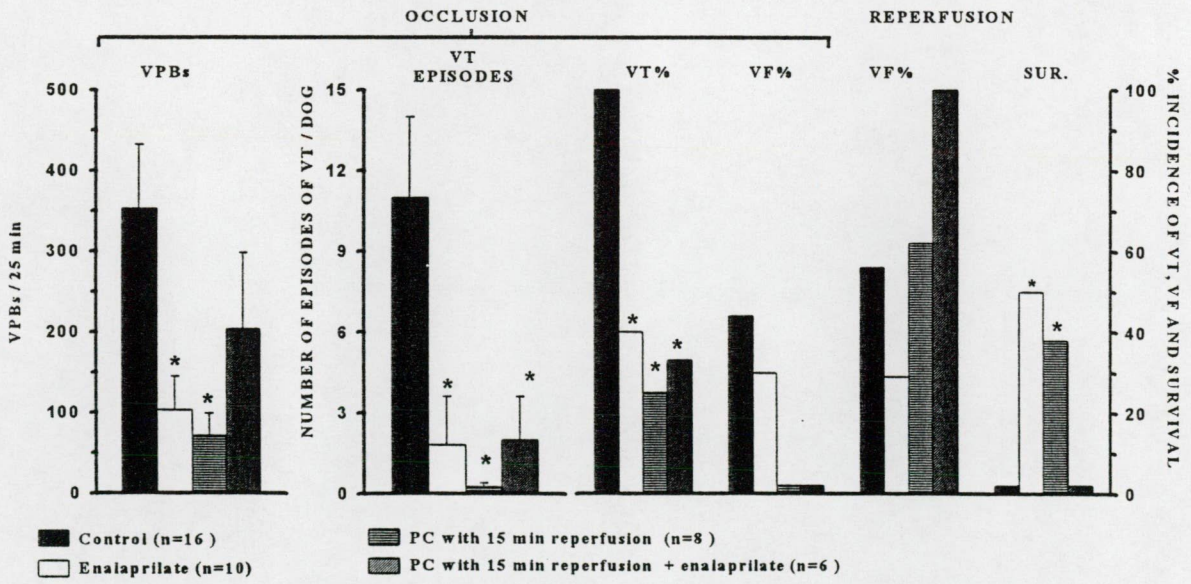


*Figure 17. The severity of ventricular arrhythmias occurring during coronary artery occlusion and subsequent reperfusion in control dogs, in dogs treated with enalaprilate, in dogs preconditioned 15 min prior to prolonged occlusion either in the presence or the absence of enalaprilate. *P < 0.05 vs control.*

When enalaprilate was given in preconditioned dogs with a 60 min reperfusion interval the severity of ventricular arrhythmias was again increased, compared to those dogs which were not preconditioned but treated with enalaprilate, and it was similar to those dogs which were subjected to preconditioning without drug treatment. Thus, the number of VPBs (252 ± 72), the number of episodes of VT (2.5 ± 1.2), the incidence of VT (54%) and the incidence of VF (38%) during occlusion were increased and survival reduced (0%) in these enalaprilate treated preconditioned dogs (Figure 18).

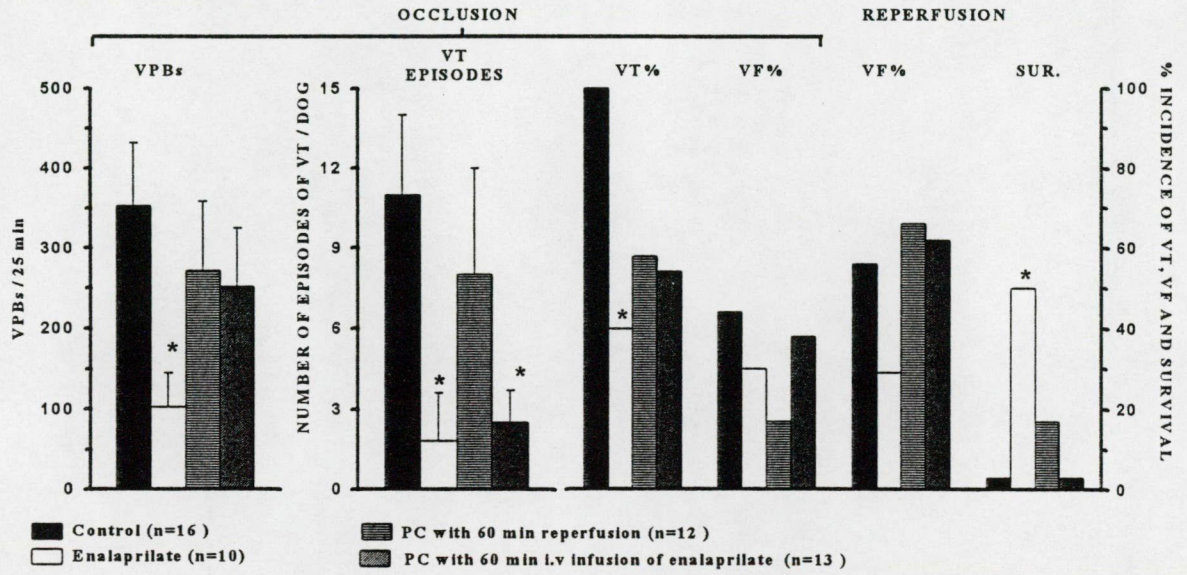


*Figure 18. The severity of ventricular arrhythmias occurring during coronary artery occlusion and subsequent reperfusion in control dogs, in dogs treated with enalaprilate, and in dogs preconditioned 60 min prior to prolonged occlusion either in the presence or the absence of enalaprilate. *P < 0.05 vs control.*

In contrast, the antiarrhythmic effect of Z13752A was still present when it was given in preconditioned dogs either with 15 or 60 min reperfusion intervals. Thus, the number of VPBs (86 ± 28 and 56 ± 24), the number of episodes of VT (0.33 ± 0.33 and 0.33 ± 0.18) and the incidences of both VT (14% and 25%) and VF (16% and 8%) during prolonged occlusion were significantly reduced. However, the protective effect of Z13752A against reperfusion induced ventricular arrhythmias was abolished in these preconditioned dogs. For example, if Z13752A was infused in dogs preconditioned 15 min (Figure 19) and 60 min previously (Figure 20), 67% and 59% of the dogs fibrillated during reperfusion and survival from the combined ischaemia-reperfusion insult again reduced to 17% and 33%.

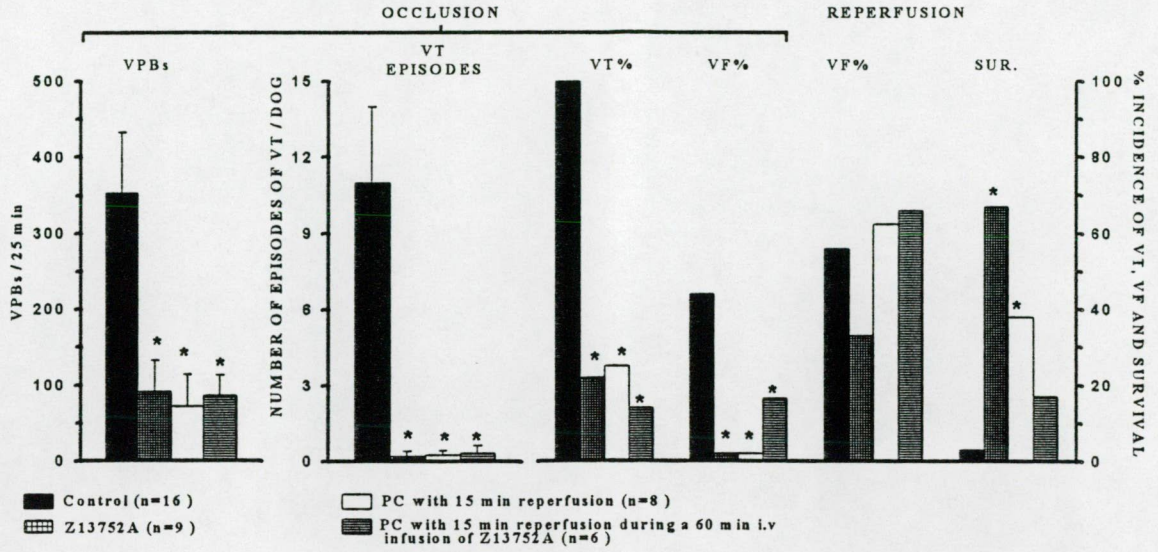


*Figure 19. The severity of ventricular arrhythmias occurring during coronary artery occlusion and subsequent reperfusion in control dogs, in dogs treated with Z13752A, and in dogs preconditioned 15 min prior to ischaemia either in the presence or the absence of Z13752A. *P < 0.05 vs control.*

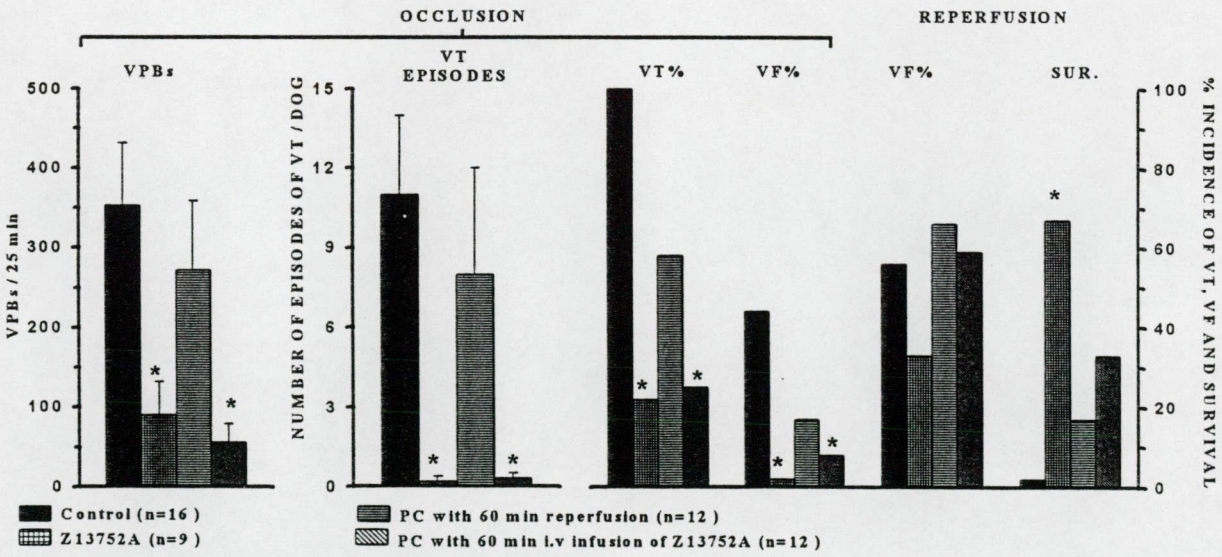


*Figure 20. The severity of ventricular arrhythmias occurring during coronary artery occlusion and subsequent reperfusion in control dogs in dogs treated with Z13752A, and in dogs preconditioned 60 min previously either in the presence or the absence of Z13752A. *P < 0.05 vs control.*

3.4. Evaluation of the cardioprotective effects of atrial natriuretic peptide during myocardial ischaemia and reperfusion in anaesthetised dogs

3.4.1. The haemodynamic effects of atrial natriuretic peptide

The maximum haemodynamic changes following ANP infusion, measured after the commencement of the infusion were reductions in arterial blood pressure (systolic 119 ± 7 to 98 ± 7 mmHg; diastolic 77 ± 5 to 62 ± 5 mmHg, mean 91 ± 6 to 74 ± 6 mmHg, $P < 0.05$) and in positive and negative $LVdP/dt_{max}$ (3417 ± 17 to 3155 ± 177 mmHg s^{-1} and 2875 ± 228 to 2534 ± 217 mmHg s^{-1}, respectively, $P < 0.05$). There were no significant changes in heart rate or in LVEDP. The most significant haemodynamic effect of ANP was a transient increase (of 24 ± 5 ml min^{-1}, from 110 ± 10 to 134 ± 13 ml min^{-1}) in the left circumflex diastolic coronary blood flow and a decrease in the coronary resistance (of 0.27 ± 0.05 mmHg ml^{-1}, from 0.78 ± 0.11 to 0.51 ± 0.09 mmHg ml^{-1}) measured 5 min after the onset of the ANP infusion.These changes, however, returned to the initial values prior to the commencement of the coronary artery occlusion.

3.4.2. Haemodynamic changes resulting from coronary artery occlusion in control dogs and in dogs given atrial natriuretic peptide

These results are summarised in Table 5. Occlusion of the LAD resulted in similar haemodynamic changes both in the controls and ANP treated dogs, except that the increase in LVEDP and the decrease in negative dP/dt_{max} following occlusion was significantly less marked in the ANP treated dogs (Table 5).

Occlusion of the LAD resulted in an immediate and sustained increase in blood flow through the circumflex branch of the left coronary artery. This compensatory increase in blood flow was significantly higher in the ANP treated dogs than in the controls (Table 5).

Table 5. Haemodynamic changes following LAD coronary artery occlusion in dogs pretreated with either saline or atrial natriuretic peptide (ANP).

	Control (n=14)		ANP (n=11)	
	initial value	max. change	initial value	max. change
Arterial blood pressure				
systolic (mmHg)	118 ± 4	-14 ± 2.5*	104 ± 6	-9 ± 2*
diastolic (mmHg)	84 ± 4	-12 ± 3*	70 ± 5	-7 ± 2*
mean (mmHg)	96 ± 4	-12 ± 3*	81 ± 5	-8 ± 4*
LVSP (mmHg)	123 ± 8	-15 ± 4*	109 ± 4	-9 ± 2*
LVEDP (mmHg)	5.5 ± 0.43	12.0± 0.79*	5.00 ± 0.00	9.00 ± 0.9*#
$LVdP/dt_{max}$:				
(+ve: mmHg s^{-1})	2359 ± 157	-589 ± 127*	3221 ± 174	-512 ± 95*
(-ve: mmHg s^{-1})	2655 ± 231	-630 ± 135*	2592 ± 226	-278 ± 73*#
Heart rate (beats min^{-1})	154 ± 5	5 ± 1	137 ± 5	3 ± 1
Coronary (LCX) diastolic blood flow (ml min^{-1})	82 ± 8	14 ± 3*	104 ± 8	33 ± 6*#
Coronary (LCX) diastolic resistance (mmHg ml^{-1} min^{-1})	1.13 ± 0.12	-0.23 ± 0.12	0.72 ± 0.10	-0.23 ± 0.05*

**P< 0.05 vs initial value; # P< 0.05 vs control.*

3.4.3. The effects of atrial natriuretic peptide on the severity of myocardial ischaemia

The severity of myocardial ischaemia, assessed from changes in epicardial ST-segment elevation (Figure 21a) and the degree of inhomogeneity of electrical activation (Figure 21b) were significantly less marked in dogs given ANP than in the controls.

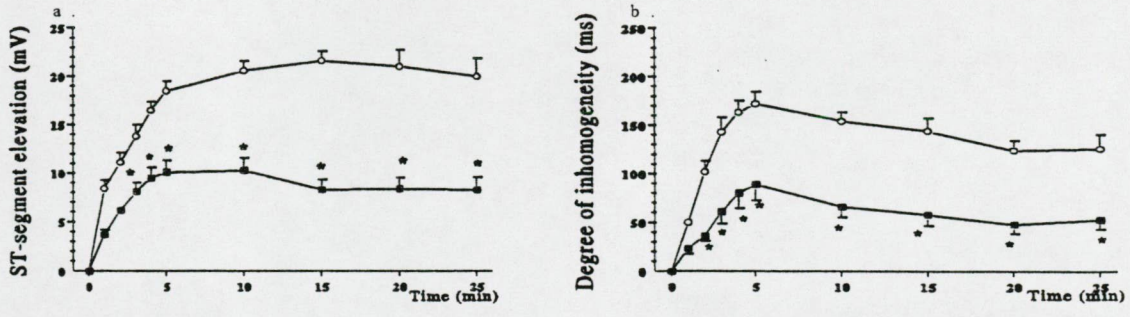


*Figure 21. Changes in epicardial ST-segment (mV, a) and in the degree of inhomogeneity of electrical activation (ms, b) during a 25 min occlusion of the LAD coronary artery in control dogs (open circles) and in dogs given ANP (filled squares). *P < 0.05 vs controls.*

3.4.4. The effects of atrial natriuretic peptide on ventricular arrhythmias following coronary artery occlusion and reperfusion

The severity of ventricular arrhythmias resulting from coronary artery occlusion and reperfusion, in control and in ANP treated dogs is illustrated in Figure 22. In this study, there was a mean of 416 ± 87 VPBs in the control dogs during coronary artery occlusion, and all the dogs exhibited VT at some stages during the period of ischaemia with a mean of 12.1 ± 4.2 episodes of VT per dogs. Eight of the 14 dogs (57%) fibrillated during the occlusion period and the remaining six dogs (43%) died following reperfusion. Thus, no control dog survived the combined ischaemia-reperfusion insult.

These ischaemia and reperfusion induced arrhythmias were much less pronounced in dogs given ANP (Figure 22). There was a mean of only 26 ± 12 VPBs and 6 out of 11 dogs had VT with a mean of 0.7 ± 0.03 VT episodes and only two dogs (18%) treated with ANP fibrillated during reperfusion. Thus, 64% of the ANP-treated dogs survived the combined ischaemia-reperfusion insult (Figure 22).

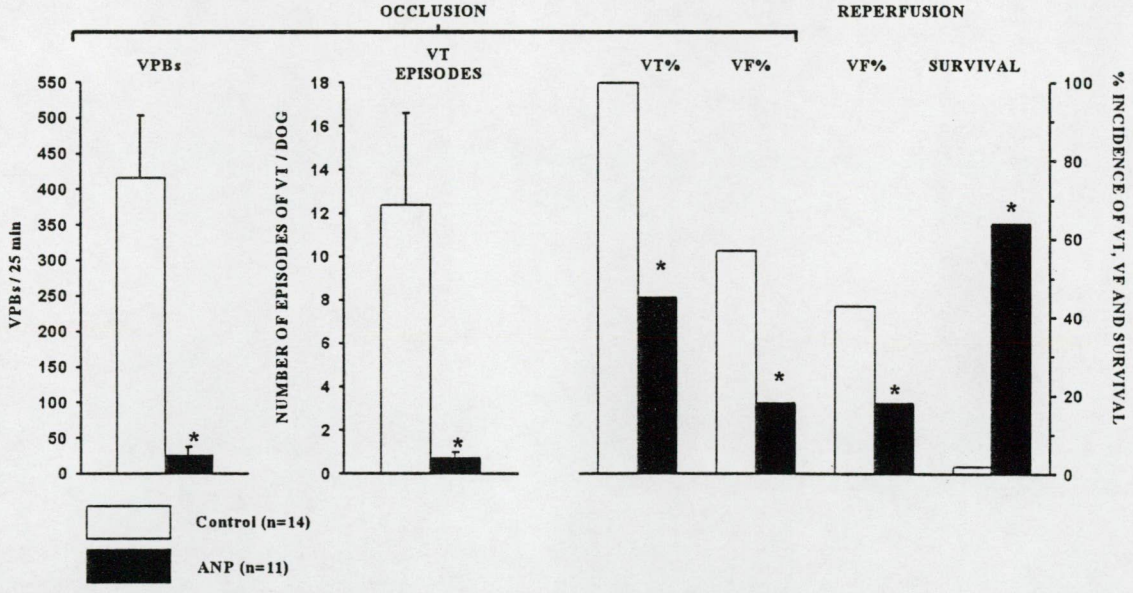


*Figure 22. Severity of ventricular arrhythmias during a 25 min occlusion of the LAD and the overall survival following the combined occlusion-reperfusion insult in control and in ANP treated dogs. *P < 0.05 vs control.*

4. DISCUSSION

4.1. Cardioprotection induced by a single brief period of coronary artery occlusion

In this part of study we examined whether one brief (5 min) period of preconditioning occlusion can protect the myocardium against those life-threatening ventricular arrhythmias that occur during a subsequent more prolonged ischaemic insults and, whether the duration of this protection is similar to that seen when preconditioning is induced by two periods of coronary artery occlusion of the same duration.

The results of the present study demonstrate that in anaesthetised dogs a single 5 min period of coronary artery occlusion provides protection against the ischaemia and reperfusion-induced ventricular arrhythmias and, that this protection is as marked as with two periods of preconditioning occlusion (6). The protection is most marked if the time interval between the preconditioning occlusion and the prolonged ischaemia is 5 min. After this time the antiarrhythmic effect starts to fade and 60 min after the preconditioning stimulus the protection is almost completly lost. Thus, the duration of this antiarrhythmic protection is almost identical with that of seen when preconditioning is induced by two 5 min occlusion; i.e. the antiarrhythmic protection last less than 60 min (6).

It is well established that short periods (3-5 min) of coronary artery occlusion in experimental animals (rats, dogs, pigs) and also in humans reduce the severity of ventricular arrhythmias that occur when the same artery is occluded for a longer period (6,43,99). For example, in dogs Végh and colleagues reported that one or two short periods of preconditioning occlusion significantly reduce the number of ectopic beats and the incidences of ventricular tachycardia and ventricular fibrillation resulting from a subsequent prolonged ischaemia and increase survival after the reperfusion of the ischaemic myocardium (6). They have also demonstrated that the optimum period for the preconditioning occlusion is 3 min in rats and 5 min in dogs (6). They have also pointed out that the protection against arrhythmias is largely lost 60 min after the commencement of preconditioning (6). In rabbits, Miura and colleagues (40) showed that a single episode of preconditioning occlusion for 2 min results in only a slight protection. However, when this occlusion was repeated twice, the reduction in infarct size was as the same as with preconditioning for 5 min. These findings suggest that the cardioprotective effect of preconditioning can be enhanced by increasing the number of the occlusion if their duration is 2 or 3 min but it is likely that a single 5 min period of ischaemia can almost result in maximal protection at least in rabbits and in dogs. In contrast, Lawson and colleagues showed in rat isolated blood perfused hearts that an increase in the number of preconditioning cycles (5 min of each) results in a "dose-dependent" reduction in the number of ventricular premature beats and in the incidences of ventricular tachycardia and ventricular fibrillation (36). These results might indicate species-dependent and end-point-dependent differences in the generation of cardioprotection associated with ischaemic preconditioning.

In summary, the present studies demonstrate that in anaesthetised dogs one brief (5 min) period of preconditioning occlusion results in marked protection against ventricular arrhythmias resulting from a subsequent, more prolonged period of ischaemia. Although this protection is marked it fades within 60 min. The time course of this antiarrhythmic protection is similar to that seen with two 5 min periods of occlusion (6) in the canine.

4.2. The cardioprotective effects of enalaprilate and Z13752A

The aim of this part of the study was to explore whether elevation of bradykinin levels by other means than ischaemic preconditioning, such as the inhibition of bradykinin breakdown, may result in protection against arrhythmias in our canine model of ischaemia/reperfusion. For this purpose, we have used the ACE inhibitor, enalaprilate and also the combined ACE/NEP inhibitor Z13752, since both drugs can elevate bradykinin levels.

Our result showed that both enalaprilate and Z13752A, given intravenously in anaesthetised dogs prior to an ischaemic insult resulted in significant haemodynamic changes (e.g. reduction in arterial blood pressure, LVSP, negative $LVdP/dt_{max}$ and coronary vascular resistance as well as increase in coronary blood flow). Furthermore, in these treated dogs there was a less marked increase in LVEDP during coronary artery occlusion. Both enalaprilate and Z13752A significantly reduced the severity of myocardial ischaemia, as assessed by changes in epicardial ST-segment elevation and in the degree of inhomogeneity of electrical activation, as well as by evaluating the severity of ventricular arrhythmias that resulted from a 25 min occlusion and then reperfusion of the left anterior descending coronary artery. For example, compared to the control group in which no dog survived the combined ischaemia-reperfusion insult, 50% of the dogs treated with enalaprilate and 67% of the animals given Z13752A survived. This degree of protection against arrhythmias is similar to that described previously, in this model, with ischaemic preconditioning (5), by cardiac pacing (24) and following the local intracoronary infusion of bradykinin (60).

The cardioprotective effects of ACE inhibitors are well demonstrated in both experimental animals (67,68) and in humans (69,70). The evidence that ACE inhibitors reduce the incidence and the severity of ventricular arrhythmias comes first from studies, performed in rat isolated perfused hearts (100,101). Clinical studies (102) also supports these findings, showing that in heart failure patients the severity of arrhythmias is less pronounced if they are treated with captopril or enalaprilate than in patient without ACE inhibitor therapy. The evidence that the cardioprotective effects of ACE inhibitors, at least in part, are mediated by bradykinin derived from those studies in which the protective effects of ACE inhibitors could be abolished by the bradykinin B_2 receptor antagonists icatibant (HOE-140) (64). This resulted in an assumption that the most likely explanation, for the cardioprotective effects of ACE inhibitors, is their ability to prevent the breakdown of bradykinin (103). The Groningen group of van Glist and de Langen (104-107) have extensively investigated the protective effects of various ACE

inhibitors in a pig model of ischaemia and reperfusion. They have showed that long term therapy with ACE inhibitors increased survival from the combined ischaemia and reperfusion insult (104,105) and it was more difficult to induce ventricular arrhythmias in the presence of an ACE inhibitor (106,107). These authors also concluded that the antiarrhythmic effect of ACE inhibitors are mediated through the elevation of bradykinin.

There has been just one study, in Lewis inbred rats, that has examined the effects of an inhibitor of neutral endopeptidase 24.11 on myocardial reperfusion injury (76). Using the Ciba-Geigy inhibitor CGS 24592 Yang and colleagues (76) showed a reduction in infarct size and this was similar to that resulted from ramiprilat administration. The protection was abolished by icatibant but unaffected by the ANF receptor antagonist HS-142-1. Their conclusion was that the infarct size reduction, following NEP inhibition, was mediated by kinins. Although the primary endpoint of these authors was to examine the effect of NEP inhibitor of the infarct size, the fact that the ventricular premature complexes which did arise following reperfusion were reduced, albeit not significantly, by this NEP inhibitor is again suggestive of a role for kinins in cardioprotection.

The cardioprotective effect of elevated levels of bradykinin resulting from inhibition of cardiac NEP activity has been recently demonstrated in isolated human cardiac membranes (74). In these preparations, in which there is a low enzymatic activity of ACE, bradykinin metabolism is mediated mostly by NEP. These results suggest that inhibition of cardiac NEP activity could be cardioprotective by elevating the local concentration of bradykinin in the heart.

In the present experiments the most likely explanation for the protective effects of Z13752A is potentiation of the cardioprotective effects of bradykinin by inhibition of its breakdown. Although ACE inhibition presumably plays a role, since Z13752A inhibits both enzymes, the evidence from the IC50 values {0.0032 μM against ACE; 0.0018 μM against NEP; (78,79)} and from the present experiments showing a more marked potentiation of bradykinin vasodilator than of inhibition of angiotensin vasopressor responses in the presence of Z13752A (Figure 9 and 10), suggests a predominant effect on neutral endopeptidase 24.11. Indeed, responses to angiotensin II itself were potentiated by the drug (Figure 9), as in the human studies of Richards et al. (108), an effect attributed by them to reduced angiotensin II clearance. The fact that the protection against arrhythmias was completely abolished by icatibant, a selective antagonist of bradykinin at B_2 receptors, and that this drug also abrogated the changes in ST-segment elevation and in the degree of inhomogeneity of electrical activation within the ischaemic area, both indices of ischaemia severity, again supports the view that the cardioprotection observed is largely kinin-mediated. We do not know if this protection, like that afforded by bradykinin itself (62), is ultimately due to nitric oxide (NO) and prostacyclin generated and released as a result of an effect of bradykinin on endothelial B_2

receptors. However, it is known that NEP inhibition leads to an increase in NO production in canine isolated coronary microvessels, and that this is mediated by kinins (109).

Besides kinin breakdown, NEP is also concerned with the breakdown of other peptides (67) such as endothelin and ANP. Z13752A when infused intravenously to dogs led to significant elevation of ANP levels (Morazzoni et al. unpuplished) and in our experiment ANP when infused intravenously in the model we have used in the present study also reduces ischaemia and arrhythmia severity during occlusion and reperfusion and could conceivably play a role in the cardioprotective effects of Z13752A. Also there is evidence that bradykinin can upregulate ANP receptors (110), therefore elevation of bradykinin levels may potentiat the cardioprotective effects of ANP.

Although selective ANP receptor antagonists are available we have no means of examining such a role for ANP in this particular large animal model. The finding that icatibant abolishes the cardioprotection resulting from Z13752A administration however would argue against this possibility.

In summary, enalaprilate a pure ACE inhibitor, and Z13752A a combined ACE/NEP inhibitor protect the heart against the severe consequences of ischaemia and evidence is adduced to suggest that this protection is mediated by bradykinin.

We believe that these results add weight to the hypothesis (111) that bradykinin acts as an endogenous myocardial protective substance (36) and that it plays a role in the protection afforded by ischaemic preconditioning. This hypothesis, the evidence for which has been recently reviewed (112), suggests that brief (preconditioning) periods of ischaemia result, like clinical coronary angioplasty, in the enhanced release of bradykinin from the heart. This then acts on endothelial B_2 receptors and stimulates the generation and release of other mediators (Figure 1), which, like bradykinin itself, are able to protect the heart against the consequences of prolonged ischaemia. That NO is a particularly important mediator is borne out by the marked attenuation of the cardioprotective effects of bradykinin, given by intracoronary administration, by inhibitors of the L-arginine-NO pathway (62).

4.3. The effect of pharmacological and mechanical preconditioning on arrhythmias; the role of bradykinin

This part of the study aimed to examine whether the protection against arrhythmias, resulting from preconditioning can be increased by an additional pharmacological stimulus. We started from the assumption that, if bradykinin is involved in the antiarrhythmic effect of ischaemic preconditioning, then enhancement of bradykinin levels by the administration of the ACE inhibitor enalaprilate or the combined ACE/NEP inhibitor Z13752A would increase the protection resulting from preconditioning.

In our experiments both enalaprilate and Z13752A (Figure 12) as well as preconditioning with a single, brief period (5 min) coronary artery occlusion (Figure 8) results in protection

against ventricular arrhythmias that occur during a subsequent prolonged ischaemia and reperfusion. However, these stimuli applied together, failed to enhance or prolong the protection resulting from either ischaemic preconditioning or drug treatment alone (Figures 17-20). Thus, both enalaprilate and Z13752A, which are alone protective, were unable to provide additional protection in preconditioned dogs. Indeed, the protective effects of these drugs (particularly of enalaprilate which is less potent than Z13752A), against ischaemia and reperfusion-induced ventricular arrhythmias, were markedly attenuated in preconditioned dogs.

Ribuot and colleagues (113) showed in anaesthetized dogs that 1 ng kg^{-1} min^{-1} dose of bradykinin, given in intracoronary infusion, 15 min before and throughout the 60 min occlusion of the LAD significantly reduced the amount of noradrenaline released following reperfusion of the ischaemic myocardium. This was accompanied by a significant reduction in the incidence of reperfusion-induced sustained VT. They concluded that the protective effects of bradykinin against reperfusion-induced arrhythmias could be associated with a reduction in cardiac noradrenaline release. Similarly, Végh and colleagues (60) showed in anaesthetised dogs, that the intracoronary infusion of bradykinin in a dose of 25 ng kg^{-1} min^{-1}, given 10 min before and throughout the entire occlusion period results in significant reduction in the severity of myocardial ischaemia (reduced ST-segment elevation and degree of inhomogeneity of electrical activation) and supresses ventricular arrhythmias. This reduction in ischaemia and arrhythmia severity following bradykinin was similar to that seen with ischaemic preconditioning. This group has also demonstrated that the protective effect of ischaemic preconditioning against ventricular arrhythmias is abolished by icatibant, indicating the involvement of bradykinin and the subsequent activation of bradykinin B_2 receptors as the consequence of preconditioning (61). Recently, Leesar and colleagues (114) showed that in patients undergoing coronary angioplasty the intracoronary infusion of 2.5 μg min^{-1} dose of bradykinin for 10 min induced similar protection than preconditioning. Furthermore, in control patient, the ST-segment shift, on the intracoronary and surface electrocardiogram, was significantly greater during the first balloon inflation than that during the second or the third inflation; consistent with ischaemic preconditioning. However, in bradykinin-treated patients, ST-segment shift during the first balloon occlusion was significantly smaller than in the control group, and there were no appreciable differences in ST-segment shifts during the three inflation. They concluded that bradykinin preconditions the human myocardium against ischaemia "*in vivo*".

There is also some evidence that ACE inhibitors can potentiate the effect of a subthreshold preconditioning stimulus "*in vitro*" and "*in vivo*". For example Morris and colleagues (115) showed that in human trabeculae a subthreshold preconditioning stimulus and the ACE inhibition alone did not enhance the recovery from a 90 min period of stimulated ischaemia. However, when these two stimuli were combined a marked protection was observed and this could be blocked by icatibant. Similarly, Miki and colleagues (116) reported that neither the

subthreshold preconditioning stimulus nor captopril treatment alone limits myocardial infarct size in rabbits subjected to a 30 min period of coronary occlusion and subsequent 3 h reperfusion. However, combination of these two stimuli potentiated the infarct size limiting effect and that this protection was also abolished by icatibant.

In our experiments in dogs which were subjected either to a single 5 min preconditioning occlusion (if the reperfusion period was less than 60 min) or to drug treatment we have found a significant reduction in the severity of arrhythmias, resulted from a 25 min occlusion of the LAD and that survival from the combined occlusion reperfusion insult was markedly increased. However, combination of these two stimuli failed to give further protection, the antiarrhythmic effects resulted from drug treatment was even attenuated in the presence of preconditioning. We do not know why the protection resulted from enalaprilate and Z13752A administration, especially against reperfusion-induced ventricular arrhythmias, disappeares in the presence of preconditioning in this canine model. There is some evidence that high levels of bradykinin can facilitate, rather than alleviate reperfusion arrhythmias in guinea pigs and in human myocardial ischaemia models (117). According to this study, bradykinin released during myocardial ischaemia accumulates in the sympathetic nerve endings and this may facilitate exocytotic and carrier mediated noradrenaline release. This enhanced noradrenaline release than contributes to coronary vasoconstriction and to the generation of ventricular arrhythmias following reperfusion. This unfavourable effect of bradykinin was abolished by the bradykinin B_2 receptor antagonist, icatibant (117). However, icatibant was not able to inhibit noradrenaline release unless enalaprilate or a combined kininase I and kininase II inhibitor was present, indicating that under these conditions endogenous bradykinin levels at the nerve ending may not be high enough to facilitate ischaemic noradrenaline release. Furtheremore, Chulak and colleagues (118) recently showed the modulatory effect of bradykinin on electrically-induced noradrenaline release in isolated atria from normal and B_2 knockout transgenic mice preincubated with noradrenaline. They showed that the lower concentrations of bradykinin 1, 3 and 10 nM did not significantly alter the outflow of noradrenaline whereas the higher concentrations of bradykinin 30 and 100 nM enhanced the release of noradrenaline. This facilitatory effect of bradykinin was inhibited by icatibant but unaffected by the bradykinin B_1 receptor antagonist. In the presence of enalaprilate or mergepta (kininase I), already the lower dose of bradykinin (10 nM) significantly increased the stimulation-induced outflow of noradrenaline. They concluded that the facilitatory effect of bradykinin on noradrenaline release in the mouse atria is mediated by presynaptic bradykinin B2 receptor which is linked to protein kinase C. Similarly, Silva and colleagues (119) showed that bradykinin concentration-dependently facilitated the release of noradrenaline evoked by electrical stimulation from the rat ventricle wall and that this was further enhanced in the presence of captopril. They have also shown that the bradykinin facilitated noadrenaline release and its enhancement by captopril was abolished by icatibant and also by the removal of the endocardium. They

concluded from these results that bradykinin is able to facilitate noradrenaline release from the sympathetic nerve endings through the activation of B_2 receptors, located in endocardial cells. Although we do not know whether a similar mechanism could explain that the antiarrhythmic protection, resulted from preconditioning and enalaprilate or Z13752A treatment, is markedly attenuated if these two stimuli are applied together, but it seems very likely that under these conditions an enhanced noradrenaline release may occur and this might contribute to the generation of ventricular arrhythmias. The validation of this hypothesis, in our canine model, requires further investigation.

4.4. New findings

1. We have showed that one brief (5 min) period of preconditioning occlusion can protect the myocardium against those life-threatening ventricular arrhythmias that occur during a subsequent more prolonged ischaemia-reperfusion insult. The most marked protection occurs 5 min after preconditioning and then the antiarrhythmic effect starts to fade. Thus, the duration of this antiarrhythmic effects is similar to that seen when preconditioning is induced by two 5 min occlusion; i.e. the antiarrhythmic protection lasts less than 60 min.

2. We have demonstrated that the ACE inhibitor enalaprilate and the combined ACE/NEP inhibitor Z13752A, both administrated intravenously 60 min prior to coronary artery occlusion, suppress the various types of ischaemia-induced ventricular arrhythmias and reduce the incidence of VF following reperfusion in anesthetised dogs.

3. However, if these drugs are given to preconditioned dogs the antiarrhythmic protection, resulted either from ischaemic preconditioning or drug treatment, is largely lost.

4. We have described for the first time a protective effect of ANP against ischaemia and reperfusion-induced ventricular arrhythmias in dogs.

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