The effect of acute simvastatin administration on the ischaemia and reperfusion-induced ventricular arrhythmias in anesthetized dogs

PhD Thesis

Gábor Kisvári

Supervisor: Prof. Dr. Ágnes Végh

Department of Pharmacology and Pharmacotherapy

Albert Szent-Györgyi Medical Centre

University of Szeged, Hungary

2016

LIST OF PUBLICATIONS

Full papers

1. **Kisvári G**, Kovács M, Gardi J, Seprényi G, Kaszaki J, Végh Á. The effect of acute simvastatin administration on the severity of arrhythmias resulting from ischaemia and reperfusion in the canine: Is there a role for nitric oxide? *Eur J Pharmacol.* **2014;** 5;732:96-104. IF: **2.13**

2. **Kisvári G**, Kovács M, Seprényi G, Végh Á. The activation of PI 3-kinase/Akt pathway is involved in the acute effects of simvastatin against ischaemia and reperfusion-induced arrhythmias in anaesthetised dogs. *Eur J Pharmacol.* **2015;** 15;769:185-94. IF: **2.54**

Full papers not related to the thesis

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

Abstracts for oral presentation

1. **Kisvári G,** Tuboly E, Kahán Zs. Egészséges, fokozott emlőrák kockázatú és illesztett kontroll személyek hormonprofil vizsgálata. III. Szegedi Emlőrák Szimpózium, Szeged, Hungary, 2009. Abstract book 21-22.

2. **Kisvári G.**, Kovács M, Kaszaki J, Végh Á. Acute administration of simvastatin reduces the severity of ventricular arrhythmias in anaesthetized dogs. Bilateral Cooperation of Doctoral Schools Timisoara-Szeged, Timisoara, Romania, 2011. Abstract book 40.

3. **Kisvári G**, Kaszaki J, Végh Á. Acute administration of simvastatin reduces ischemia/reperfusion-induced ventricular arrhythmias in anesthetized dogs. The 20th International Students' Scientific Conference for students and Young Doctors, Gdansk, Poland, 2012. Abstract book 17-18.

Abstracts for poster presentation

1. **Kisvári G**, Kovács M, Kaszaki J, Végh Á. The effect of acute administration of simvastatin on ischemia/reperfusion-induced ventricular arrhythmias in anesthetized dogs. International Society for Heart Research European Section Annual Meeting, Haifa, Izrael, 2011. Abstract book 32-34.

2. **Kisvári G**, Kovács M, Kaszaki J, Végh Á. The effect of acute simvastatin administration on ventricular arrhythmias in a canine model of ischaemia and reperfusion. 75th Anniversary of Albert Szent-Györgyi's Nobel Prize Award, Szeged, Hungary, 2012. Abstract book 49-50.

3. **Kisvári G**, Kaszaki J, Végh Á. Acute simvastatin administration reduces ischemia/reperfusion-induced ventricular arrhythmias in anesthetized dogs. Sudden Cardiac Death and Cardioprotection, Timisoara, Romania, 2012. Abstract book 11-12

4. **Kisvári G**, Seprényi Gy, Végh Á. Wortmannin abolishes the protective effect of simvastatin against the ischaemia and reperfusion-induced ventricular arrhythmias in the anaesthetized canine. International Society for Heart Research European section Annual Meeting, Barcelona, Spain, 2014. Abstract book 57.

TABLE OF CONTENTS

LIST OF ABBREVIATION

ACE	Angiotensin converting enzyme
BS	Blood sample
CBF	Coronary blood flow
DABP	Diastolic arterial blood pressure
DHE	Dihydroethidium
DMSO	Dimethylsulphoxide
eNOS	Endothelial nitric oxide synthase
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HR	Heart rate
iNOS	Inducible nitric oxide synthase
LAD	Left anterior descending coronary artery
LCX	Left circumflex coronary artery
LDL	Low density lipoprotein
L-NAME	NG-nitro-L-arginine methyl ester
LV	Left ventricle
LVEDP	Left ventricular end-diastolic pressure
LVSP	Left ventricular systolic pressure
MABP	Mean arterial blood pressure
mitoK$_{ATP}$	Mitochondrial ATP-sensitive potassium channels
NAC	N-acetyl-L-cysteine
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase
NOx	Plasma nitrate/nitrite concentrations
peNOS	Phosphorylated eNOS
PKC	Protein kinase C
PVDF	Polyvynildene-fuoride
SABP	Systolic arterial blood pressure
SCD	Sudden cardiac death
SEM	Standard error of mean
TS	Tissue sample
VF	Ventricular fibrillation
VFT	Ventricular fibrillation threshold
VPB	Ventricular premature beat
VT	Ventricular tachycardia

SUMMARY

Ventricular tachyarrhythmia is one of the main causes responsible for sudden cardiac death. The prevention and treatment of these severe tachyarrhythmias is still remained a big challenge of cardiology in the industrialized countries. There is increasing evidence that statins, which represent the first-line therapy for hyperlipidaemia, may possess lipid-independent pleiotropic actions, which are associated with an increased release of nitric oxide (NO) through the activation of eNOS, and contribute to cardioprotection following chronic statin treatment. There is lack of evidence, however, whether statins administered acutely influence the severity of arrhythmias, resulting from ischaemia and reperfusion (I/R).

Therefore, the aim of the present study was to examine the effects and mechanisms of acute statin administration on arrhythmias resulting from a 25 min coronary artery occlusion and reperfusion (I/R) in chloralose/urethane anaesthetized dogs. In a group of dogs activated simvastatin (0.1 mg/kg) was administrated in slow intracoronary injection just prior to the occlusion of the left anterior descending coronary artery. The severity of ischaemia (degree of inhomogeneity of electrical activation, epicardial ST-segment) and of arrhythmias, as well as the plasma NOx levels in blood samples taken from the coronary sinus were assessed. From the myocardial tissue samples the activity of eNOS (Western blot) and superoxide production (confocal microscopy) were determined.

We have shown that compared to the control group, in which the dogs were treated only with the solvent of simvastatin, the administration of simvastatin significantly decreased the severity of ischaemia and of ventricular arrhythmias, increased the activity of eNOS and the plasma concentration of NO metabolites, and significantly reduced the production of superoxide during reperfusion. We have also pointed out that the protective effects of simvastatin are mediated through the rapid activation of eNOS, most probably via the stimulation of PI-3 kinase/Akt pathway, since the inhibition of NOS by L-NAME, and the inhibition PI-3 kinase by wortmannin abolished the protective effects of simvastatin.

Thus we conclude that the antiarrhythmic effect of acute simvastatin administration can certainly be associated with an increased NO bioavailability during occlusion, due to the rapid activation of eNOS via the stimulation of the PI3/Akt pathway by simvastatin.

1. INTRODUCTION

1.1. Sudden cardiac death and preconditioning

The ways of prevention sudden cardiac death (SCD) due to fatal ventricular tachyarrhythmias resulting from acute myocardial ischemia have been in the focus of research for a long time. One of the earliest papers on this issue came from Harris [1] in 1950, who showed that ventricular fibrillation resulting from coronary artery occlusion is less likely to occur in dogs if a coronary artery is occluded in two stages; i.e. the complete occlusion is preceded by a partial occlusion of the same coronary artery. Somewhat later, Gülker and his colleagues [2] examined the effect of multiple coronary artery occlusion on the ventricular fibrillation threshold (VFT), and found that the decrease in VFT became increasingly less with repeated coronary occlusions. Similarly, in 1983 Barber [3], using serial short (5 min) periods of occlusion of the anterior descending coronary artery, showed that the number of ventricular ectopic beats during the second occlusion was significantly less than during the first, similar period of occlusion, if the reperfusion interval between the occlusions was 3 min. This effect, however, abolished if the reperfusion interval between the occlusions was increased to 40 min [3].

The seminal paper of Murry and his colleagues in 1986 [4] was the first publication, which provided evidence that the heart is able to adapt to ischaemic stress; i.e. short periods of ischaemia increase the tolerance of the heart to a subsequent more severe and prolonged period of ischaemia. This phenomenon was termed as ‘ischaemic preconditioning’ [4]. They showed that the metabolic changes and the size of infarct, resulting from a 40 min occlusion of the left circumflex coronary artery, is considerably reduced, if this prolonged occlusion was preceded by four 5 min periods of occlusion 20 min earlier [4]. Soon after this first description of the preconditioning phenomenon, in 1987 Shiki and Hearse [5] reported that repeated, similar periods (5 min) of occlusions markedly reduce the reperfusion-induced ventricular arrhythmias, if the reperfusion interval between the occlusions did not exceed 20 min. Extending the time interval between the occlusions to hours or to days the protection against arrhythmias was ceased [5]. If ischaemic preconditioning is considered as it originally defined by Murry and his colleagues [4], then the first evidence for the antiarrhythmic effect of ischaemic preconditioning was provided by Komori and his colleagues in 1990 [6], and somewhat later in 1992, using a large animal model, Végh and her colleagues [7]. These

studies clearly showed in two species (rats and dogs) that preconditioning provides marked protection against the ischaemia and reperfusion-induced serious, often fatal ventricular arrhythmias [6, 7].

There appears to be no doubt that ischaemic preconditioning is a general phenomenon; the protection can be induced in all species thus far investigated, including humans, suggesting possible clinical exploitation of the phenomenon. However, these expectations have been disappointed by two observations; i.e. (1) that the protection resulting from preconditioning is short-lived (it lasts only for one or two hours, then it disappears), and (2) that coronary artery occlusion, apart from some acute surgical and/or interventional cardiologic manoeuvres, cannot be applied as a preconditioning stimulus in human. These disappointments were, however, largely solved by two observations, giving further stimulus to continue 'preconditioning' research. For example, it has turned out that there is a second phase of the cardioprotection, which occurs 20-24 hours after the initial preconditioning stimulus. This "delayed" or "second window of protection" has raised a considerable interest and facilitated further investigations for the exploration of this phenomenon [8-10]. Furthermore, in 1991 Végh and her colleagues [11] provided the first evidence that not only the brief ischaemic episodes, but rapid pacing of the heart through the right ventricle, may evoke protection against the ischaemia and reperfusion-induced arrhythmias. Also, they showed that heavy physical exercise is an effective stimulus to induce a preconditioning-like cardioprotection [12]. Since then it becomes obvious that protection, similar to that results from ischaemic preconditioning, can be induced by several stimuli, like partial coronary artery occlusion, hypoxia, increased myocardial stretch, or by various drugs that may use similar pathways to elicit protection than preconditioning itself.

The mechanisms involved in the preconditioning-induced adaptive phenomenon have also raised considerable interest both in experimental and clinical point of view. A number of hypotheses have been raised to explain the mechanisms involved in cardioprotection associated with preconditioning. Among these, perhaps the most accepted one is, which emphasizes the involvement of endogen substances in the induction and mediation of the protection [13]. According to this, endogenous substances (both protective and injurious) are generated and released from the myocardium in response to the transient ischaemic stress, resulting from the preconditioning stimulus. These substances are then activating their

receptors, stimulate various signalling pathways and modify myocardial function, leading to cardioprotection.

The first endogen substances that related to the preconditioning-induced protection were prostacyclin [14] and adenosine [15, 16]. For example, in 1990 Végh and her colleagues [14] demonstrated in anaesthetized dogs that the inhibition of the cyclooxygenase pathway by meclofenamate markedly attenuates the antiarrhythmic effect of preconditioning. Almost at the same time the Downey's group provided evidence that adenosine, plays a crucial role in the preconditioning-induced cardioprotective effect [15]. They proposed that adenosine by activating the Gi-protein coupled adenosine A1 receptor leads to the translocation of protein kinase C (PKC) from the cytosol to the membrane, resulting in the opening of sarcolemmal ATP-sensitive potassium channels [16, 17]. The role of ATP channels in the preconditioning-induced cardioprotection is supported that the protection was abolished by the ATP-sensitive potassium channel blocker glibenclamide [17].

There is some evidence that reactive oxygen species (ROS) may also play a trigger role in the preconditioning-induced protection [18, 19]. The short periods of preconditioning ischaemia and reperfusion insult, result in early ROS generation via the opening of the mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}), and the influx of potassium ion influx into the mitochondria [20].

Besides the 'adenosine hypothesis' proposed by the Downey's group [15], our research group, in the Department of Pharmacology and Pharmacotherapy at the University of Szeged, suggested an alternative hypothesis for mechanisms involved in the preconditioning-induced protection against arrhythmias. It was hypothesized that, more than likely, several mediators are generated and released in response to the preconditioning stimuli, not only from the cardiac myocytes, but also form the vascular endothelium. These endothelium-derived substances would modify arrhythmogenesis by direct communication with cardiac myocytes. It was shown that bradykinin, which is rapidly formed in the blood during hypoxia [21-23] plays an important trigger role in the preconditioning-induced protection, since it acting on the endothelial bradykinin B_2 receptors triggers the generation and release of other mediators, such as prostacyclin[14] and nitric oxide [24]. These diffusible mediators reaching the myocytes activate mechanisms, which are ultimately lead to the protection [25]. The

involvement of bradykinin in the cardioprotective effect of preconditioning was confirmed by others as well [26, 27].

Our research group was the first to propose that nitric oxide (NO) plays a mandatory role in the cardioprotective effect of preconditioning. The most likely scenario is that NO by stimulating soluble guanylyl cyclase elevates cGMP, which could modify arrhythmogenesis, for example, by suppressing calcium entry into the cell through L-type calcium channels [28]. There is abundant evidence that NO plays a major role, both as a trigger and a mediator, in the preconditioning-induced early and delayed protection [10, 29-31].

Under physiologic conditions NO is synthesized by NOS enzymes, among which the endothelial isoform (eNOS) has the major role in NO formation to regulate vascular tone, inhibit platelet aggregation, or to achieve negative inotropic effect on the myocardium [32, 33]. The other two isoforms, such as the neuronal (nNOS) and the inducible (iNOS) enzymes may also have a role in NO production both under physiological and pathophysiological conditions. These isoforms differ in their localisation, dependence on Ca^{2+}, as well as in their expression and activities. Both eNOS and nNOS are Ca^{2+}-dependent enzymes, they are activated, when the level of intracellular Ca^{2+} becomes elevated [34]. In contrast, the activation of iNOS does not require calcium; it is activated by other factors, such as cytokines or tumour necrosis factor and largely involved in myocardial depression associated with sepsis [35]. Interestingly, it has been proved that the activation of iNOS, by producing NO, is the part of that pathway, which is involved in the delayed phase of the cardioprotection afforded by preconditioning [31, 34, 36, 37].

Most of the effects of NO, such as vasodilatation, inhibition of platelet aggregation, reduced myocardial contractility, are mediated through the activation of guanylyl cyclase and the resulting elevation of cGMP [28, 38]. Furthermore, NO regulates calcium homeostasis within the cell by enhancing calcium uptake into the sarcoplasmic reticulum [39], directly inhibits Ca^{2+} influx across L-type calcium channels of the cardiomyocytes [40], modifies noradrenaline release from nerve endings [41], regulates free radical formation and protects the myocardium against reperfusion injury [42, 43]. Some of these effects are not mediated by cGMP; there is increasing evidence that NO may modify processes and functions by a cGMP-independent way, with S- nitrosylation [44]. Nevertheless, all these above mentioned effects

and mechanisms of NO may somehow contribute to the antiarrhythmic effect of preconditioning.

Another important step in the research of preconditioning was the recognition that certain drugs may induce protection similar to that of preconditioning. The idea of the “drug-induced preconditioning „came from the assumption that if endogenous substances are, indeed, involved in the preconditioning-induced protection, and that their synthesis and the release become impaired under pathophysiological conditions, then drugs, which are able to replace or modify the production of these mediators, would elicit protection. One of the first attempts to pharmacological preconditioning was the use of adenosine and its analogues like acadesine [45]. However, the clinical exploitation of these drugs is largely limited by the strong hypotensive and bradycardiac effects, and that tachyphylaxis may develop after their administration. There was, however, more success with the use of organic nitrites and nitrates as well as the angiotensin converting enzyme (ACE) inhibitors. These drugs have been using for a long time in the treatment of angina pectoris, hypertension and heart failure. The recognition that nitrites and nitrates act by donating NO (NO donors) and that ACE participates in the degradation of bradykinin, make these drugs candidate for pharmacological preconditioning. Indeed, we have substantial evidence that the K_{ATP} channel opener and NO donor nicorandil [46] and the ACE-NEP inhibitor Z13752A [47] by reducing the degradation of bradykinin, mimic the antiarrhythmic effects of preconditioning. More recently we showed that the inorganic sodium nitroprusside [48] and sodium nitrite [49] by donating NO provides marked protection against arrhythmias, resulting from ischaemia and reperfusion.

Beyond the abovementioned drugs, clinical studies suggest that statins may provide protection against the severe ventricular arrhythmias and sudden cardiac death [50-52]. These drugs were originally developed for the treatment of hypercholesterolemia, but it turned out that some of the cardiovascular protective effects are independent from their lipid lowering effect.

1.2. Statins in the treatment of cardiovascular diseases

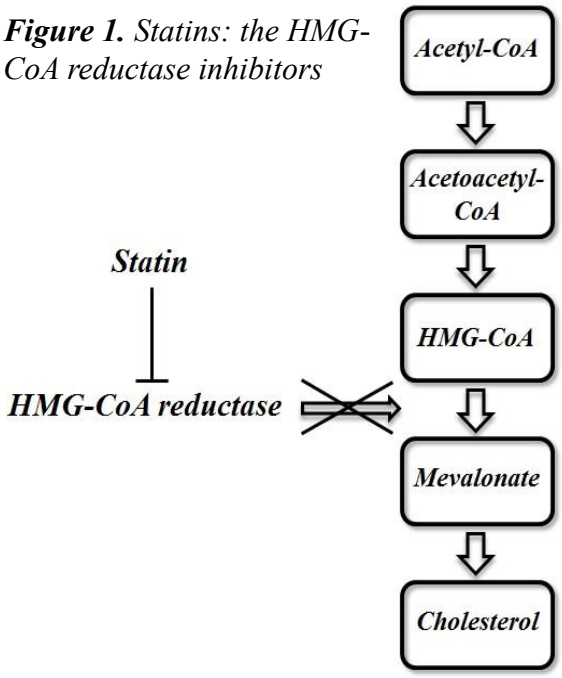

Figure 1. *Statins: the HMG-CoA reductase inhibitors*

In the 1950s it became obvious that the elevated level of cholesterol in plasma mainly contributes to the cardiovascular diseases [53]. Since in those times there was no clear clinical evidence for the benefit of cholesterol level reduction, the interest for developing cholesterol lowering drugs was marginalized. In the following years numerous studies confirmed the therapeutic advantage of the decrease of cholesterol synthesis, pointing out that a reduction in the elevated plasma levels of LDL with diet and drugs decreases the risk of cardiovascular diseases [54]. One of the possibilities by which cholesterol formation can be reduced is the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which is a rate limiting enzyme of cholesterol synthesis (Figure 1). The first HMG-CoA reductase inhibitor was ML236B (Compacting), which had been found in a fermentation broth of Penicillium citrinum during the search for an antimicrobial agent [55]. This substance has been shown to reduce cholesterol level by 30% in rats [55]. Somewhat later, in a fermentation broth of Aspergillus terreus, an even more potent inhibitor of HMG-CoA reductase was found, that we now know as lovastatin [56]. Since then, the research for novel drugs with lipid lowering effect has been accelerated; as a result, several drugs, termed as statins (simvastatin, pravastatin, fluvastatin, atorvastatin, rosuvastatin), were developed [57].

Recently statins are widely used to lower cholesterol level. Their primary effect is the competitive inhibition of HMG-CoA reductase, which is the rate-limiting enzyme of the mevalonate pathway, producing cholesterol and isoprenoid (Figure 1). Mitigation of cholesterol synthesis leads to enhanced synthesis of LDL receptors, resulting in increased absorption of low-density lipoprotein from the plasma, and decreased cholesterol level. Since they have also a salutary effect on the lipid composition of plasma, due to an increase in high density lipoprotein (HDL) cholesterol and a decrease in triglyceride levels, statins play an

important role in the treatment of patients with hyperlipidemia, and subsequently, with cardiovascular diseases.

Statins can be classified on the basis of their effectiveness to reduce lipid levels (rosuvastatin = atorvastatin > simvastatin > lovastatin > pravastatin). The differences are derived from the strength of binding between statins and HMG-CoA reductase [58]. Statins may also classify according to their lipid solubility; e.g. simvastatin, atorvastatin, lovastatin and fluvastatin are lipophilic, while rosuvastatin and pravastatin hydrophilic, and to their chemical structure; i.e. whether the certain drug is a lactone pro-drug, which is converted to active beta-hydroxyl form in the gut, like simvastatin and lovastatin, or exists in active form having an open lactone ring, like atorvastatin, fluvastatin, pravastatin and rosuvastatin [59]. In respect of manufacturing, we distinguish synthetic products (atorvastatin, cerivastatin, fluvastatin and rosuvastatin) and molecules that are produced by fermentation (e.g. pravastatin from *Nocardia autotrophica*, simvastatin from *Aspergillus terreus*) [57-61].

1.3. Pleiotropic effects of statins

There is increasing experimental and clinical evidence that statins, beyond their lipid lowering effects, are able to reduce the incidence of fatal ventricular arrhythmias, which are the major cause of mortality in various cardiovascular diseases [50-52, 62, 63]. Since cardioprotection by statins was also observed under normocholesterolemic conditions [64-67], it was proposed that these drugs might have cholesterol-independent, pleiotropic effects as well [68, 69]. These include plaque stabilization and improvement in vascular endothelial function [70], reduction in oxidative stress-induced injury [71-73], inhibition of inflammatory [74, 75] and thrombogenic responses [69]. Most of these effects are thought to play a role in the anti-arrhythmic action of statins [66, 76, 77], and would explain the observed reduction in cardiac death in patients with statin treatment [78, 79]. The salutary effects, unrelated to the cholesterol lowering action of statins, are proposed to involve both nitric oxide (NO)-dependent and NO-independent mechanisms [65, 80]. For example, statins have been found to increase the generation of NO via eNOS activation [81-83], enhance the formation of prostanoids [84] and induce heme-oxygenase-1 [85].

Perhaps, the most likely mechanism by which statins provide protection against ischaemia and reperfusion-induced arrhythmias is their ability to increase NO synthesis [65,

82, 86]. There is substantial previous evidence that NO plays an essential role in both the early and delayed anti-arrhythmic effects of preconditioning, induced either by brief periods of coronary artery occlusion [29], cardiac pacing [30, 31] or heavy physical exercise [12]. It was also demonstrated that these preconditioning stimuli, via the activation of eNOS, enhance NO production and modify myocardial function during ischaemia and reperfusion [87]. More recently it was showed that pacing induces an immediate increase in eNOS activation and NO production, but it also causes an up-regulation of eNOS gene and protein expressions 12 and 24h later [88].

Although the precise mechanism through which statins yield cardioprotection is still not well established, clinical and experimental studies suggest that NO, generated via the activation of eNOS, may play a mandatory role [89-91]. The mechanisms by which statins may activate eNOS are even less understood; the prevention of the oxLDL-mediated down-regulation of eNOS mRNA and protein levels [92], as well as the up-regulation of eNOS by blocking the synthesis of those isoprenoid intermediates of the cholesterol synthesis pathway that are involved in the posttranslational modification of proteins which regulate eNOS expression [81, 93], were suggested as potential mechanisms for eNOS activation during chronic statin administration. There are, however, only a few studies which have attempted to examine the acute effects of statins; these were related to the assessment of infarct size [94-96] and post-ischaemic contractile dysfunction [97]. One of the possible pathways that has been proposed to activate eNOS through phosphorylation within minutes, would be the PI 3-kinase/Akt signalisation [95, 98]. Indeed, Kureishi and his colleagues [98] have demonstrated that following acute simvastatin administration, the activation of the PI 3-kinase/Akt/eNOS cascade is involved in the increase of NO synthesis in endothelial cells. Similarly, Wolfrum and his colleagues [95] showed that in anaesthetised rats subjected to coronary artery occlusion, the administration of simvastatin just prior to reperfusion, markedly reduced infarct size, increased PI 3-kinase activity, as well as Akt Ser473 and eNOS Ser1177 phosphorylation [95]. There is, however, no study which has examined how a single dose of simvastatin influences the acute ischaemia and reperfusion induced severe ventricular arrhythmias. To answer this question, two studies were designed and performed.

2. AIMS OF THE STUDY

The purpose of the present thesis was to answer the question whether a single dose of simvastatin would modify the acute ischaemia and reperfusion induced severe, often life-threatening ventricular arrhythmias. If so, what might be the mechanism? We have designed two separate studies in order to obtain answers for these questions, and the results were published in the enclosed publications. Our questions were as follows:

a) Despite the number of clinical evidence that chronic statin treatment in patients reduces the mortality due to sudden cardiac death, there is no sufficient information as to whether the acute simvastatin administration would elicit a similar salutary effect. Therefore, we designed studies in which we have examined in our established canine model, whether a single bolus injection of simvastatin prior to an ischaemia and reperfusion insult would modify the severity of ventricular arrhythmias. We have also examined whether nitric oxide (NO) is involved in the acute effect of simvastatin.

b) Since we have found that the acute administration of simvastatin provides marked protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias and that this antiarrhythmic effect involves the generation of NO by simvastatin, in a separate study we have now examined whether this NO formation is mediated through the rapid activation eNOS via the stimulation of the PI 3-kinase/Akt signalisation. For this purpose we used the PI-3-kinase inhibitor wortmannin prior to the administration of simvastatin.

3. MATERIALS AND METHODS

3.1. Experimental animals and ethical concerns

Adult mongrel dogs of both sexes with a mean body weight of 23 ± 1 kg were used. The origin and upkeep of these dogs were in accord with Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3.2. Surgical preparation

The animals were anaesthetized with pentobarbital (30 mg kg^{-1} intravenous sodium pentobarbitone, Sigma, St. Louis, MO, USA). The right femoral artery was prepared and catheterized, through which the dogs were further anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively; Sigma, St. Louis, MO, USA) to maintain anaesthesia. The animals were then intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus, Natick, MA, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits [7]. Body temperature was measured from the mid-oesophagus and maintained by a heating pad at 37 ± 0.5^{o}C.

Polyethylene catheters were inserted into the right femoral artery for monitoring arterial blood pressure (systolic and diastolic), and via the left carotid artery into the left ventricle (LV) for the measurement of systolic (LVSP) and end-diastolic (LVEDP) pressures. From the LV pressure curves changes in positive and negative dP/dt_{max} were calculated.

The chest was opened at the fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. Distal to the occlusion site a smaller side branch of the same artery was also prepared and cannulated for the local administration of drugs (simvastatin, L-NAME, wortmannin and DMSO) and vehicle. Another catheter was positioned through the right jugular vein into the coronary sinus to obtain blood samples for the assessment of plasma nitrate/nitrite (NOx) levels. In some dogs from each group, the left circumflex (LCX) coronary artery was also prepared to measure coronary blood flow (CBF; ml/min^{-1}) by means of a transit time Doppler flow probe (Hugo Sachs Electronics, Germany).

The severity of myocardial ischaemia was evaluated by changes in the epicardial ST-segment and in the degree of inhomogeneity of electrical activation. These were measured by a composite electrode (containing also four unipolar electrodes by which changes in ST-segment [mV] were detected) positioned within the potentially ischaemic area as described previously [7]. The greatest delay in activation within the ischaemic area following coronary artery occlusion was expressed in ms. All parameters, together with a chest lead electrocardiogram, were measured with a Plugsys Haemodynamic Apparatus (Hugo Sachs Electronics, Germany) and recorded on a Graphtec Thermal Array Recorder (Hugo Sachs Electronics, Germany).

Ventricular arrhythmias were assessed according to the Lambeth conventions [99] with that modification as outlined previously [7]. In brief, the total number of ventricular premature beats (VPBs), the incidence and the number of episodes of ventricular tachycardia (VT; defined as a run of four or more consecutive VPBs at a rate faster than the resting heart rate), and the incidence of ventricular fibrillation (VF) were assessed during the occlusion period. During reperfusion, only the incidence of VF, which is a fatal event in this species, was determined. Dogs that were alive 1-2 min after reperfusion were considered to be survivors.

The risk area following coronary artery occlusion was assessed by injecting Patent Blue V dye into the re-occluded artery using the same method that has been described in detail elsewhere [7].

3.3. Measurement of plasma nitrate/nitrite (NOx) levels

These were performed as described previously [100]. Plasma nitrate/nitrite (NO_x) concentrations were determined by means of the Griess reaction in blood samples taken from the coronary sinus at various time intervals as illustrated in Figure 2. After preparation of blood samples, the absorbance of the azo compound was measured spectrophotometrically at a wavelength of 540 nm and the total nitrate/nitrite (NO_x) concentration ($\mu mol\ l^{-1}$) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma, St Louis, MO, USA).

3.4. Determination of eNOS phosphorylation by Western blot

Freshly excised tissue samples from the ischaemic and non-ischaemic regions of the left ventricular myocardial wall were immediately frozen in liquid nitrogen and stored at −80°C.

100 µg of protein extracts were resolved using 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and blotted on polyvinylidene fluoride membranes. The blots were immunolabeled overnight with a monoclonal mouse anti-eNOS primary antibody (pS1177, BD Biosciences) diluted to 1:2500, followed by 1 h incubation with an HRP-conjugated anti-mouse rabbit secondary antibody (Dako, Danmark) in a dilution of 1:8000. Band densities were detected with the ECL Plus kit (GE Healthcare, Buckinghamshire, UK) and developed on Amersham Hyperfilm^{TM} (GE Healthcare, Buckinghamshire, UK). Pixel intensities of each band were measured using ImageJ software (NIH). Three parallel Western blots were performed for the statistical analysis using Bonferroni correction. On the blots each examined group was compared to the sham-controls. For verifying equal loading, PVDF membranes were labelled with Coomassie-Blue.

An integrated optical density value (the sum of each pixel value corrected to the background) was formed by drawing equal size boxes around the bands. The intensities obtained from both the total and peNOS bands were normalised to this integrated value.

3.5. Determination of the functional activity of eNOS by radio immunoassay

This was performed using a NOS activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) based on the biochemical conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline by NOS. From the tissue samples (100 mg) membrane proteins were isolated, homogenized in ice-cold homogenization buffer (Cayman Chemical, Ann Arbor, MI, USA), and centrifuged at 2000 g for 15 min. The supernatant was then ultra-centrifuged at 50000 g for 45 min and the pellet (membrane fraction) was re-suspended in the homogenization buffer. A liquid scintillation counter was used to determine eNOS activity by measuring the amount of the radio-labelled citrulline formed during the reaction, and expressed as the percentage of the total counts corrected with the background counts per minute.

3.6. Assessment of superoxide production

This was determined by dihydroethidium (DHE; Sigma-Aldrich) fluorescence staining. Tissue blocks, excised from the ischaemic myocardial wall were embedded in optimal cutting temperature compounds. Cryosections (20 µm) were produced, stained with DHE (1µmol l^{-1}, dissolved in pH 7.4 phosphate buffer solution), and incubated at 37^{o}C for 30 min in a dark humidified chamber. A negative control was obtained by blocking the reaction with N-acetyl-L-cysteine (NAC, 100 mmoll^{-1}, Sigma-Aldrich). Both from the stained and the negative

control samples 10 to 15 serial images were captured by a confocal laser scanning microscope (Olympus FV1000). The intensity of the fluorescent signals were analysed by ImageJ software (NIH) and expressed in arbitrary units.

3.7. Preparation of simvastatin solution

Before the application of simvastatin (Sigma, St Louis, MO, USA) it has to be converted into an active form. A stock solution containing 25 mg simvastatin, dissolved in 625 µl ethanol and 937.5 µl 0.1 N NaOH, was prepared and incubated at 50^{o}C for 2 hours. Then the pH of the solution was adjusted to 7.0 with 1N HCl and stored at -20°C until use. Immediately prior to the experiments an aliquot was taken and diluted in distilled water to obtain the appropriate dose.

3.8. Experimental protocols

3.8.1. Experimental protocol for the assessment of acute simvastatin administration on the ischaemia and reperfusion-induced arrhythmias

Dogs were randomly selected to form four experimental groups (Figure 2). Control dogs (n = 16) were administered the solvent of simvastatin (0.5 ml min^{-1}) in intracoronary injection (over 5 min) and subjected to a 25 min occlusion and then reperfusion of the descending branch of the left coronary artery (LAD). Fifteen dogs were received activated simvastatin in a dose of 0.1 mg kg^{-1} by the same route, 5 min prior to the onset of the occlusion. In another two groups, L-NAME (Sigma) was given in a dose of 5 mg kg^{-1} also in slow intracoronary injection, 15 min before the solvent (L-NAME; n = 12) and the simvastatin (n = 11) administration. Eight dogs were used as sham-operated controls (SC; not included in the protocol figure).These dogs were only instrumented, without subjecting them to any treatment, and from which, after the euthanasia, tissue samples were harvested for measuring eNOS phosphorylation and superoxide production. At the end of the experiments the hearts were stopped by an excess of the anaesthetic, and myocardial tissue samples were collected from both the ischaemic and the non-ischaemic regions of the left ventricular wall for further analyses. In at least 5 dogs of each group sample taking was performed at the end of the 25 min occlusion period, whereas in dogs that had been subjected to reperfusion, tissue samples were collected either 5 min after reperfusion (these animals were considered as survivors) or at the time when the fibrillation was observed.

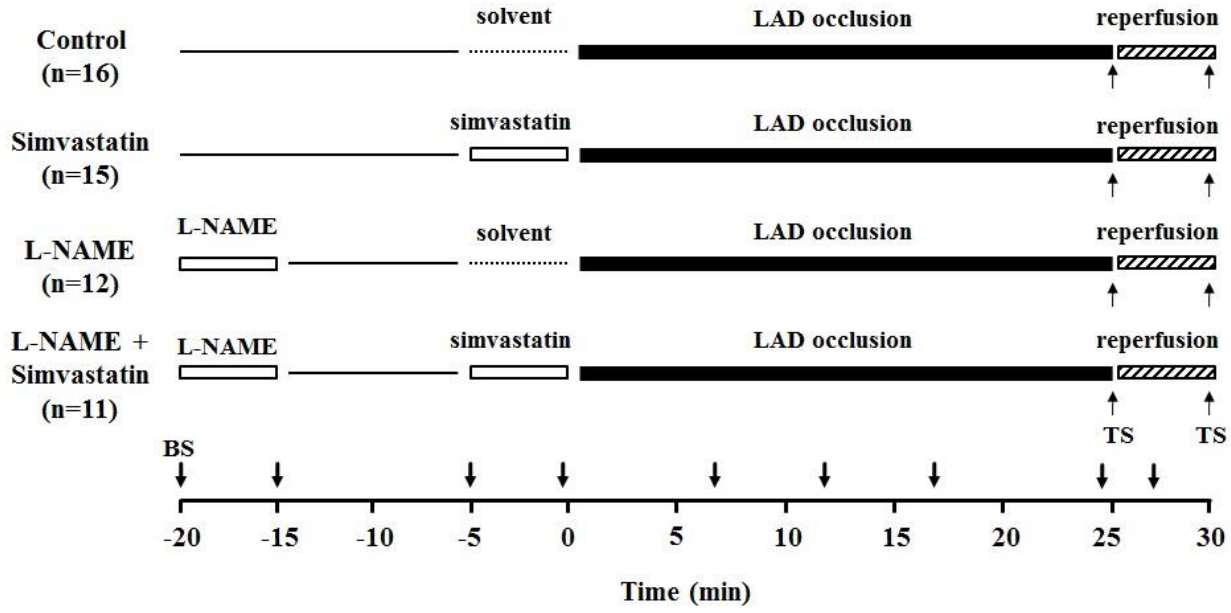

Figure 2. *Experimental protocol for the evaluation of the antiarrhythmic effect of acute simvastatin administration. Blood samples (BS) were taken at various time intervals (indicated by arrows) from the coronary sinus, and tissue samples (TS) were collected at the end of the ischaemic period or 5 min after reperfusion for further biochemical analyses.*

3.8.2. *Experimental protocol for the assessment the role of PI 3-kinase/Akt pathway in protection provided by the acute simvastatin administration*

In the second series of the experiment, the dogs were randomly selected into five experimental groups (Figure 3).The control (C; n=14) and the simvastatin (S; n=12) groups were similar to the groups mentioned above. In two other groups, wortmannin alone (W; n=12, Sigma) or together with simvastatin (W+S; n=11) was given in a dose of 1.5 $mgkg^{-1}$ in slow intracoronary injection, 15 min before the administration of the solvent and simvastatin. Additional 13 dogs were received 0.1% dimethylsulphoxide (DMSO), the solvent of wortmannin. Data obtained from 8 dogs in the control and from 6 dogs in the simvastatin groups were already used in the first series of experiments.

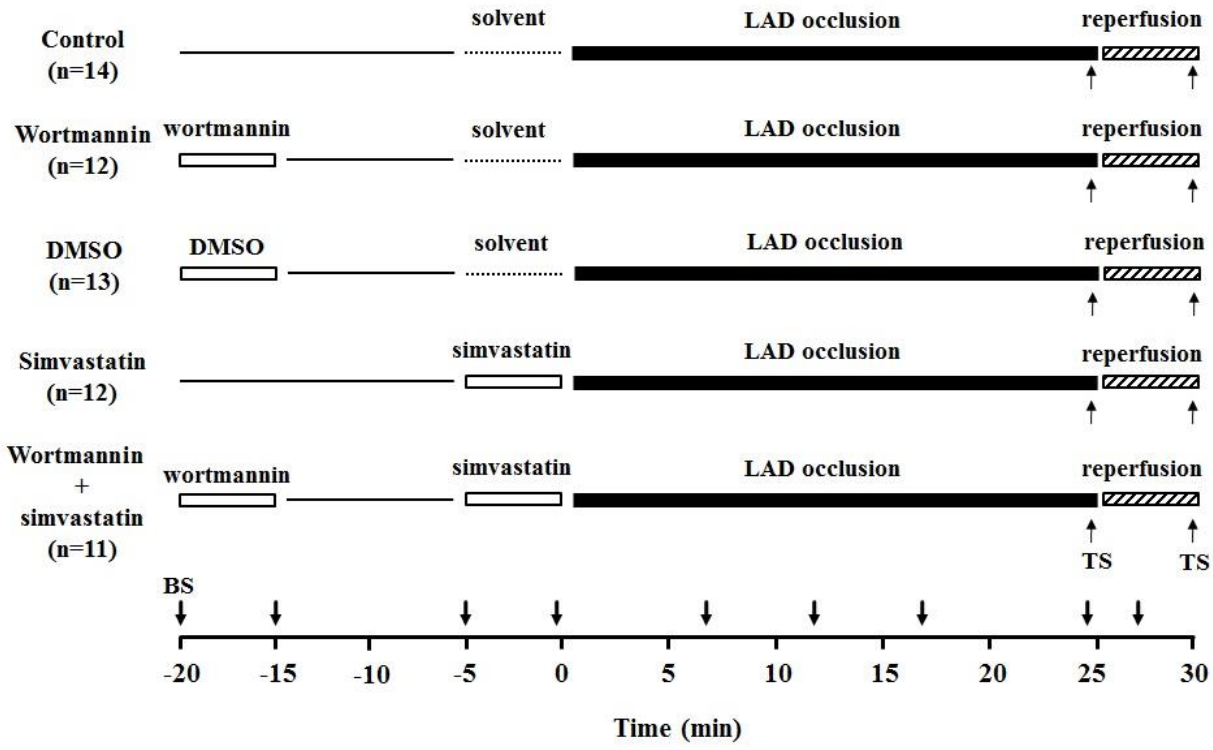

Figure 3. *Experimental protocol for the exploration of the IP-3/Akt pathway in the antiarrhythmic effect of simvastatin (TS = tissue sample, BS = blood sample).*

3.9. Statistical analysis

All data are expressed as means ± S.E.M. and the differences between means were compared by ANOVA for repeated measures and by the one-way ANOVA as appropriate, using the Fisher *post hoc* test. VPBs and episodes of VT were compared using the Kruskal–Wallis test. The incidences of arrhythmias (such as VT and VF) and survival from the combined ischaemia and reperfusion insult were compared by the Fisher's exact test. Differences between groups were considered significant at $P < 0.05$.

4. RESULTS

4.1. Evaluation of the effect of acute simvastatin administration on ischaemia and reperfusion-induced arrhythmias

4.1.1. Haemodynamic changes following the administration of solvent, simvastatin and L-NAME, as well as after coronary artery occlusion

These are summarized in Table 1 and Table 2. Local intracoronary injection of simvastatin and the solvent of simvastatin did not substantially modify the haemodynamic parameters and blood flow, measured on the LCX coronary artery. In contrast, the administration of L-NAME significantly elevated arterial blood pressure and reduced heart rate without substantially modifying the other haemodynamic parameters (Table 1). These haemodynamic changes in the L-NAME treated dogs were still present before the onset of the LAD occlusion.

Occlusion of the LAD resulted in significant reductions in arterial blood pressure, LVSP, positive and negative dP/dt_{max} and an increase in LVEDP, whereas the HR remained substantially unchanged. These alterations were almost similar in all the examined groups. The compensatory blood flow changes, occurring on the LCX when the LAD was occluded, were not significantly modified by the administration of simvastatin and L-NAME (Table 2).

Table 1. *Haemodynamic changes following the administration of solvent, simvastatin and L-NAME*

	Solvent		Simvastatin		L-NAME	
	Baseline	**Change**	**Baseline**	**Change**	**Baseline**	**Change**
SABP (mmHg)	140 ± 10	3 ± 3	148 ± 8	-4 ± 2	149 ± 7	19 ± 5 *
DABP (mmHg)	105 ± 6	0 ± 5	101 ± 5	-2 ± 2	104 ± 5	29 ± 5 *
MABP (mmHg)	117 ± 6	1 ± 4	117 ± 6	-3 ± 2	119 ± 5	25 ± 4 *
LVSP (mmHg)	154 ± 8	3 ± 3	158 ± 8	0 ± 2	164 ± 7	13 ± 4 *
LVEDP (mmHg)	4.6 ± 0.7	0.2 ± 0.3	5.6 ± 0.4	0 ± 0.3	5.1 ± 0.7	0.4 ± 0.3
+dP/dt (mmHg·s^{-1})	2316 ± 225	99 ± 61	2795 ± 219	-67 ± 118	2444 ± 242	-124 ± 189
-dP/dt (mmHg·s^{-1})	2092 ± 260	91 ± 103	2415 ± 183	-107 ± 81	2172 ± 122	-43 ± 43
HR (beats·min^{-1})	166 ± 9	5 ± 2	173 ± 5	-3 ± 1	181 ± 8	-15 ± 3
mean CBF$_{LCX}$ (mL·min^{-1})	30 ± 1	1 ± 1	30 ± 2	2 ± 1	32 ± 2	-3 ± 2

Data are means ±SEM calculated from n = 11–16 experiments. Data, presented as changes, were determined 5 min after starting the infusion of the solvent, simvastatin and L-NAME.*P < 0.05 compared to baseline value. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; CBF, coronary blood flow.

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

	Solvent		Simvastatin		L-NAME		L-NAME+Simvastatin	
	Baseline	**Max.change**	**Baseline**	**Max. change**	**Baseline**	**Max.change**	**Baseline**	**Max.change**
SABP (mmHg)	143 ± 10	-11 ± 2*	145 ± 6	-10 ± 2*	166 ± 5	-8 ± 2*	163 ± 8	-10 ± 3*
DABP (mmHg)	104 ± 6	-11 ± 3*	99 ± 3	-7 ± 2*	133 ± 4	-8 ± 3*	129 ± 7	-11 ± 3*
MABP (mmHg)	117 ± 6	-12 ± 3*	114 ± 4	-7 ± 2*	144 ± 5	-8 ± 3*	142 ± 8	-11 ± 2*
LVSP (mmHg)	144 ± 11	-10 ± 3*	145 ± 7	-9 ± 2*	177 ± 5	-9 ± 2*	180 ± 11	-14 ± 5*
LVEDP (mmHg)	4.7 ± 0.7	9.2 ± 1.5*	5.5 ± 0.3	8.5 ± 1.0*	5.5 ± 0.7	10.0 ± 2.1*	5.0 ± 0.5	9.0 ± 1.0*
+dP/dt (mmHg·s^{-1})	2236 ± 212	-568 ± 99*	2670 ± 164	-499 ± 137*	2252 ± 174	-553 ± 165*	2526 ± 317	-566 ± 109*
-dP/dt (mmHg·s^{-1})	2183 ± 253	-338 ± 161*	2349 ± 145	-378 ± 68*	2197 ± 174	-491 ± 125*	2317 ± 205	-350 ± 76*
HR (beats·min^{-1})	171 ± 9	5 ± 5	170 ± 4	5 ± 3	166 ± 9	3 ± 4	174 ± 11	7 ± 2
mean CBF$_{LCX}$ (mL·min^{-1})	31 ± 2	9 ± 2	30 ± 2	10 ± 2	29 ± 1	9 ± 1	30 ± 2	8 ± 2

Data are means ±SEM calculated from n = 11–16 experiments. *$P < 0.05$ compared to baseline value. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; CBF, coronary blood flow.

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

This is shown in Figure 4. In control dogs, occlusion of the LAD resulted in a high number of VPBs (289 ± 34) and episodes of VT (5.6 ± 1.3) that occurred in 88% of these dogs. Furthermore, 56% of the dogs fibrillated during occlusion and no control dog survived reperfusion. Local administration of simvastatin, just prior to the occlusion, significantly reduced these arrhythmias (VPBs: 94 ± 25, episodes of VT: 0.3 ± 0.2, incidence of VT: 20%, VF: 0%; $P < 0.05$ compared with controls) during occlusion, and increased survival from 0% to 33% compared to the controls. Inhibition of the L-arginine-NO pathway with L-NAME did not substantially modify arrhythmia severity during occlusion and reperfusion, but it significantly attenuated the antiarrhythmic effect of simvastatin. Thus in the presence of L-NAME the number of VPBs (438 ± 49), the incidence (100%) and number of episodes of VT (6.8 ± 2.9) were again increased in the simvastatin treated dogs, and as in the control group, no dog survived the combined ischaemia and reperfusion insult. Interestingly, L-NAME, however, did not affect the protective effect of simvastatin against the occlusion-induced ventricular fibrillation; i.e. as with simvastatin alone, no dog fibrillated during occlusion in the L-NAME+simvastatin group.

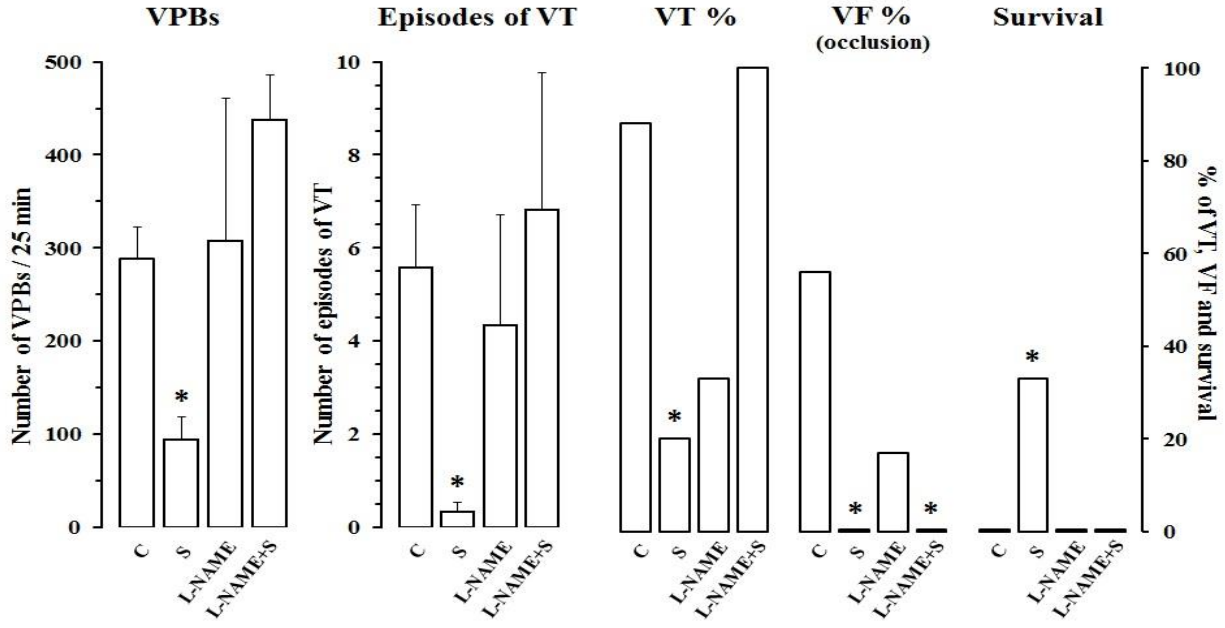

Figure 4. *The severity of ventricular arrhythmias during a 25 min occlusion and reperfusion of the LAD in control (C) and simvastatin (S) treated dogs, and in dogs given L-NAME alone or together with simvastatin (L-NAME+S). Values are means ± S.E.M. *P < 0.05 compared to the controls.*

4.1.3. Changes in the severity of myocardial ischaemia during coronary artery occlusion

This was assessed by two parameters: i.e. changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment In control dogs, occlusion of the LAD resulted in immediate and significant increases both in the epicardial ST-segment (Figure 5A) and the degree of inhomogeneity of electrical activation (Figure 5B). These changes were significantly less pronounced following simvastatin administration. Although L-NAME itself did not modify these indices of ischaemia severity, it abolished the anti-ischaemic effects of simvastatin.

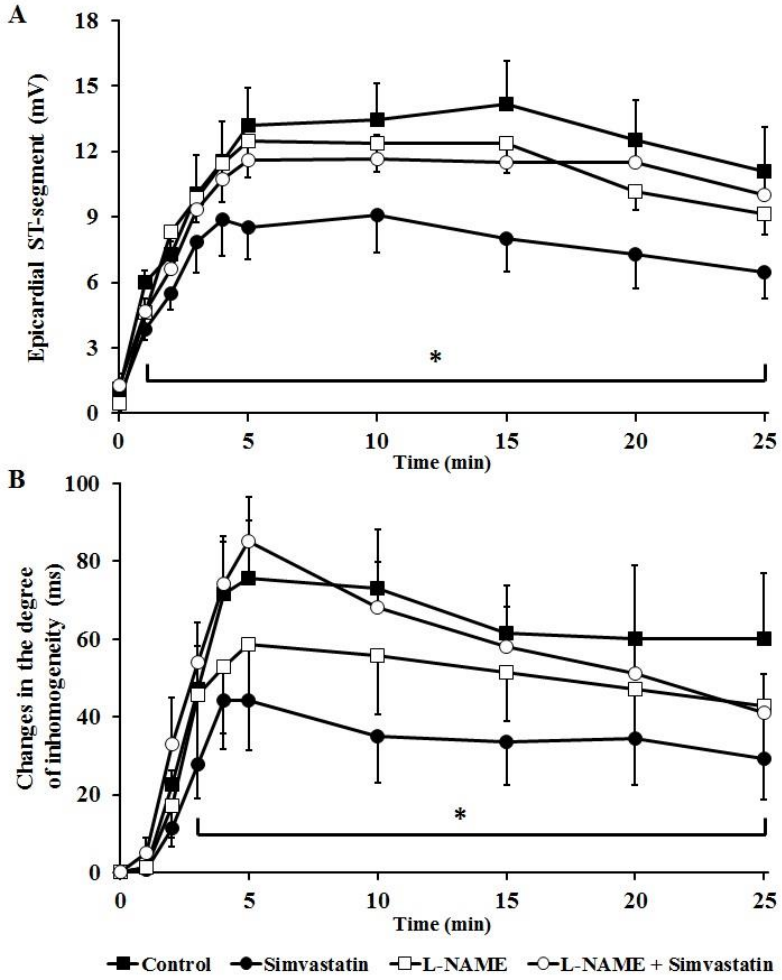

Figure 5. *Changes in the epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 25 min occlusion of the LAD. Values are means ± S.E.M.* $^{*}P < 0.05$ *compared to the controls.*

4.1.4. Changes in NOx levels during coronary artery occlusion and reperfusion

These are shown in Figure 6. A single bolus injection of simvastatin alone or together with L-NAME, as well as the solvent of simvastatin, did not affect the plasma NOx levels prior to the occlusion. In contrast, L-NAME itself slightly decreased NOx, which was further declined during the occlusion. When the LAD was occluded in control animals, a transient elevation occurred in the plasma NOx levels (around 7 min of the ischaemia), after which NOx started to decrease and became significantly lower than the initial baseline values. In contrast, the administration of simvastatin elevated NOx levels almost over the entire occlusion period. This effect was completely abolished by the prior administration of L-NAME. Reperfusion of the ischaemic myocardium evoked similar increases in NOx levels in all groups.

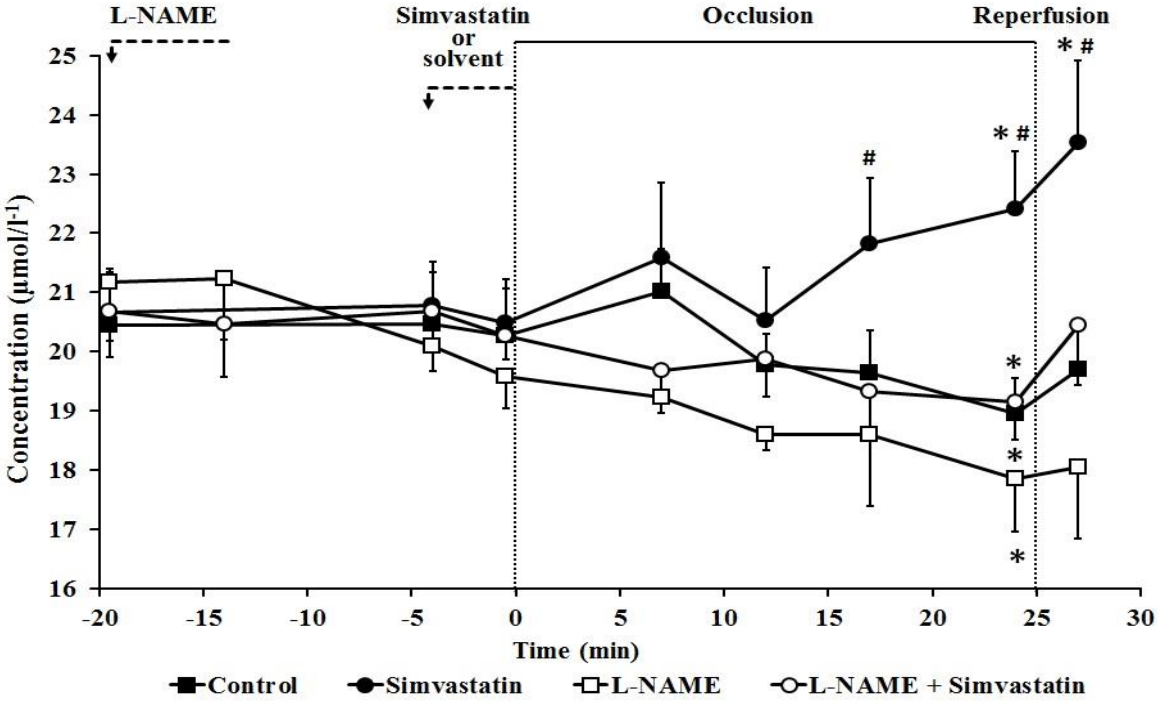

Figure 6. *Changes in plasma nitrite/nitrate (NOx) levels in the blood of the coronary sinus. Values are means ± S.E.M. *P < 0.05 compared to the initial baseline values, and $^{\#}P < 0.05$ compared to the control group.*

4.1.5. Determination of eNOS activity

The activity of eNOS was assessed by measuring eNOS phosphorylation with Western blot (Figure 7A), whereas the functional activity of the enzyme was determined using radio immunoassay (Figure 7B). Compared to the sham-operated controls (n = 5), occlusion of the LAD significantly reduced the activity of eNOS by the end of the occlusion period. In contrast, simvastatin preserved or even increased both the phosphorylation and the functional activity of the enzyme. Although L-NAME alone did not modify the ischaemia-induced reduction in eNOS activity, it abolished the simvastatin-induced activation, but not the phosphorylation (Figure 7B) of the enzyme. After reperfusion, the function of eNOS was rapidly regained in the control dogs, maintained in the simvastatin treated dogs, and remained inhibited in dogs given L-NAME.

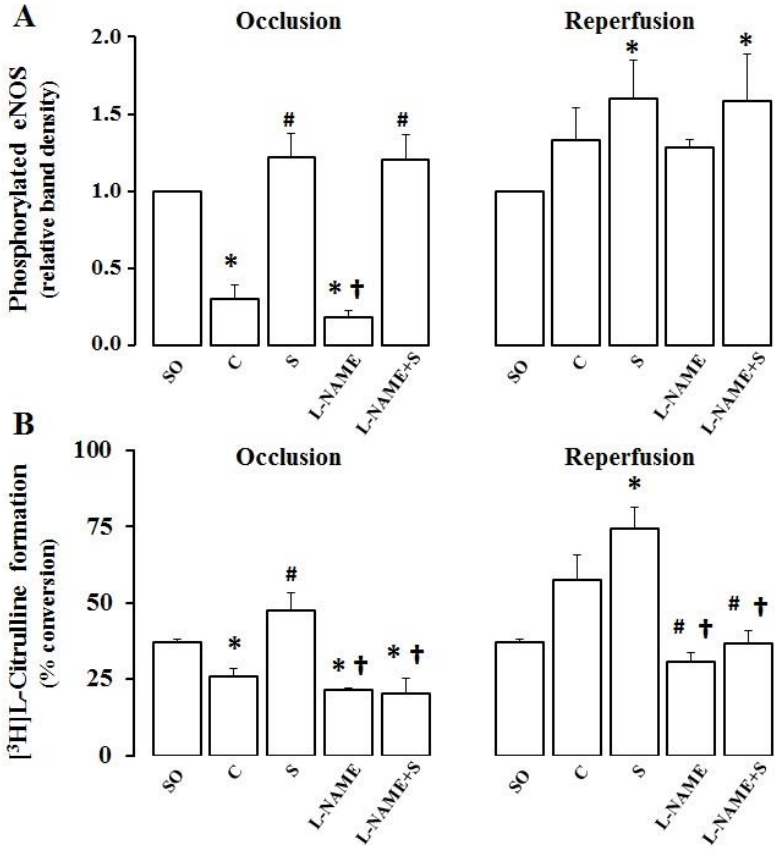

Figure 7. *Changes in NOS phosphorylation (A), and functional activity of eNOS (B) in control (C) and simvastatin (S) treated dogs, and in dogs given L-NAME alone or together with simvastatin (L-NAME+S). Values are means ± S.E.M. *P < 0.05 cp. SO group, #P < 0.05 cp..control group, and †P < 0.05 cp. simvastatin group.*

4.1.6. Changes in myocardial superoxide production following reperfusion

These are shown in Figure. 8. Compared with the sham controls, in dogs subjected to ischaemia and reperfusion a marked increase in superoxide production occurred soon after the reopening of the coronary artery. This ischaemia and reperfusion-induced generation of superoxide was significantly suppressed by the administration of simvastatin; an effect which was reversed by L-NAME.

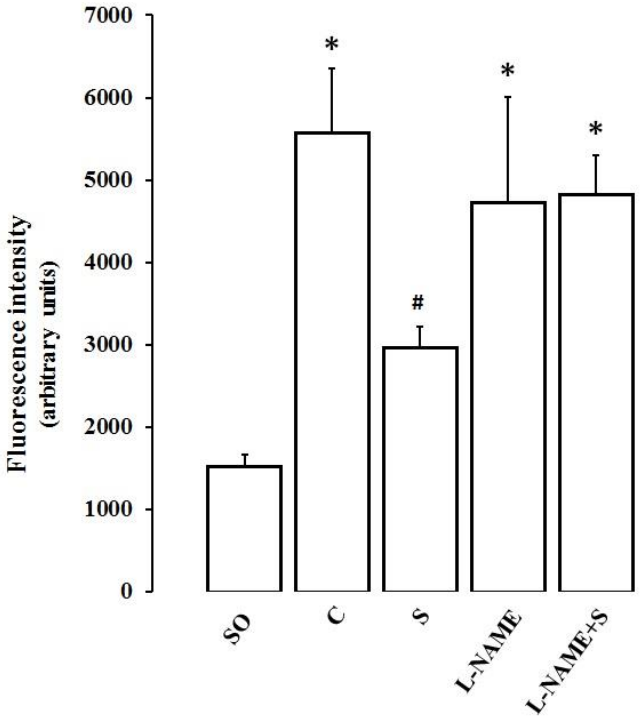

Figure 8. *Tissue superoxide production following a 25 min occlusion and reperfusion of the LAD in sham-operated (SO), in ischaemic control (C) and in simvastatin (S) treated dogs, as well as in dogs given L-NAME alone or together with simvastatin (L-NAME+S). Values are means ± S.E.M. *P < 0.05 compared to the SO group and $^{\#}P < 0.05$ compared to the control (C) group.*

4.1.7. Changes in QTc interval following coronary artery occlusion

This was measured in order to assess the potential direct electrophysiological effects of simvastatin. The results are illustrated in Figure 9. In control dogs a marked increase developed in QTc interval within 5 min of the occlusion and this was maintained during the whole ischaemic period. Such a prolongation of the QTc interval was abrogated with simvastatin no matter whether L-NAME was present or not.

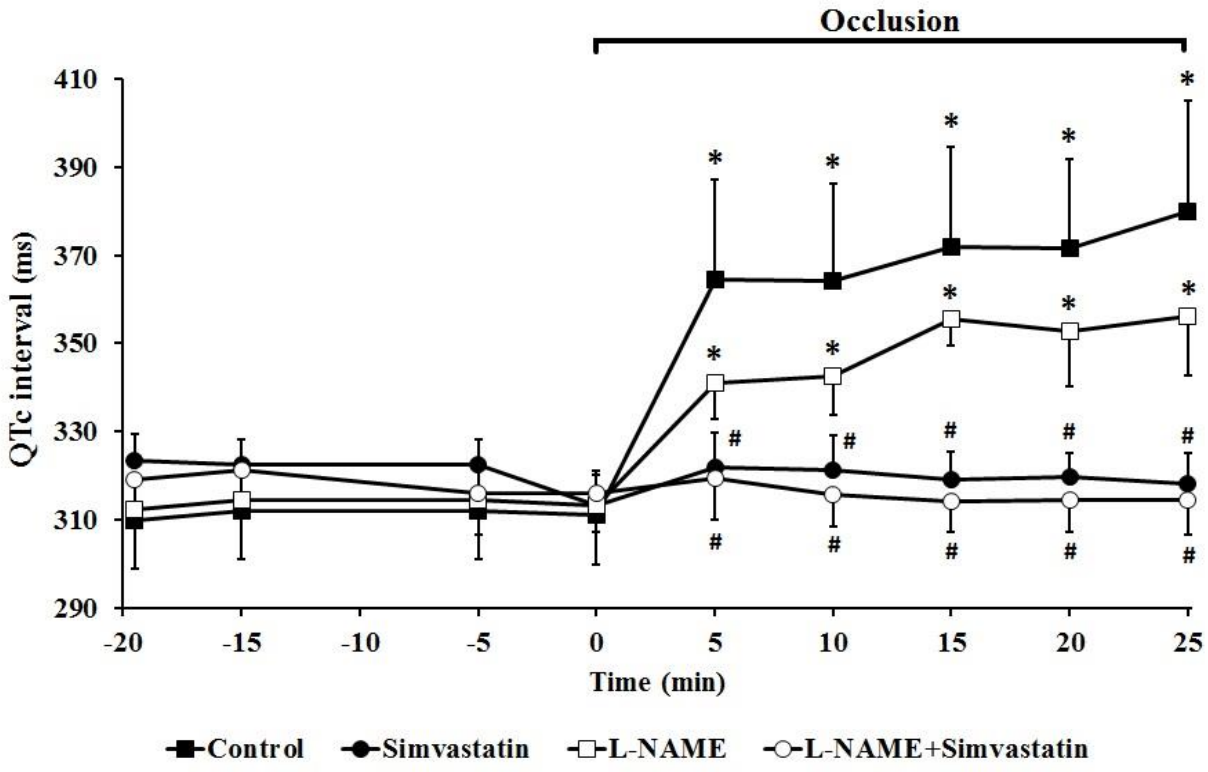

Figure 9. *Changes in the QTc interval determined prior to and during a 25 min occlusion of the LAD. Values are means ± S.E.M. *P < 0.05 compared to the initial baseline values, and #P < 0.05 compared to the control group.*

4.1.8. Area at risk

There were no significant differences in the area at risk among the groups. Thus the risk area was 38 ± 2% in the controls, 40 ± 3 % in the simvastatin, 37 ± 3 % in the L-NAME, and 39 ± 2% in the L-NAME+simvastatin groups.

4.2. Examination of the role of the IP3-kinase pathway in the simvastatin-induced rapid activation of eNOS

This was examined by the use of the specific IP3-kinase inhibitor wortmannin.

4.2.1. Haemodynamic changes following the administration of solvent, simvastatin, wortmannin and DMSO, as well as after coronary artery occlusion

These data are summarised in Tables 3 and 4. There were no considerable changes in the haemodynamic parameters following the local intracoronary injection of simvastatin and wortmannin, as well as of the vehicle of these drugs (Table 1). Although occlusion of the LAD resulted in significant reductions in arterial blood pressure, LVSP, positive and negative dP/dt_{max}, and an increase in LVEDP in all groups, these changes were not substantially different among the groups. The compensatory blood flow changes, occurring during the occlusion of the LAD on the LCX coronary artery, were not significantly modified by any drug treatment. Furthermore, in anaesthetised dogs HR was unchanged during coronary artery occlusion in all groups (Table 4).

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

This is illustrated in Figure 10. In control dogs occlusion of the LAD resulted in high number of VPBs (310 ± 45) and episodes of VT (7.1 ± 1.4) that occurred in 93%of the dogs. Furthermore, 50% of these control dogs exhibited VF during the occlusion and all the remaining dogs died on reperfusion, thus there were no survivors in this group from the combined ischaemia and reperfusion insult. In contrast, the single dose of simvastatin markedly reduced the number of ectopic beats (62 ± 14) and episodes of VT (0.3 ± 0.2), the incidences of VT (17%) and VF (0%) during occlusion, and 67% of the animals survived reperfusion. The administration of wortmannin in the simvastatin treated dogs significantly reduced this antiarrhythmic protection.

Table 3. *Haemodynamic changes following solvent, wortmannin, DMSO and simvastatin administration.*

	Solvent		Wortmannin		DMSO		Simvastatin	
	Baseline	**Change**	**Baseline**	**Change**	**Baseline**	**Change**	**Baseline**	**Change**
SABP (mmHg)	143 ± 7	3 ± 2	131 ± 4	0 ± 2	135 ± 6	2 ± 2	151 ± 7	-4 ± 2
DABP (mmHg)	105 ± 5	0 ± 4	96 ± 3	0 ± 2	93 ± 5	0 ± 1	103 ± 4	-2 ± 2
MABP (mmHg)	118 ± 5	1 ± 3	109 ± 3	0 ± 2	108 ± 5	0 ± 1	119 ± 5	-4 ± 1
LVSP (mmHg)	154 ± 7	2 ± 2	139 ± 4	1 ± 2	146 ± 5	0 ± 2	163 ± 7	-1 ± 2
LVEDP (mmHg)	4.3 ± 0.6	0.5 ± 0.3	4.0 ± 0.5	0.5 ± 0.5	3.3 ± 0.4	0.1 ± 0.2	5.3 ± 0.4	0 ± 0.2
+dP/dt (mmHg/s)	2351 ± 184	90 ± 50	2186 ± 160	-75 ± 43	2398 ± 116	61 ± 55	2787 ± 180	-79 ± 98
-dP/dt (mmHg/s)	2266 ± 213	40 ± 81	2443 ± 159	-5 ± 112	2676 ± 181	65 ± 71	2506 ± 216	-28 ± 50
HR (beats/min)	163 ± 7	4 ± 2	156 ± 5	-3 ± 1	156 ± 4	1 ± 1	169 ± 5	-3 ± 1
mean CBF_{LCX} (ml/min)	42 ± 5	-1 ± 2	25 ± 3	3 ± 2	28 ± 4	-1 ± 1	32 ± 8	1 ± 2

Data are means ± S.E.M. calculated from n = 11–14 experiments. Data, presented as changes, were determined 5 min after starting the infusion of the solvent, wortmannin, DMSO and simvastatin. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; CBF, coronary blood flow.

Table 4. *Haemodynamic changes during a 25 min occlusion of the LAD.*

	Control		Wortmannin		DMSO		Simvastatin		Wortmannin+Simvastatin	
	Baseline	**Max.change**	**Baseline**	**Max.change**	**Baseline**	**Max.change**	**Baseline**	**Max. change**	**Baseline**	**Max.change**
SABP (mmHg)	146 ± 7	-10 ± 2*	135 ± 5	-11 ± 2*	137 ± 7	-11 ± 2*	148 ± 5	-11 ± 2*	155 ± 8	-10 ± 2*
DABP (mmHg)	105 ± 5	-10 ± 3*	99 ± 4	-8 ± 1*	93 ± 6	-9 ± 2*	101 ± 3	-7 ± 2*	116 ± 7	-7 ± 2*
MABP (mmHg)	119 ± 5	-10 ± 3*	111 ± 4	-9 ± 2*	108 ± 6	-10 ± 2*	117 ± 4	-8 ± 2*	129 ± 7	-8 ± 2*
LVSP (mmHg)	146 ± 9	-10 ± 2*	148 ± 6	-10 ± 2*	150 ± 7	-15 ± 2*	152 ± 7	-11 ± 2*	164 ± 8	-11 ± 2*
LVEDP (mmHg)	4.5 ± 0.6	9.3 ± 1.3*	4.4 ± 0.6	9.6 ± 1.0*	3.4 ± 0.5	13.2 ± 1.0*	5.2 ± 0.3	9.0 ± 0.9*	4.5 ± 0.6	9.6 ± 1.1*
+dP/dt (mmHg/s)	2310 ± 157	-495 ± 81*	2143 ± 161	-346 ± 66*	2586 ± 155	-534 ± 109*	2673 ± 135	-495 ± 114*	2411 ± 181	-358 ± 93*
-dP/dt (mmHg/s)	2316 ± 200	-425 ± 139*	2446 ± 215	-540 ± 82*	2656 ± 210	-624 ± 111*	2563 ± 183	-504 ± 91*	2518 ± 159	-426 ± 113*
HR (beats/min)	166 ± 7	4 ± 4	154 ± 7	1 ± 2	162 ± 5	3 ± 2	167 ± 4	4 ± 2	162 ± 5	4 ± 2
mean CBF_{LCX} (ml/min)	41 ± 5	13 ± 2	26 ± 3	13 ± 4	30 ± 3	11 ± 2	32 ± 2	10 ± 1	39 ± 6	12 ± 2

Data are means ±S.E.M. calculated from n = 11–14 experiments. [a]P< 0.05 compared to baseline value. SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; MABP, mean arterial blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; HR, heart rate; CBF, coronary blood flow.

Thus in the presence of wortmannin the number of VPBs (30 ± 759), the incidence (73%) and number of episodes of VT (4.3 ± 1.3) were again increased, and as with the controls (C and DMSO groups), no dog in the W+S group survived reperfusion. However, wortmannin, like L-NAME before (see 4.1.2) did not modify the effect of simvastatin on the ischaemia-induced VF; i.e. as with simvastatin alone, no dog in the W+S group died in VF during the occlusion period. Compared to the controls (C), neither wortmannin, nor DMSO, the vehicle of wortmannin, affected the severity of arrhythmias resulted from a 25 min LAD occlusion and reperfusion. Thus in these groups the number of VPBs was 202 ± 64 and 246 ± 36, further 67% and 69% of these dogs exhibited 3.8 ± 1.7 and 3.8 ± 1.6 episodes of VT, and 25% and 23% of the animals showed VF during the occlusion. In these groups none of the dogs survived reperfusion.

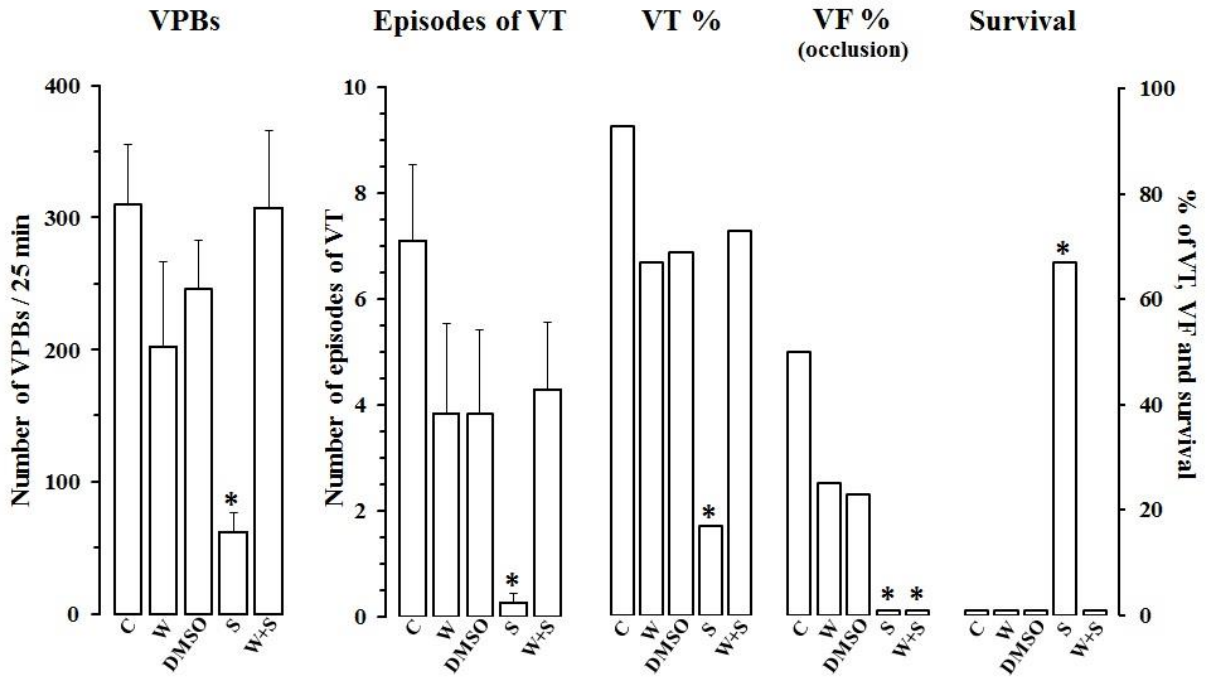

Figure 10. *The severity of ventricular arrhythmias during a 25 min occlusion and reperfusion of the LAD in control (C) and in simvastatin (S) treated dogs, as well as in dogs in which DMSO and wortmannin (W) was given alone or together with simvastatin (W+S). Values are means ± S.E.M.* $^{*}P < 0.05$ *compared to the controls.*

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

This was assessed by changes in the epicardial ST-segment (Figure 11A) and in the degree of inhomogeneity of electrical activation (Figure 11B). In the control groups (C and DMSO) there were marked increases in both parameters over the entire occlusion period. Simvastatin markedly suppressed these indices of ischaemia severity, which effect was completely abolished in the presence of wortmannin. Inhibition of the PI 3-kinase by wortmannin had no significant effect on these ischaemia-induced inhomogeneity and ST-segment changes.

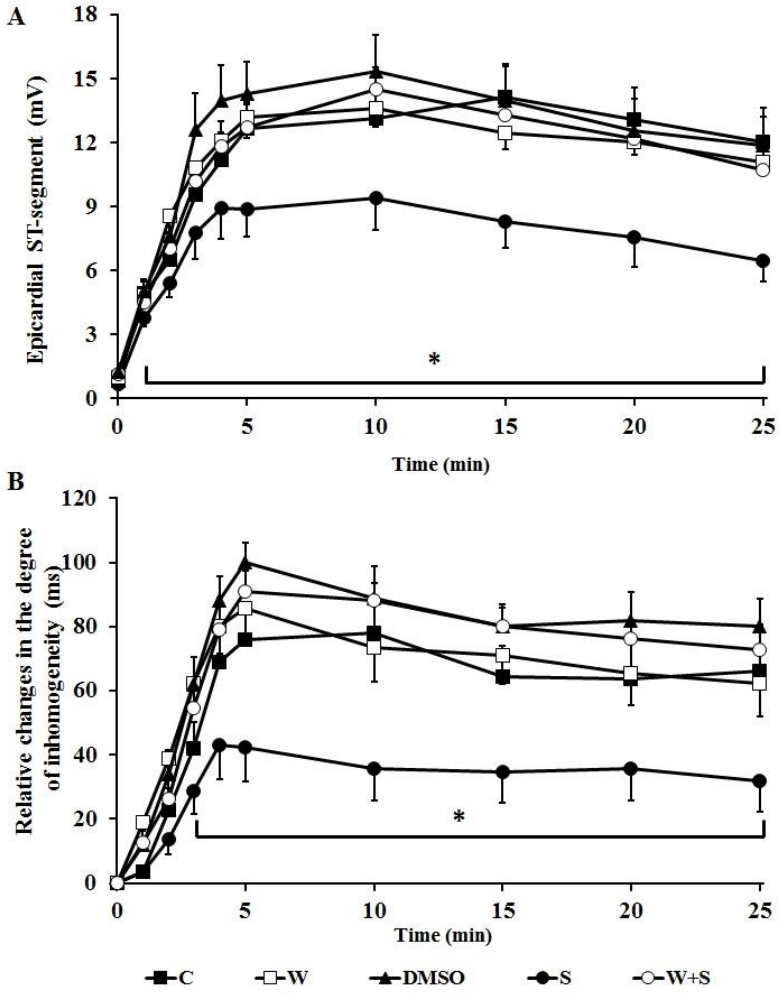

Figure 11. *Changes in epicardial ST-segment (A) and in the degree of inhomogeneity of electrical activation (B) during a 25 min occlusion of the LAD. Values are means ± S.E.M.* $^{*}P < 0.05$ *compared to the controls.*

4.2.4. Determination of eNOS activity

Compared to the sham controls (instrumented but not subjected to ischaemia; SC), in dogs, infused either with the solvent of simvastatin (C) or DMSO and subjected to occlusion, there was a significant decrease in eNOS phosphorylation (Figure 12A). The administration of simvastatin preserved, or even slightly increased the activity of eNOS during the occlusion. This effect was abolished in the presence of wortmannin (W+S group), although wortmannin (W group) alone did not affect the ischaemia-induced changes in NOS phosphorylation. When the coronary artery was re-opened, it can be seen that NOS regained very rapidly (these data were collected at different times of the reperfusion according to the occurrence of VF within the 5 min observation period; Figure 12B). Thus following reperfusion the phosphorylation of eNOS was increased in almost all groups, except those that had been received wortmannin (W and W+S groups) prior to the 25 min occlusion and reperfusion of the LAD.

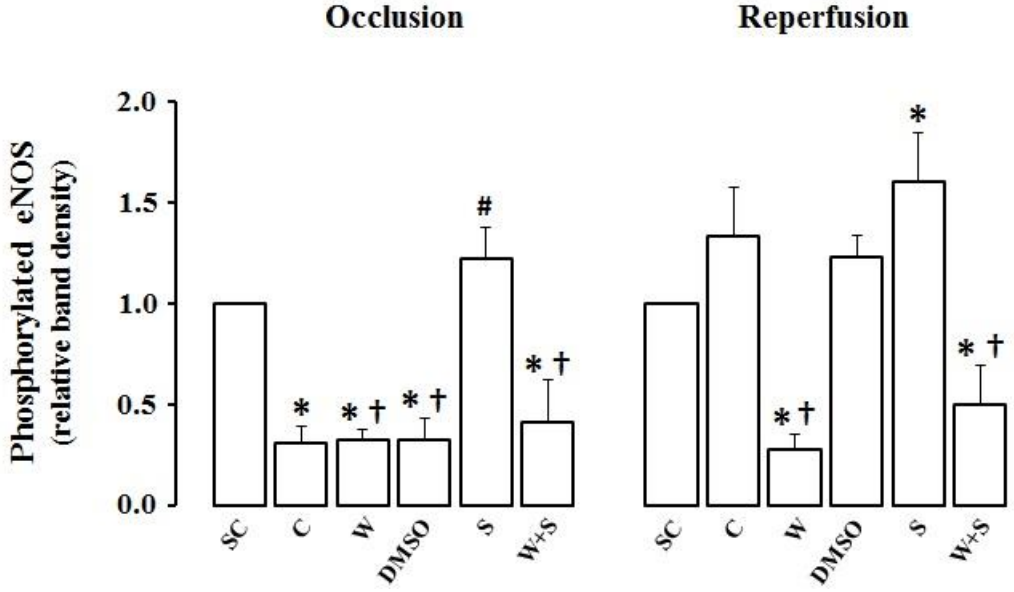

Figure 12. *Changes in the phosphorylated eNOS content, determined by Western blot at the end of the coronary artery occlusion, and 5 min after reperfusion. Values are means ± S.E.M. *P < 0.05 compared to the SC group, #P < 0.05 compared to the ischaemic control (C) group, and †P < 0.05 compared to the simvastatin group.*

4.2.5. Changes in NOx levels during coronary artery occlusion and reperfusion

These are illustrated in Figure 13. In control dogs occlusion of the LAD resulted in a transient, but significant increase in NOx (peaked around 7 min of the occlusion), followed by a continuous decrease up to the end of the 25 min occlusion period. This ischaemia-induced reduction in NOx was prevented by the application of a single dose of simvastatin. The administration of wortmannin in control dogs abolished the early increase of NO metabolites, and it completely abrogated the NO preserving effect of simvastatin over the entire occlusion period. DMSO had no effect on NO bioavailability during occlusion and reperfusion.

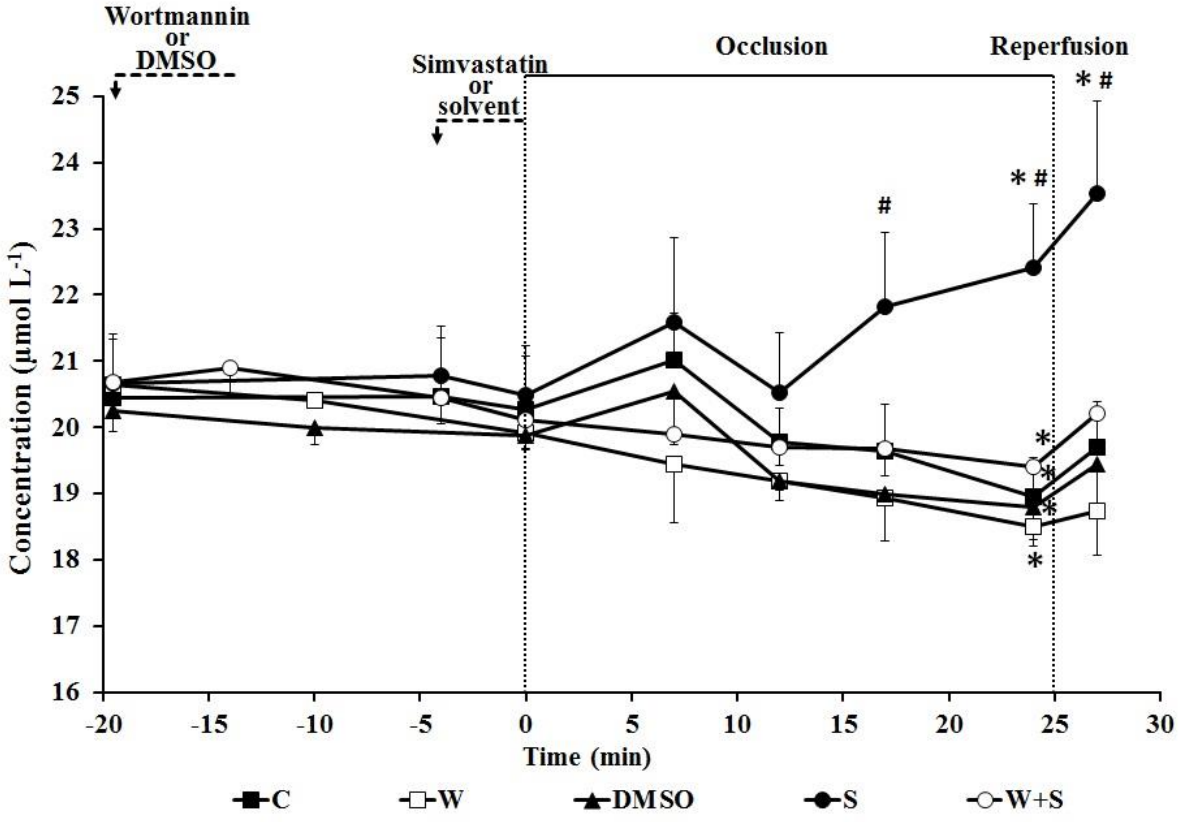

Figure 13. *Changes in plasma nitrite/nitrate (NOx) levels in the blood of the coronary sinus. Values are means ± S.E.M. *P < 0.05 compared to the initial baseline values, and $^{\#}P < 0.05$ compared to the control group.*

4.2.6. Changes in myocardial superoxide production following reperfusion

In dogs that had been subjected to reperfusion following a 25 min period of ischaemia, myocardial superoxide production was determined within 5 min of the reperfusion, irrespective whether these dogs died or survived. The results are illustrated in Figure 14. Compared to the sham controls, in dogs subjected to occlusion and reperfusion, there was a marked increase in superoxide production, which was significantly reduced by simvastatin. The administration of DMSO, as well as of wortmannin itself, did not affect the ischaemia/reperfusion-induced generation of superoxide, but wortmannin reversed the effect of simvastatin on superoxide production.

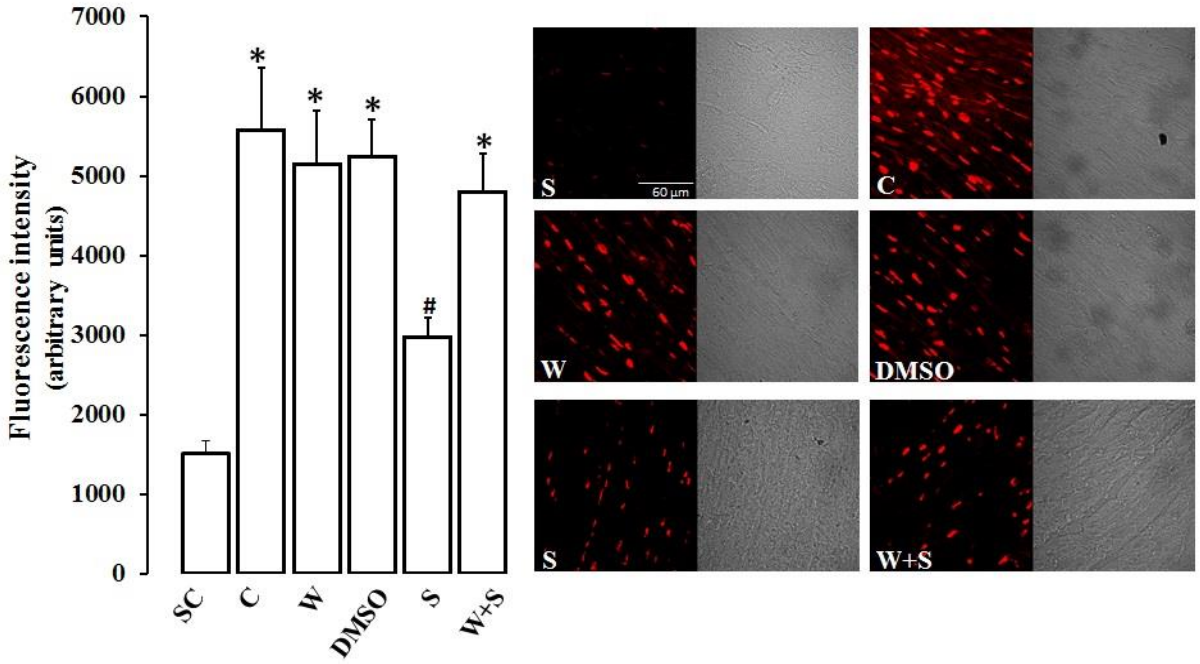

Figure 14. *Tissue superoxide production following a 25 min occlusion and reperfusion of the LAD. Values are means ± S.E.M. *P < 0.05 compared to the SC group and $^{\#}P < 0.05$ compared to the control (C) group.*

4.2.7. Changes in QTc interval following coronary artery occlusion

As we have previously described, simvastatin prevents the ischaemia-induced prolongation of the QTc interval, indicating a possible direct electrophysiological effect of the drug (see 4.1.7.). We have now shown that similar to L-NAME, in the presence of wortmannin the effect of simvastatin on the QTc interval was still maintained (Figure 15).

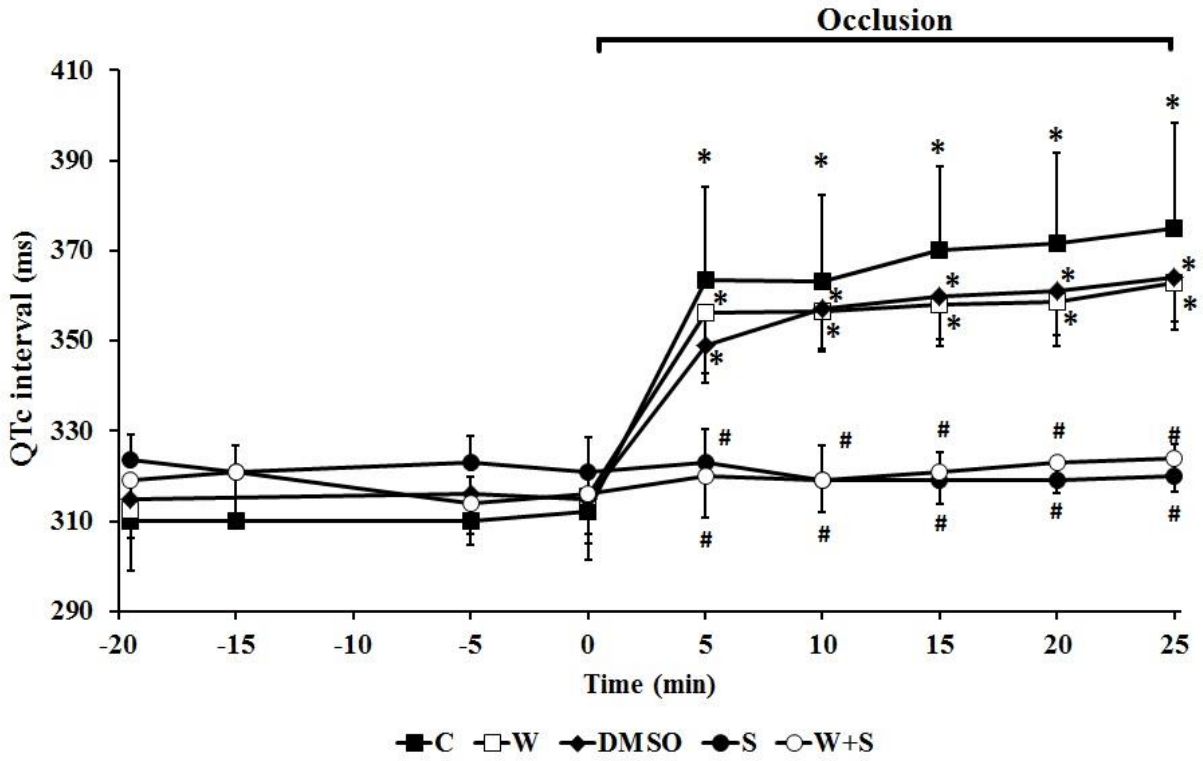

Figure 15. *Changes in the QTc interval determined prior to and during a 25 min occlusion of the LAD. Values are means ± S.E.M. *P < 0.05 compared to the initial baseline values, and $^{\#}P$ < 0.05 compared to the control group.*

4.2.8. Area at risk

There were no significant differences in the area at risk among the groups. Thus the risk area was 37 ± 3% in the controls, 38 ± 2% in the simvastatin, 39 ± 2% in the wortmannin, 37 ± 2% in the wortmannin+simvastatin and 39 ± 3% in the DMSO groups.

5. DISCUSSION

5.1. New findings

1. We have provided evidence that a single dose of simvastatin, applying just prior to a 25 min period of coronary artery occlusion in anaesthetized dogs, results in profound protection against those severe ventricular arrhythmias that are, in most instances, responsible for sudden cardiac death. We have also pointed out that this antiarrhythmic effect of simvastatin, at least in part, is due to an increased nitric oxide production, resulting from the rapid activation of the endothelial nitric oxide synthase (eNOS) by simvastatin, since the protective effect of simvastatin was almost completely abolished in the presence of the NOS enzyme inhibitor L-NAME.

2. We have also shown that the simvastatin-induced rapid eNOS activation results from the stimulation of the IP3/Akt pathway, since the inhibition of the IP3-kinase with wortmannin, attenuates eNOS activation and, consequently, abolishes the antiarrhythmic protection.

5.2. The effect of acute simvastatin administration on ischaemia and reperfusion ventricular arrhythmias. The role of nitric oxide.

The evidence that statins may reduce the incidence of life-threatening ventricular arrhythmias and prevent sudden cardiac death, comes mainly from large clinical studies with chronic statin treatment [51, 52 66, 101]. There has been much less information, apart from a few clinical investigations [102, 103], whether these drugs, following acute administration, would influence the consequences of ischaemia and reperfusion, including the life threatening ventricular arrhythmias [77, 96]. Furthermore, since many of the beneficial effects of statins have been shown to involve increased synthesis of nitric oxide [81, 93], and since we have a number of previous evidence that NO plays an essential role in the protection against the ischaemia-induced early arrhythmias [29, 30], we have now examined whether the effect of simvastatin on arrhythmias is also due to the activation of eNOS and the subsequent increase in NO formation.

The results show that in anaesthetized dogs the administration of a single dose of simvastatin markedly reduced the number of VPBs and episodes of VT, the incidence of VT and VF that resulted from a 25 min occlusion of the LAD and increased survival from the

combined ischaemia and reperfusion insult, compared with the untreated controls. This protection against arrhythmias was similar to that we have obtained previously, in the same model, with ischaemic preconditioning [7], and with the administration of NO donors [48, 100, 104]. Furthermore, simvastatin significantly reduced the severity of ischaemic changes during coronary artery occlusion. These salutary effects of simvastatin administration were associated with increased eNOS activity since the phosphorylation of eNOS (determined by Western blot) and the functional activity of this enzyme (determined by RIA) were significantly higher during ischaemia and reperfusion in the simvastatin treated dogs than in the solvent treated controls (Figure 7). This activation of eNOS by simvastatin resulted in an increased generation of NO during occlusion (Figure 6) and also a decrease in superoxide production following reperfusion (Figure 8). As we have shown previously, the preservation of NO bioavailability during coronary artery occlusion by preconditioning or by donating NO, attenuates the reperfusion-induced marked increases of superoxide production [100], since NO may regulate the generation of superoxide during reperfusion [43]. These effects are certainly can be associated with the antiarrhythmic effect of simvastatin. Furthermore, the fact that L-NAME prevented the simvastatin activated enzyme to form NO (inhibits the L-arginine-NO pathway without affecting enzyme phosphorylation) and attenuated or even abolished most of the salutary effects of simvastatin, supports the hypothesis that the eNOS activated NO formation plays a crucial role in the protective effect of simvastatin against arrhythmias.

We have raised the question, how the activation of eNOS takes place following a single dose of simvastatin. There are a few studies in the literature, which show that statins can rapidly activate eNOS by stimulating the phoshatidylinositide 3-kinase/Akt (PI 3-kinase/Akt) pathway [93, 95, 119]. For example, in the study of Wolfrum and colleagues [95], simvastatin given in anaesthetised rats 3 min prior to reperfusion markedly reduced infarct size that resulted from a 30 min coronary artery occlusion and a subsequent 180 min period of reperfusion. They also showed that simvastatin increased myocardial PI 3-kinase activity, and enhanced Akt_{Ser473} and $eNOS_{Ser1177}$ phosphorylation. Since the infarct size reducing effect of simvastatin was abolished by the administration of the PI 3-kinase inhibitor wortmannin and of the NOS inhibitor L-NAME, they concluded that the activation of PI 3-kinase/Akt/eNOS cascade plays an important role in the acute cardioprotective effects of simvastatin [95].

On the basis of the abovementioned study, in the second part of the experiments we examined, whether the antiarrhythmic effect of acute simvastatin administration involves the rapid activation of the PI 3-kinase/Akt/eNOS signalling pathway. For this purpose we have also used wortmannin, a potent and specific inhibitor of PI 3-kinase, and administered in dogs prior to the bolus injection of simvastatin. We have found that in the presence of wortmannin most of the salutary effects of simvastatin were attenuated, or even abolished. Thus the number of VPBs, the number of episodes of VT and the incidence of VT during occlusion were again increased, and compared with the simvastatin treated group, in which 40 % of the dogs survived reperfusion, in the wortmannin + simvastatin group (W + S) no animal survived the combined ischaemia and reperfusion insult. The pre-treatment with wortmannin significantly attenuated the simvastatin induced activation of eNOS and the subsequent NO production, as well as the decreases in superoxide generation. The marked anti-ischaemic effect of simvastatin was also abolished by the administration of wortmannin.

There is still ongoing debate as to whether the reduction in tachyarrhythmias (VT and VF) that are responsible in most instances for sudden cardiac death, is due to the anti-ischaemic effect of statins, or there might be a more direct electrophysiological action of these drugs, which would explain their marked antiarrhythmic effect. A recent review from Beri et al. [105] has addressed this question by collecting and systematically evaluating data published over a 13-year period on the reduction of VT and VF events, as well as sudden cardiac death in patients suffering from various cardiovascular diseases and treated with statins. They concluded that the anti-arrhythmic/anti-fibrillatory effects of statins most probably result from an anti-ischaemic rather than a direct anti-arrhythmic effect, since a definitive reduction in sudden cardiac death occurred only in patients with ischaemic-type cardiovascular diseases, such as coronary artery disease or ischaemic cardiomyopathy [105]. These anti-ischaemic effects of statins are supposed to mediate through nitric oxide and can indirectly influence the generation of arrhythmias [105].

Although we have found that simvastatin results in pronounced anti-ischaemic effect, since the increases in the epicardial ST-segment and in the degree of inhomogeneity during occlusion were significantly less marked in the simvastatin treated dogs than in the controls, we suppose that simvastatin might possesses a direct anti-arrhythmic effect as well. The evidence for this assumption may come from two observations; i.e. (*i*) despite the abolition of

the anti-ischaemic effect of simvastatin by L-NAME (Figure 5) and wortmannin (Figure 11), the protective effect of simvastatin against the ischaemia-induced ventricular fibrillation was still present, and (*ii*) neither L-NAME nor wortmannin did not influence the reduction in QTc interval, resulted from simvastatin administration (Figure 15). However, interestingly, both inhibitors abolished the protective effect of simvastatin against the reperfusion-induced VF. The explanations for this dichotomy could be many and varied, including differences in the underlying mechanisms of the various arrhythmia types induced by the acute ischaemia [106, 107], as well as differences in the local and systemic regulatory influences of NO on arrhythmia mechanisms. These latter may involve, for example, the modulation of the effect of autonomic tone on the myocardium [41] and of gap junction function [48], as well as the regulation of free radical formation by NO [43, 100].

Nevertheless, the fact that L-NAME and wortmannin did not influence the protective effect of simvastatin against the occlusion-induced ventricular fibrillation suggests that in the anti-fibrillatory effect of simvastatin, there might be an NO-independent and, perhaps, a more direct electrophysiological mechanism. This assumption is supported by the results of the QTc interval measurements. These show that simvastatin almost completely inhibited the ischaemia-induced prolongation of the QTc interval, and this effect was not modified by the administration of L-NAME or wortmannin. Although we cannot ascertain the precise mechanism of this phenomenon only from the measurement of QTc intervals, recent electrophysiological studies suggest that statins influence impulse conduction, improve cardiac repolarization [108, 109] and suppress cardiac excitability [110] perhaps by directly and selectively affecting ion channels in cardiomyocytes (e.g. Kv4.3; [111]). Certainly, long-term statin treatment by modulating the lipid portions of the sarcolemma, which contain the ion channel regulatory proteins and signalling molecules (lipid rafts), influence the ion channel conduction and ion transport [112, 113], but it is not known whether such a mechanism would also account for the acute administration of statins. Nevertheless, considering that polyunsaturated fatty acids, which alter the structure of the sarcolemmal phospholipids, were able to evoke immediate antiarrhythmic effect [114-116], we may speculate that statins perhaps also possess such acute modulator properties on the lipid portions of the membrane. This, by causing favourable changes in ion transport or by stabilizing the membrane, would lead to arrhythmia suppression during ischaemia.

In contrast, the fact that L-NAME and wortmannin abrogated the protective effect of simvastatin against the reperfusion-induced VF suggests that this action depends more on NO bioavailability than the ischaemia-induced VF [48]. Considering the electrophysiological differences between the ischaemia-induced and the reperfusion-induced arrhythmias [106], and that, in this latter, the products of the oxidative stress and the calcium overload [117] play a mandatory role, it seems more than likely that simvastatin through an NO-dependent way [118] reduces superoxide production and hence the occurrence of VF during reperfusion. Consequently, this protection disappears by inhibiting the formation of NO.

Summarizing our results, we have shown that a single bolus injection of simvastatin markedly reduces the ischaemic changes and the incidence and severity of ventricular arrhythmias that results from a 25 min occlusion and reperfusion in anaesthetized dogs. Furthermore, we have demonstrated that the simvastatin-induced marked antiarrhythmic effect largely depends on the generation of NO that results from the rapid activation of eNOS via the stimulation of the PI 3-kinase/Akt pathway. The evidence for this is that most of the salutary effects of simvastatin (except the protection against the occlusion-induced VF) are attenuated or even abolished, if PI 3-kinase, one of the upstream components of NOS activation is inhibited with wortmannin. Thus we think that the activation of PI 3-kinase/Akt/eNOS cascade and the subsequent increased formation of NO play a crucial role in the antiarrhythmic effect of acute simvastatin administration, but a direct and presumably NO-independent mechanism cannot be ruled out.

Considering these findings our hypothesis is that simvastatin, like a short (5 min) period of ischaemia rapidly activates NOS and results in enhanced NO formation. However, the ischaemia-induced elevation in NO formation is transient, if the ischaemia is not reperfused, but it is maintained over a longer period (e.g. 25 min), then the activity of NOS, and consequently, the NO production, are markedly reduced [120]. Furthermore, we suppose that both simvastatin and a short period of ischaemia/reperfusion insult (i.e. preconditioning, [120]), is able to rapidly activate NOS, and in this case the enzyme remains to be activated and provides increased NO bioavailability over the entire prolonged period of the ischaemia [120]. The fact that in both cases the NOS activation can be blocked by L-NAME [29, 95], and also by wortmannin [95, 121], suggests that there might be a common pathway in NOS activation both by simvastatin and preconditioning. Indeed, there is evidence coming mainly from *in*

vitro experiments that phosphorylation of NOS rapidly modifies NOS activity [122]. For example, it has been recently reported that in the rat isolated hearts, preconditioning, by stimulating the Akt/protein kinase A (PKA) pathway, activates eNOS via serine 1176 phosphorylation [123]. There is also evidence that the PI 3-kinase inhibitor wortmannin abolished the preconditioning-induced phosphorylation of protein kinase B (Akt) and the increase in NO production in Langendorff-perfused rat hearts [121].

We are convinced that NO bioavailability during occlusion plays an essential role in arrhythmia generation both during occlusion and reperfusion [29, 48, 100, 120]. Thus any manoeuvre, which increases or, at least, maintains NO formation during occlusion may protect the myocardium against arrhythmias, most probably through the regulation of those local and systemic mechanisms by NO that are implicated in arrhythmia generation, such as the autonomic tone [41], gap junctional function [48, 124], or the formation of free radicals [43, 100], etc. The latter is particularly important in the generation of the reperfusion-induced severe ventricular arrhythmias, and NO by reducing free radical formation (most likely superoxide) may suppress the occurrence of arrhythmias during reperfusion [100]. Since both L-NAME and wortmannin abolished the suppressing effect of simvastatin on the reperfusion-induced superoxide production and subsequently increased the incidence of VF during reperfusion, it seems more than likely that this effect of simvastatin is NO-dependent and it attains via the activation of the PI 3-kinase/Akt/eNOS pathway. On the other hand, those of our findings which show that the inhibition of NO formation does not influence the protective effect of simvastatin against the occlusion-induced VF indicates an NO-independent, direct electrophysiological mechanism in the anti-fibrillatory effect of simvastatin.

6. REFERENCES

[1] Harris, A. S. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. *Circulation* 1, 1318–1328 (1950).

[2] Gülker, H., Krämer, B., Stephan, K. & Meesmann, W. Changes in ventricular fibrillation threshold during repeated short-term coronary occlusion and release. *Basic Res. Cardiol.* 72, 547–562 (1977).

[3] Barber, M. J. Effect of time interval between repeated brief coronary artery occlusions on arrhythmia, electrical activity and myocardial blood flow. *J. Am. Coll. Cardiol.* 2, 699–705 (1983).

[4] Murry, C. E., Jennings, R. B. & Reimer, K. A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74, 1124–1136 (1986).

[5] Shiki, K. & Hearse, D. J. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am. J. Physiol.* 253, H1470–1476 (1987).

[6] Komori, S., Végh, Á., Szekeres, L. & Parratt, J. R. Preconditioning reduces the severity of ischaemia and reperfusion-induced arrhythmias in both anaesthetised rats and dogs. *J. Physiol.* 423, 16 (1990).

[7] Végh, Á., Komori, S., Szekeres, L. & Parratt, J. R. Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. *Cardiovasc. Res.* 26, 487–495 (1992).

[8] Kuzuya, T., Hoshida, S., Yamashita, N., Fuji, H., Oe, H., Hori, M., Kamada, T., Tada, M. Delayed effects of sub-lethal ischemia on the acquisition of tolerance to ischemia. *Circ. Res.* 72, 1293–1299 (1993).

[9] Marber, M. S., Latchman, D. S., Walker, J. M. & Yellon, D. M. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88, 1264–1272 (1993).

[10] Végh, Á., Papp, J. G. & Parratt, J. R. Prevention by dexamethasone of the marked antiarrhythmic effects of preconditioning induced 20 h after rapid cardiac pacing. *Br. J. Pharmacol.* 113, 1081–1082 (1994).

[11] Végh, Á., Szekeres, L. & Parratt, J. R. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc. Res.* 25, 1051–1053 (1991).

[12] Babai, L., Szigeti, Z., Parratt, J. R. & Végh, Á. Delayed cardioprotective effects of exercise in dogs are aminoguanidine sensitive: possible involvement of nitric oxide. *Clin. Sci.* 102, 435–445 (2002).

[13] Végh, Á. Endogén protektív mechanizmusok a prekondícionálás antiarrhythmiás hatásában. *Cardiol. Hung. 26 (Suppl. 6),* 33–46 (1997).

[14] Végh, Á., Szekeres, L. & Parratt, J. R. Protective effects of preconditioning of the ischaemic myocardium involve cyclooxygenase products. *Cardiovasc. Res.* 24, 1020–1023 (1990).

[15] 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* 84, 350–356 (1991).

[16] Downey, J. M., Cohen, M. V., Ytrehus, K. & Liu, Y. Cellular mechanisms in ischemic preconditioning: the role of adenosine and protein kinase C. *Ann. N. Y. Acad. Sci.* 723, 82–98 (1994).

[17] Auchampach, J. A. & Gross, G. J. Adenosine A1 receptors, KATP channels, and ischemic preconditioning in dogs. *Am. J. Physiol.* 264, H1327–1336 (1993).

[18] Tritto, I., D'Andrea, D., Eramo, N., Scognamiglio, A., De Simone, C., Violante, A., Esposito, A., Chiariello, M. & Ambrosio, G. Oxygen radicals can induce preconditioning in rabbit hearts. *Circ. Res.* 80, 743–748 (1997).

[19] Baines, C. P., Goto, M. & Downey, J. M. Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J. Mol. Cell. Cardiol.* 29, 207–216 (1997).

[20] Pain, T., Yang, X. M., Critz, S. D., Yue, Y., Nakano, A., Liu, G. S., Heusch, G., Cohen, M. V. & Downey, J. M. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ. Res* .87, 460–466 (2000).

[21] Végh, Á., Szekeres, L. & Parratt, J. R. Local intracoronary infusions of bradykinin profoundly reduce the severity of ischaemia-induced arrhythmias in anaesthetized dogs. *Br. J. Pharmacol.* 104, 294–295 (1991).

[22] Végh, Á., Papp, J. G. & Parratt, J. Attenuation of the antiarrhythmic effects of ischaemic preconditioning by blockade of bradykinin B2 receptors. *Br. J. Pharmacol.* 113, 1167–1172 (1994).

[23] Parratt, J.R., Vegh, A., Zeitlin, I. J., Ahmad, M., Oldroyd, K., Kaszala, K. & Papp, J. G. Bradykinin and endothelial-cardiac myocyte interactions in ischemic preconditioning. *Am. J. Cardiol* .80, 124A–131A (1997).

[24] Végh, Á., Papp, J. G., Szekeres, L. & Parratt, J. R. Prevention by an inhibitor of the L-arginine-nitric oxide pathway of the antiarrhythmic effects of bradykinin in anaesthetized dogs. *Br. J. Pharmacol.* 110, 18–19 (1993).

[25] Végh Á., & Parratt J.R. Ischaemic preconditioning markedly reduces the severity of ischaemia and reperfusion-induced arrhythmias; role of endogenous myocardial protective substances. In : Myocardial Preconditioning. Eds: Wainwright, C.L. & Parratt, J.R. Chapter 3, pp 35-60, Springer, Berlin, 1996.

[26] Goto, M., Liu, Y., Yang, X. M., Ardell, J. L., Cohen, M. V. & Downey, J. M. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ. Res.* 77, 611–621 (1995).

[27] Chahine, R., Adam, A., Yamaguchi, N., Gaspo, R., Regoli, D. & Nadeau, R. Protective effects of bradykinin on the ischaemic heart: implication of the B1 receptor. *Br. J. Pharmacol.* 108, 318–322 (1993).

[28] Végh, Á., Papp, J. G., Szekeres, L. & Parratt, J. The local intracoronary administration of methylene blue prevents the pronounced antiarrhythmic effect of ischaemic preconditioning. *Br. J. Pharmacol.* 107, 910–911 (1992).

[29] Végh, Á., Szekeres, L. & Parratt, J. Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. *Br. J. Pharmacol.* 107, 648–652 (1992).

[30] Kis, A., Végh, Á., Papp, J. G. & 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.* 31, 1229–1241 (1999).

[31] Kis, A., Végh, Á., Papp, J. & Parratt, J. Pacing-induced delayed protection against arrhythmias is attenuated by aminoguanidine, an inhibitor of nitric oxide synthase. *Br. J. Pharmacol.* 127, 1545–1550 (1999).

[32] Palmer, R. M., Ashton, D. S. & Moncada, S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333, 664–666 (1988).

[33] Radomski, M. W., Palmer, R. M. & Moncada, S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet l.*2, 1057–1058 (1987).

[34] Andrew, P. J. & Mayer, B. Enzymatic function of nitric oxide synthases. *Cardiovasc. Res.* 43, 521–531 (1999).

[35] Parratt, J. R. Nitric Oxide and Cardiovascular Dysfunction in Sepsis and Endotoxaemia: An Introduction and an Overview. In: Shock, Sepsis, and Organ Failure — Nitric Oxide. Eds: Schlag, G. & Redl, H. 1–29, Springer, Berlin, Heidelberg, 1995.

[36] Bolli, R., Manchikalapudi, S., Tang, X. L., Takano, H., Qiu, Y., Guo, Y., Zhang, Q. & Jadoon, A. K. The Protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase : evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic reconditioning. *Circ. Res* .81, 1094–1107 (1997).

[37] Végh, Á., György, K., Rastegar, M. A., Papp, J. G. & Parratt, J. R. Delayed protection against ventricular arrhythmias by monophosphoryl lipid-A in a canine model of ischaemia and reperfusion. *Eur. J. Pharmacol.*382, 81–90 (1999).

[38] Denninger, J.W. & Marletta M.A. Guanylate cyclase and the NO/cGMP signalling pathway. *Biochim. Biophys. Acta,* 1411, 334-350 (1999).

[39] Cohen, R. A., Weisbrod, R. M., Gericke, M., Yaghoubi, M., Bierl, C. & Bolotina, V. M. Mechanism of nitric oxide-induced vasodilatation: refilling of intracellular stores by sarcoplasmic reticulum Ca^{2+} ATP-ase and inhibition of store-operated Ca^{2+} influx. *Circ. Res.* 84, 210–219 (1999).

[40] Sun, J., Picht, E., Ginsburg, K. S., Bers, D. M., Steenbergen, C. & Murphy, E. Hyper-contractile female hearts exhibit increased S-nitrosylation of the L-type Ca^{2+} channel alpha1 subunit and reduced ischemia/reperfusion injury. *Circ. Res.* 98, 403–411 (2006).

[41] Schwarz, P., Diem, R., Dun, N. J. & Förstermann, U. Endogenous and exogenous nitric oxide inhibits norepinephrine release from rat heart sympathetic nerves. *Circ. Res.* 77, 841–848 (1995).

[42] Burwell, L. S. & Brookes, P. S. Mitochondria as a target for the cardioprotective effects of nitric oxide in ischemia-reperfusion injury. *Antioxid. Redox Signal.* 10, 579–599 (2008).

[43] Iwase, H., Robin, E., Guzy, R. D., Mungai, P. T., Vanden Hoek, T. L., Chandel, N. S., Levraut, J. & Schumacker, P. T. Nitric oxide during ischemia attenuates oxidant stress and cell death during ischemia and reperfusion in cardiomyocytes. *Free Radic. Biol. Med.* 43, 590–599 (2007).

[44] Sun, J., Aponte, A. M., Kohr, M. J., Tong, G., Steenbergen, C. & Murphy, E.Essential role of nitric oxide in acute ischemic preconditioning: S-nitros(yl)ation versus sGC/cGMP/PKG signaling? *Free Radic. Biol. Med.* 54, 105–112 (2013).

[45] Tsuchida, A., Yang, X. M., Burckhartt, B., Mullane, K. M., Cohen, M. V. & Downey, J. M. Acadesine extends the window of protection afforded by ischaemic preconditioning. *Cardiovasc. Res.* 28, 379–383 (1994).

[46] Végh, Á., Györgyi, K., Papp, J. G., Sakai, K. & Parratt, J. R. Nicorandil suppressed ventricular arrhythmias in a canine model of myocardial ischaemia. *Eur. J. Pharmacol.* 305, 163–168 (1996).

[47] Rastegar, M. A., Marchini, F., Morazzoni, G., Vegh, A., Papp, J. G. & 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.* 129, 671–680 (2000).

[48] Gönczi, M., Papp, R., Kovács, M., Seprényi, G. & Végh, A. Modulation of gap junctions by nitric oxide contributes to the anti-arrhythmic effect of sodium nitroprusside? *Br. J. Pharmacol.* 156, 786–793 (2009).

[49] Kovács, M., Kiss, A., Gönczi, M., Miskolczi, G., Seprényi, G., Kaszaki, J., Kohr, M. J., Murphy, E. & Végh, Á. Effect of sodium nitrite on ischaemia and reperfusion-induced arrhythmias in anaesthetized dogs: is protein S-nitrosylation involved? *PloS One* 10, e0122243 (2015).

[50] Pedersen, T. R., Kjekshus, J., Berg, K., Haghfelt, T., Faergeman, O., Faergeman, G., Pyörälä, K., Miettinen, T., Wilhelmsen, L., Olsson, A.G. & Wedel, H. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). 1994. *Atheroscler. Suppl.*5, 81–87 (2004).

[51] Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N. Engl. J. Med.*339, 1349–1357 (1998).

[52] Horwich, T. B., MacLellan, W. R. & Fonarow, G. C. Statin therapy is associated with improved survival in ischemic and non-ischemic heart failure. *J. Am. Coll. Cardiol.* 43, 642–648 (2004).

[53] Dawber, T. R., Moore, F. E. & Mann, G. V. II. Coronary Heart Disease in the Framingham Study. *Int. J. Epidemiol.* 44, 1767–1780 (1957).

[54] Gotto, A. Lowering Blood Cholesterol to Prevent Heart Diseases. *Nutr. Rev.* 43, 283–291 (1985).

[55] Endo, A., Kuroda, M. & Tsujita, Y. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinium. *J. Antibiot. (Tokyo)* 29, 1346–1348 (1976).

[56] Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. & Springer, J. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. *Proc. Natl. Acad. Sci. U. S. A.* 77, 3957–3961 (1980).

[57] Tobert, J. A. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nat. Rev. Drug Discov.* 2, 517–526 (2003).

[58] Shepherd, J., Hunninghake, D. B., Barter, P., McKenney, J. M. & Hutchinson, H. G. Guidelines for lowering lipids to reduce coronary artery disease risk: a comparison of rosuvastatin with atorvastatin, pravastatin, and simvastatin for achieving lipid-lowering goals. *Am. J. Cardiol* .91, 11C–17C; discussion 17C–19C (2003).

[59] Kerpel-Fronius, S. A statinok biztonságos alkalmazásánka klinikai farmakológiai alapjai. *LAM* 433–442 (2005).

[60] Endo, A. The discovery and development of HMG-CoA reductase inhibitors. *J. Lipid Res.* 33, 1569–1582 (1992).

[61] Endo, A. The origin of the statins. 2004. *Atheroscler. Suppl.* 5, 125–130 (2004).

[62] Gould, A. L., Davies, G. M., Alemao, E., Yin, D. D. & Cook, J. R. Cholesterol reduction yields clinical benefits: meta-analysis including recent trials. *Clin. Ther.* 29, 778–794 (2007).

[63] Sever, P. S., Poulter, N. R., Dahlof, B., Wedel, H., Beevers, G., Caulfield, M., Collins, R., Kjeldsen, S. E., Kristinsson, A., McInnes, G., Mehlsen, J., Nieminen, M. S., O'Brien, E. T. & Ostergren, J.; ASCOT Investigators. The Anglo-Scandinavian Cardiac Outcomes Trial lipid lowering arm: extended observations 2 years after trial closure. *Eur. Heart J.* 29, 499–508 (2008).

[64] Lefer, A. M., Campbell, B., Shin, Y. K., Scalia, R., Hayward, R. & Lefer, D. J. Simvastatin Preserves the Ischemic-Reperfused Myocardium in Normocholesterolemic Rat Hearts. *Circulation* 100, 178–184 (1999).

[65] Lefer, A. M., Scalia, R. & Lefer, D. J. Vascular effects of HMG CoA-reductase inhibitors (statins) unrelated to cholesterol lowering: new concepts for cardiovascular disease. *Cardiovasc. Res.* 49, 281–287 (2001).

[66] Chen, J., Nagasawa, Y., Zhu, B. M., Ohmori, M., Harada, K., Fujimura, A. & Hashimoto, K. Pravastatin prevents arrhythmias induced by coronary artery ischemia/reperfusion in anesthetized normocholesterolemic rats. *J. Pharmacol. Sci.* 93, 87–94 (2003).

[67] Adameova, A., Harcarova, A., Matejikova, J., Pancza, D., Kuzelova, M., Carnicka, S., Svec, P., Bartekova, M., Styk, J. & Ravingerová, T. Simvastatin alleviates myocardial contractile dysfunction and lethal ischemic injury in rat heart independent of cholesterol-lowering effects. *Physiol. Res. Acad. Sci. Bohemoslov.* 58, 449–454 (2009).

[68] Calabrò, P. & Yeh, E. T. H. The pleiotropic effects of statins. *Curr. Opin. Cardiol.* 20, 541–546 (2005).

[69] Liao, J. K. & Laufs, U. Pleotropic effects of statins. *Annu. Rev. Pharmacol. Toxicol.* 45, 89–118 (2005).

[70] O'Driscoll, G., Green, D. & Taylor, R. R. Simvastatin, an HMG coenzyme A reductase inhibitor, improves endothelial function within 1 month. *Circulation* 95, 1126–1131 (1997).

[71] Ceylan, A., Karasu, C., Aktan, F., Güven, C., Can, B. & Ozansoy, G. Effects of simvastatin treatment on oxidant/antioxidant state and ultrastructure of diabetic rat myocardium. *Gen. Physiol. Biophys* .22, 535–547 (2003).

[72] Jones, S. P. Simvastatin attenuates oxidant-induced mitochondrial dysfunction in cardiac myocytes. *Circ. Res.* 93, 697–699 (2003).

[73] Bloom, H. L., Shukrullah, I., Veledar, E., Gutmann, R., London, B. & Dudley, S. C. Statins Decrease Oxidative Stress and ICD Therapies. *Cardiol. Res. Pract* .2010, 1–7 (2010).

[74] Shimizu, K. Direct Anti-Inflammatory Mechanisms Contribute to Attenuation of Experimental Allograft Arteriosclerosis by Statins. *Circulation* 108, 2113–2120 (2003).

[75] Endres, M. Statins: potential new indications in inflammatory conditions. *Atheroscler. Suppl.*7, 31–35 (2006).

[76] Kostapanos, M., Liberopoulos, E., Goudevenos, J., Mikhailidis, D. & Elisaf, M. Do statins have an antiarrhythmic activity? *Cardiovasc. Res.* 75, 10–20 (2007).

[77] Xing, D., Murry, D. J., Schmidt, M. S., Hohl, R. J. & Martins, J. B. Lovastatin specifically prevents focal ischemic ventricular tachycardia due to triggered activity. *Heart Rhythm Off. J. Heart Rhythm Soc* .4, 629–637 (2007).

[78] De Sutter, J., Tavernier, R., De Buyzere, M., Jordaens, L. & De Backer, G. Lipid lowering drugs and recurrences of life-threatening ventricular arrhythmias in high-risk patients. *J. Am. Coll. Cardiol.* 36, 766–772 (2000).

[79] Zhao, J. L., Yang, Y. J., Zhang, Y. H., You, S. J., Wu, Y. J. & Gao, R. L.Effect of statin therapy on reperfusion arrhythmia in patients who underwent successful primary angioplasty. *Clin. Res. Cardiol. Off. J. Ger. Card. Soc.* 97, 147–151 (2008).

[80] Wright, D. G. & Lefer, D. J. Statin mediated protection of the ischemic myocardium. *Vascul. Pharmacol.* 42, 265–270 (2005).

[81] Laufs, U., Fata, V. L. & Liao, J. K. Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. *J. Biol. Chem.* 272, 31725–31729 (1997).

[82] Mital, S., Zhang, X., Zhao, G., Bernstein, R. D., Smith, C. J., Fulton, D. L., Sessa, W. C., Liao, J. K. & Hintze, T. H. Simvastatin upregulates coronary vascular endothelial nitric oxide production in conscious dogs. *Am. J. Physiol. Heart Circ. Physiol.* 279, H2649–2657 (2000).

[83] Landmesser, U., Engberding, N., Bahlmann, F. H., Schaefer, A., Wiencke, A., Heineke, A., Spiekermann, S., Hilfiker-Kleiner, D., Templin, C., Kotlarz, D., Mueller, M.,Fuchs, M., Hornig, B., Haller, H. & Drexler, H. Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularisation, left ventricular function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. *Circulation* 110, 1933–1939 (2004).

[84] Birnbaum, Y., Ye, Y., Rosanio, S., Tavackoli, S., Hu, Z. Y., Schwarz, E. R. & Uretsky, B. F.Prostaglandins mediate the cardioprotective effects of atorvastatin against ischemia-reperfusion injury. *Cardiovasc. Res.* 65, 345–355 (2005).

[85] Lee, T. S., Chang, C. C., Zhu, Y. & Shyy, J. Y. Simvastatin induces heme oxygenase-1: A novel mechanism of vessel protection. *Circulation* 110, 1296–1302 (2004).

[86] Atar, S., Ye, Y., Lin, Y., Freeberg, S. Y., Nishi, S. P., Rosanio, S., Huang, M. H., Uretsky, B. F., Perez-Polo, J. R. & Birnbaum, Y. Atorvastatin-induced cardioprotection is mediated by increasing inducible nitric oxide synthase and consequent S-nitrosylation of cyclooxygenase-2. *AJP Heart Circ. Physiol.* 290, H1960–H1968 (2006).

[87] Parratt, J. R. & Végh, Á. Endothelial cells, nitric oxide and ischaemic preconditioning. *Basic Res. Cardiol.* 91, 27–30 (1996).

[88] Kovács, M., Gönczi, M., Kovács, E. & Végh, Á. Time course analysis of cardiac pacing-induced gene expression changes in the canine heart. *Mol. Cell. Biochem.* 372, 257–266 (2013).

[89] Altun, I., Oz, F., Arkaya, S. C., Altun, I., Bilge, A. K., Umman, B. & Turkoglu, U. M. Effect of statins on endothelial function in patients with acute coronary syndrome: A prospective study using adhesion molecules and flow-mediated dilatation. *J. Clin. Med. Res.* 5, 354- 361 (2014).

[90] Athyros, V. G., Katsiki, N., Karagiannis, A. & Mikhailidis, D. P. Short-, mid-, and long-term benefits of peri-procedural high-intensity statin administration in patients undergoing percutaneous coronary intervention. *Curr. Med. Res. Opin.* 31, 191–195 (2015).

[91] Wang, X., Lin, Y., Luo, N., Chen, Z., Gu, M., Wang, J. & Chen, Y. Short-term intensive atorvastatin therapy improves endothelial function partly via attenuating perivascular adipose tissue inflammation through 5-lipoxygenase pathway in hyperlipidemic rabbits. *Chin. Med. J. (Engl.)* 127, 2953–2959 (2014).

[92] Hernández-Perera, O., Pérez-Sala, D., Navarro-Antolín, J., Sánchez-Pascuala, R., Hernández, G., Díaz, C., & Lamas, S. Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J. Clin. Invest.* 101, 2711–2719 (1998).

[93] Laufs, U. & Liao, J. K. Post-transcriptional Regulation of Endothelial Nitric Oxide Synthase mRNA Stability by Rho GTP-ase. *J. Biol. Chem.* 273, 24266–24271 (1998).

[94] Tiefenbacher, C. P., Kapitza, J., Dietz, V., Lee, C. H. & Niroomand, F. Reduction of myocardial infarct size by fluvastatin. *Am. J. Physiol. Heart Circ. Physiol.* 285, H59–64 (2003).

[95] Wolfrum, S., Dendorfer, A., Schutt, M., Weidtmann, B., Heep, A., Tempel, K., Klein, H. H., Dominiak, P. & Richardt, G. Simvastatin acutely reduces myocardial reperfusion injury in vivo by activating the phoshatidylinositide 3-kinase/Akt pathway. *J. Cardiovasc. Pharmacol.* 44, 348–355 (2004).

[96] Čarnická, S., Adameová, A., Nemčeková, M., Matejíková, J., Pancza, D. & Ravingerová, T. Distinct effects of acute pre-treatment with lipophilic and hydrophilic statins on myocardial stunning, arrhythmias and lethal injury in the rat heart

subjected to ischemia/reperfusion. *Physiol. Res. Acad. Sci. Bohemoslov.* 60, 825–830 (2011).

[97] Szárszoi, O., Malý, J., Ostádal, P., Netuka, I., Besík, J., Kolár, F., & Ostádal, B.Effect of acute and chronic simvastatin treatment on post-ischemic contractile dysfunction in isolated rat heart. *Physiol. Res. Acad. Sci. Bohemoslov.* 57, 793–796 (2008).

[98] Kureishi, Y., Luo, Z., Shiojima, I., Bialik, A., Fulton, D., Lefer, D. J., Sessa, W. C. & Walsh, K. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. *Nat. Med* .6, 1004–1010 (2000).

[99] Walker, M. J., Curtis, M. J., Hearse, D. J., Campbell, R. W., Janse, M. J., Yellon, D. M., Cobbe, S. M., Coker, S. J., Harness, J. B., Harron, D. W., Higgins, A. J., Julian, D. G., Lab, M. J., Manning, A. S., Northover, B. J., Parratt, J. R., Riemersa, R. A., Riva, E., Russell, D. C., Sheridan, D. J., Winslow, E. & Woodward, B. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc. Res.* 22, 447–455 (1988).

[100] Kiss, A., Juhász, L., Seprényi, G., Kupai, K., Kaszaki, J. & Végh, Á. The role of nitric oxide, superoxide and peroxynitrite in the anti-arrhythmic effects of preconditioning and peroxynitrite infusion in anaesthetized dogs. *Br. J. Pharmacol.* 160, 1263–1272 (2010).

[101] Chen, C. C., Lien, H. Y., Hsu, Y. J., Lin, C. C., Shih, C. M. & Lee, T. M. Effect of pravastatin on ventricular arrhythmias in infarcted rats: role of connexin43. *J. Appl. Physiol. Bethesda Md 1985* 109, 541–552 (2010).

[102] Kayikcioglu, M., Can, L., Evrengul, H., Payzin, S. & Kultursay, H. The effect of statin therapy on ventricular late potentials in acute myocardial infarction. *Int. J. Cardiol.* 90, 63–72 (2003).

[103] Fonarow, G. C., Wright, R. S., Spencer, F. A., Fredrick, P. D., Dong, W., Every, N. & French, W. J.; National Registry of Myocardial Infarction 4 Investigators. Effect of statin use within the first 24 hours of admission for acute myocardial infarction on early morbidity and mortality. *Am. J. Cardiol.* 96, 611–616 (2005).

[104] György, K., Végh, Á., Rastegar, M. A., Papp, J. G. & Parratt, J. R. Isosorbide-2-mononitrate reduces the consequences of myocardial ischaemia, including arrhythmia severity: implications for preconditioning. *Cardiovasc. Drugs Ther.* 14, 481–488 (2000).

[105] Beri, A., Contractor, T., Khasnis, A. & Thakur, R. Statins and the reduction of sudden cardiac death: antiarrhythmic or anti-ischemic effect? *Am. J. Cardiovasc.* 10, 155–164 (2010).

[106] Wit, A. L. & Janse, M. J. Reperfusion arrhythmias and sudden cardiac death: a century of progress toward an understanding of the mechanisms. *Circ. Res.* 89, 741–743 (2001).

[107] Zipes, D. P. & Wellens, H. J. J. Sudden Cardiac Death. *Circulation* 98, 2334–2351 (1998).

[108] Tang, Q., Huang, J., Qian, H., Chen, L., Wang, T., Wang, H., Shen, D., Wu, H. & Xiong, R. Antiarrhythmic effect of atorvastatin on autoimmune myocarditis is mediated by improving myocardial repolarization. *Life Sci.* 80, 601–608 (2007).

[109] Tekin, A., Tekin, G., Sezgin, A. T. & Müderrisoğlu, H. Short- and long-term effect of simvastatin therapy on the heterogeneity of cardiac repolarization in diabetic patients. *Pharmacol. Res.* 57, 393–397 (2008).

[110] Sicouri, S., Gianetti, B., Zygmunt, A. C., Cordeiro, J. M. & Antzelevitch, C. Antiarrhythmic effects of simvastatin in canine pulmonary vein sleeve preparations. *J. Am. Coll. Cardiol.* 57, 986–993 (2011).

[111] Su, F., Shi, M., Yan, Z., Ou, D., Li, J., Lu, Z. & Zheng, Q.Simvastatin modulates remodeling of Kv4.3 expression in rat hypertrophied cardiomyocytes. *Int. J. Biol. Sci* .8, 236–248 (2012).

[112] Maguy, A., Hebert, T. E. & Nattel, S. Involvement of lipid rafts and caveolae in cardiac ion channel function. *Cardiovasc. Res.* 69, 798–807 (2006).

[113] Vyas, A. K., Guo, H., Moss, A. J., Olshansky, B., McNitt, S. A., Hall, W. J., Zareba, W., Steinberg, J. S., Fischer, A., Ruskin, J. & Andrews, M. L.; MADIT-II Research Group.Reduction in ventricular tachyarrhythmias with statins in the Multicenter Automatic Defibrillator Implantation Trial (MADIT)-II. *J. Am. Coll. Cardiol.* 47, 769–773 (2006).

[114] Billman, G. E., Hallaq, H. & Leaf, A. Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proc. Natl. Acad. Sci.* 91, 4427–4430 (1994).

[115] Kang, J. X. & Leaf, A. Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. *Am. J. Clin. Nutr.* 71, 202S–7S (2000).

[116] Leaf, A., Xiao, Y. F., Kang, J. X. & Billman, G. E. Prevention of sudden cardiac death by n-3 polyunsaturated fatty acids. *Pharmacol. Ther.* 98, 355–377 (2003).

[117] Opie, L. H. & Coetzee, W. A. Role of calcium ions in reperfusion arrhythmias: relevance to pharmacologic intervention. *Cardiovasc. Drugs Ther.* 623–636 (1988).

[118] Di Napoli, P., Antonio Taccardi, A., Grilli, A., Spina, R., Felaco, M., Barsotti, A. & De Caterina, R. Simvastatin reduces reperfusion injury by modulating nitric oxide synthase expression: an ex vivo study in isolated working rat hearts. *Cardiovasc. Res.* 51, 283–293 (2001).

[119] Bell, R. M. & Yellon, D. M. Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. *J. Am. Coll. Cardiol.* 41, 508–515 (2003).

[120] Juhász, L., Déri, S., Kisvári, G., Kiss, A., Seprényi, G., Gardi, J. & Végh, Á. The effect of preconditioning on nitric oxide synthase activity during myocardial ischemia and reperfusion in anesthetized dogs. *Curr. Res. Cardiol.* 73–78. (2014).

[121] Tong, H., Chen, W., Steenbergen, C. & Murphy, E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. *Circ. Res* .87, 309–315 (2000).

[122] Mount, P. F., Kemp, B. E. & Power, D. A. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. *J. Mol. Cell. Cardiol.* 42, 271–279 (2007).

[123] Yang, C., Talukder, M. A. H., Varadharaj, S., Velayutham, M. & Zweier, J. L. Early ischaemic preconditioning requires Akt- and PKA-mediated activation of eNOS via serine1176 phosphorylation. *Cardiovasc. Res* .97, 33–43 (2013).

[124] Végh, Á., Gönczi, M., Miskolczi, G. & Kovács, M. Regulation of gap junctions by nitric oxide influences the generation of arrhythmias resulting from acute ischemia and reperfusion in vivo. *Frontiers in Pharmacol.* 4, 76 (2013).

7. ACKNOWLEDGEMENT

First and foremost, I would like to express my gratitude to my principal supervisor, Prof. Dr. Ágnes Végh, who helped me acquire the basic experimental skills, and I am especially grateful for her patience and daily assistance in my performing the studies, preparing for conferences, writing publications and my thesis.

I am also grateful to Prof. Dr. András Varró for providing me with the opportunity to carry out my scientific work and for initiating my scientific career in the Department of Pharmacology and Pharmacotherapy under his valuable scientific guidance.

The members of the department, especially Dr. Márton Gönczi, Gottfried Miskolczi, Dr. László Juhász, Dr. Attila Kiss and Dr. Mária Kovács have contributed immensely to my personal and professional activities. The team has been a source of friendships, good advice and collaboration. Special thanks go to Erika Bakó and Irén Biczókné. Apart from their precious professional expertise in conduting animal experiments they were both worthy friends and made working hours more colourful.

I would like to give my sincere thanks to Dr. György Seprényi, who created the possibilities for doing the fluorescent microscopy experiments in the Institute of Medical Biology.

This work would not have been possible without the help and support of my researcher colleagues in Szeged. I have been able to learn and develop considerably through this and I would never have achieved my goals without them.

I wish to thank my close and distant friends for supporting me during the years, helping me to get through challenging times. Lastly, I am very grateful to and wish to thank my family.

8. ANNEX

Reprints of full papers

1. **Kisvári G**, Kovács M, Gardi J, Seprényi G, Kaszaki J, Végh Á. The effect of acute simvastatin administration on the severity of arrhythmias resulting from ischaemia and reperfusion in the canine: Is there a role for nitric oxide? *Eur J Pharmacol*. **2014;** 5;732:96-104.

2. **Kisvári G**, Kovács M, Seprényi G, Végh Á. The activation of PI 3-kinase/Akt pathway is involved in the acute effects of simvastatin against ischaemia and reperfusion-induced arrhythmias in anaesthetised dogs. *Eur J Pharmacol.* **2015;** 15;769:185-94.