Short-term statin administration in hypercholesterolaemic rabbits resistant to postconditioning: effects on infarct size, endothelial nitric oxide synthase, and nitro-oxidative stress

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Aims
The effectiveness of postconditioning (POC) in hypercholesterolaemia is in dispute. We investigated the effects of 3-day lipophilic (simvastatin) or hydrophilic (pravastatin) statin treatment, without or with POC in normocholesterolaemic (Norm) and hypercholesterolaemic (Chol) rabbits.

Methods and results
Norm or Chol rabbits were subjected to 30 min ischaemia and randomized in two series of 12 groups each: control, simvastatin (Sim), pravastatin (Prav), POC, Sim-POC, Prav-POC, Sim-Chol, Prav-Chol, POC-Chol, Sim-POC-Chol, Prav-POC-Chol. After ischaemia, rabbits of the first series underwent 3 h reperfusion, followed by infarct size, total cholesterol, and low density lipoprotein plasma level evaluation; animals of the second series underwent 10 min reperfusion followed by tissue sampling for nitrotyrosine (NT), malondialdehyde, endothelial nitric oxide synthase (eNOS), and Akt analyses.

N-nitro-L-arginine methylester (L-NAME) was given in two additional groups (POC-L-NAME and Prav-Chol-L-NAME) for infarct size assessment. All interventions reduced infarction in Norm (24.3 ± 1.3, 25.9 ± 2.8, 27.9 ± 3.1, 23.3 ± 2.3, and 33.4 ± 3.1% in POC, Sim, Prav, Sim-POC, and Prav-POC groups, respectively, vs. 49.3 ± 1.9% in control, P < 0.05), but only Prav did so in Chol animals (25.3 ± 3.3 and 25.3 ± 3.9% in Prav-Chol and Prav-POC-Chol vs. 50.9 ± 1.7, 44.8 ± 4.3, 41.5 ± 3.5, and 49.3 ± 5.5% in Chol, Sim-Chol, POC-Chol, and Sim-POC-Chol, respectively, P < 0.05). L-NAME abolished the infarct size-limiting effect of POC and Prav-Chol. Prav induced the greatest reduction in NT, while it was the only intervention that increased myocardial eNOS and Akt in Chol rabbits (P < 0.05 vs. all others).

Conclusion
Prav, in contrast to same-dose Sim or POC, reduces infarction in Chol rabbits independently of lipid lowering, potentially through eNOS activation and nitro-oxidative stress attenuation.

Keywords
Statins • Simvastatin • Pravastatin • Postconditioning • Hypercholesterolaemia

1. Introduction
Ischaemic postconditioning reduces infarct size by attenuation of reperfusion injury in all species studied so far, including humans.1,2 A series of clinical studies have hitherto documented the effectiveness of postconditioning in mixed populations of humans with variable risk factor profiles and different treatment regimens.3,4 However, there is still some controversy in the literature regarding the effectiveness of postconditioning in hypercholesterolaemia, a common risk factor in patients with coronary artery disease.5 Some investigators showed...
that postconditioning was still effective,6 while others showed that it failed to reduce infarct size in animals with hypercholesterolaemia and atherosclerosis.7–10

The mechanisms by which hyperlipidaemia may blunt the cardioprotective effects of ischaemic conditioning are not clear.9 The expression and activity of endothelial nitric oxide synthase (eNOS) may play a key role. More specifically, hypercholesterolaemia impairs endothelial function and reduces NO synthesis, potentially by increasing the production of several oxidants such as peroxynitrite and lipid peroxidation compounds.11 Furthermore, endothelial dysfunction can be characterized by a decreased synthesis of endothelial nitric oxide and it has been showed that postconditioning increases tissue eNOS activity under normocholesterolaemia but not under hypercholesterolaemia.10

Statins are among the most widely prescribed drugs and provide additional benefits besides those derived by lipid lowering, including the improvement in endothelial function, potentially by the enhancement of eNOS expression and activity.12,13 We have previously shown that postconditioning reduced infarct size only in normo- and not in hypercholesterolaemic rabbits, while a long-term (3 weeks) simvastatin administration, besides reducing the total cholesterol and low density lipoprotein (LDL) levels, also limited the infarct size both in normo- and in hypercholesterolaemic rabbits, irrespectively of postconditioning.8

In order to elucidate further the cardioprotective role of statins and their interaction with postconditioning under hypercholesterolaemia, in the present study, we investigated whether a short-term (3 days) statin administration, which might not have a lipid-lowering action, also restored the infarct size-limiting effect of postconditioning in hypercholesterolaemic and statin-naïve rabbits. In this context, we compared two statins with different structure and properties, the lipophilic simvastatin and the most hydrophilic pravastatin. Moreover, in order to shed some light on the potential underlying mechanisms, we assessed the oxidative and nitrosative status as well as the activation of eNOS through the PI3/Akt pathway.

2. Methods

2.1 Animals

New Zealand White male rabbits with a weight between 2.6 and 3.3 kg received proper care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No.85-23, revised 1996). Approval from the Ethical Committee and the veterinary authorities of East Attica prefecture was obtained before the study was started. Normal fed rabbits were used for continuous electrocardiographic monitoring. The chest was surgically opened with a left thoracotomy and the beating heart was exposed. The pericardium was opened and a 3-0 silk suture was passed around a prominent coronary artery. Ischaemia was induced by pulling the thread through a small piece of soft tubing, which was firmly positioned against the coronary arterial wall with the aid of a small clamp. Ischaemia resulted in ST elevation on the ECG and a change in the colour of the myocardium. At the end of ischaemic period, the snare was opened, the artery refilled, and the myocardium reperfused.8

2.3 Experimental protocol

162 animals in total completed the study. In the first series of experiments, 88 rabbits were subjected to 30 min regional ischaemia of the heart, followed by 3 h reperfusion and were randomized into 12 groups as follows: Normocholesterolaemic (normally-fed) rabbits: (i) control (n = 10), no additional intervention; (ii) Sim (n = 7), simvastatin (3 mg/kg) for 3 days; (iii) Prav (n = 7), pravastatin (3 mg/kg) for 3 days; (iv) POC (n = 6), postconditioning consisted of 8 cycles of 30 s ischaemia/reperfusion immediately after completion of index ischaemia; (v) Sim-POC (n = 7), simvastatin for 3 days and postconditioning; (vi) Prav-POC (n = 8), pravastatin for 3 days and postconditioning. Hypercholesterolaemic (cholesterol-fed) rabbits: (i) Chol (n = 7), no additional intervention; (ii) Sim-Chol (n = 7), simvastatin for 3 days; (iii) Prav-Chol (n = 7), pravastatin for 3 days; (iv) POC-Chol (n = 7), postconditioning; (v) Sim-POC-Chol (n = 7), simvastatin for 3 days and postconditioning; (vi) Prav-POC-Chol (n = 8), pravastatin for 3 days and postconditioning. The study protocol of this first series of experiments is shown in Figure 1. Simvastatin and pravastatin sodium were administered by a feeding tube once daily in the evening. The dose of simvastatin was selected according to the literature.8,14 Given the fact that a long-term (3 weeks) simvastatin treatment at this dose reduced significantly the infarct size in hypercholesterolaemic animals irrespectively of postconditioning,8 we investigated whether a short-term (3 days) administration of the same regimen was still effective. Pravastatin was administered at the same dosage for comparison.

In a second series of experiments, 60 additional rabbits (five in each of the above groups) were subjected to the above interventions up to the 10th min of reperfusion when tissue samples were taken from the ischaemic area for analysis of tissue malondialdehyde (MDA) as a lipid peroxidation product, nitrotyrosine (NT) as a nitrosative stress product, and evaluation of eNOS and Akt.

Blood samples from all groups were collected at baseline for analysis of total cholesterol and LDL levels.

Two additional experimental groups (seven animals per group) were subjected to 30 min regional ischaemia of the heart, followed by 3 h reperfusion and treated with N-nitro-L-arginine methylester (L-NAME), a non-specific inhibitor of NO synthase, at a dose of 10 mg/kg 10 min before reperfusion, as previously described.15 POC-L-NAME group and Prav-Chol-L-NAME group were, respectively, subjected to the same interventions that already described for POC and Prav-Chol groups.

2.4 Risk zone and infarcted zone measurement

After the end of reperfusion, hearts were excised, mounted on an apparatus, and perfused with normal saline for 2 min for blood removal.
Then the coronary ligature was retightened at the same site and 5 mL of green fluorescent microspheres were infused for the separation of the normally perfused area from the area at risk. Hearts were kept in the refrigerator for 24 h and cut into 3 mm thick sections. The slices were stained with triphenyltetrazolium chloride at 37°C and immersed in formaldehyde. With a wavelength of 366 nm UV light, we separated the risk from the infarcted zone of the heart and we traced all the areas onto an acetate sheet in order to estimate the volumes of these areas in cm³. The tracings were subsequently scanned with the Adobe Photoshop 6.0 and measured with the Scion Image program. The areas of myocardial tissue at risk and infarcted were automatically transformed into volumes. Infarct and risk area volumes were expressed in cm³ and the percent of infarct to risk area ratio (%I/R) was calculated.

2.5 Measurement of MDA and NT
Myocardial tissue samples were frozen at −70°C until assay. On the day of analysis, tissue samples were homogenized as previously described. For the evaluation of free MDA, 1 vol of heart tissue was homogenized in 4 vol of 25 mM HEPES buffer (pH 7.5) containing 5 mM EDTA and 5 mM butylated hydroxytoluene. The protein concentration was determined using the BioRad Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The MDA concentration was determined spectrophotometrically and expressed as μM (Oxford Biomedical Research Colorimetric Assay for lipid peroxidation) with some modifications as previously described. Protein-bound MDA was calculated by subtraction of free MDA from total MDA, as previously described. NT concentration was determined using a commercially available enzyme-linked immunosorbent assay kit according to the

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/94/3/501/355963)
manufacture’s specifications (Hycult Biotechnology b.v., The Netherlands). This kit has a minimum detection level of 2 nM and measurable concentration range of 2–1500 nM.

2.6 Measurement of total cholesterol levels and LDL

Plasma cholesterol levels were determined at baseline spectrophotometrically using a commercial kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany), diagnostic reagent for quantitative in vitro determination of cholesterol in plasma. Plasma LDL levels were determined spectrophotometrically using a commercial kit (Biosystems S.A., Cholesterol LDL direct, Spain).

2.7 Immunohistochemistry

For immunohistochemical analysis, the anti-phospho eNOS (Ser1177) at a 1:100 dilution (Sigma, USA) was used. Immunohistochemistry was performed according to the indirect streptavidin–biotin–peroxidase method. In brief, 5 μm paraffin sections were placed on poly-L-lysine-coated slides, dewaxed, rehydrated, and incubated for 30 min with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Unmasking of the related proteins was carried out. The sections were incubated with the primary antibody at 4°C overnight. Biotin-conjugated secondary antibody was added at 1:200 dilution for 1 h at room temperature (RT). The next stage comprised 30 min incubation in StreptAB Complex (1:100 stock biotin solution, 1:100 stock streptavidin–peroxidase solution, Dako, Greece). For colour development, we used 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Hellas, Greece) and haematoxylin as a counterstain. The staining pattern was considered positive only if cytoplasmic signal was discerned. Images were obtained with a Zeiss-Axiolab microscope (Carl Zeiss GmbH, Germany) employing video analysis software as previously described.18

2.8 Western blot analysis

Phosphorylation states of eNOS (phospho-eNOS, Ser1177), Akt (phospho-Akt, Ser 473), and total levels of eNOS and Akt proteins were analysed by SDS–PAGE immunoelectrophoresis using antibodies (Sigma, USA and Cell Signaling Technology) as previously described.19,20 Relative densitometry was determined using a computerized software package (NIH Image) and the values for phosphorylated eNOS and Akt were normalized to the values for total eNOS and Akt.

2.9 Statistical analysis

All results are presented as mean ± standard error (SEM). Comparisons of numeric variables among the 12 groups were analysed using the one-way analysis of variance model with Bonferroni correction and with Tukey post hoc analysis. A calculated P-value of less than 0.05 was considered to be statistically significant.
3. Results

3.1 Total cholesterol and LDL levels
In cholesterol-fed animals, baseline cholesterol and LDL concentrations were significantly higher compared with normally fed animals (Figure 2). Three-day treatment with simvastatin or pravastatin did not reduce the cholesterol and LDL levels.

3.2 Infarct size
No significant differences were detected in the areas at risk among the studied groups. However, the infarct size and the %I/R ratio differed significantly among groups; in normocholesterolaemic animals, the infarct size was reduced in POC, Sim, Prav, Sim-POC, and Prav-POC groups (mean I/R, 24.3 ± 1.3, 25.9 ± 2.8, 27.9 ± 3.1, 23.3 ± 2.3, and 3.4 ± 2.5%, respectively vs. 49.3 ± 1.9% in control group, P < 0.05). The administration of L-NAME abrogated the limiting size effect of POC, 45.0 ± 2.9%, P < 0.05 vs. POC, Sim, Prav, Sim-POC, and Prav-POC groups (Figure 3A). In hypercholesterolaemic rabbits, the infarct size was reduced only in Prav-Chol and Prav-POC-Chol (mean I/R, 25.7 ± 3.3% and 25.3 ± 3.9% in Prav-Chol and Prav-POC-Chol vs. 50.9 ± 1.7, 44.8 ± 4.3, 41.5 ± 3.5, and 49.3 ± 5.5% in Chol, Sim-Chol, POC-Chol, and Sim-POC-Chol, P < 0.05) (Figure 3B). The administration of L-NAME abrogated the reduction in infarct size of Prav (41.9 ± 1.6 vs. 25.7 ± 3.3% in Prav-Chol group, P < 0.05).

3.3 Myocardial MDA and NT
At the 10th min of reperfusion, in normocholesterolaemic animals, total, free, and protein-bound myocardial MDA concentrations (Figure 4A) were significantly lower in POC and in all groups treated with simvastatin and pravastatin compared with control (P < 0.05). In hypercholesterolaemic animals, treatment with either simvastatin or pravastatin reduced significantly myocardial total, free, and protein-bound MDA, compared with Chol and POC-Chol groups (P < 0.05).

In normocholesterolaemic animals, a significant reduction in myocardial NT was noted in POC and in all groups treated with simvastatin and pravastatin compared with control (P < 0.05, Figure 2C). In hypercholesterolaemic animals, treatment with either simvastatin or pravastatin reduced significantly myocardial NT, whereas a further significant decrease was noted in groups treated with pravastatin compared with those treated with simvastatin (P < 0.05).

3.4 Western blot analysis and immunohistochemistry
In normocholesterolaemic animals, POC resulted in a significant increase in phosphorylation of both myocardial eNOS and Akt at the...
10th min of reperfusion compared with control (Figure 5A and C). In contrast, in hypercholesterolaemic animals, POC showed no phosphorylation of either eNOS or Akt in the ischaemic region of myocardium (Figure 5B and D). Treatment with simvastatin and pravastatin in normocholesterolaemic animals resulted in phosphorylation of eNOS and Akt, whereas the degree of phosphorylation in animals treated with pravastatin was higher than in those treated with simvastatin (Figure 5A and C). Although the animals treated with simvastin had a significantly lower degree of phosphorylation than those treated with pravastatin, when simvastatin was combined with POC, this difference did not reach statistical significance. In hypercholesterolaemic animals, phosphorylation of both eNOS and Akt was observed only in the groups treated with pravastatin (Figure 5B and D). None of the treatments change myocardial total eNOS content.

Western blot results were confirmed by immunohistochemical analysis, which showed a consistent distribution of eNOS in the 12 groups (Figure 6). A strong cytoplasmic expression of phosphorylated eNOS was observed in endothelial cells of cardiac vessels in groups POC, Prav, Prav-POC, Prav-Chol, and Prav-POC-Chol, a median expression in Sim and Sim-POC, and a low expression in control, Chol, POC-Chol, Sim-Chol, and Sim-POC-Chol. These results were in parallel with the results obtained with western blot analysis, indicating that the phosphorylation of eNOS was expressed in the endothelial cells of the heart.

4. Discussion

The present study demonstrates for the first time that 3-day pravastatin administration, although it bears no lipid lowering action, reduces infarct size both in normo- and in hyper-cholesterolaemic statin-naive rabbits exposed to ischaemia and reperfusion, while it also enhances the phosphorylation of eNOS and Akt and confers significant attenuation of nitro-oxidative stress. In contrast, simvastatin at the same dose with pravastatin and postconditioning reduce infarct size and activate eNOS and Akt only in normocholesterolaemic animals.

An initial report in 1997 suggested that statins exert cardioprotective effects independently of their lipid-lowering action.21 Regarding cardioprotection, Bao et al.15 showed that acute pravastatin administration at 5 mg/kg reduced infarct size in rabbits. We also found that 3-day pravastatin treatment at 3 mg/kg/day was effective not only in normocholesterolaemic but also in hypercholesterolaemic rabbits. In contrast, Ueda et al.22 found that 8-week pravastatin treatment did not reduce infarct size in rabbits that were fed with cholesterol enriched diet for 16 weeks. Those conflicting results may be related to the longer duration of hypercholesterolaemia and thus to the extent of atherosclerosis. We have previously shown that 6 weeks of cholesterol feeding induces atherosclerosis in rabbits.7

Hypercholesterolaemia blunts the infarct size-limiting effect of postconditioning possibly by decreasing cardiac NO content.5 Hypercholesterolaemia decreases NO bioavailability with down-regulation
of eNOS, in association with increased production of oxygen-derived free radicals that may inactivate NO. More specifically, increased plasma LDL inhibits the active transport of L-arginine (L-ARG) by endothelial cells, uncoupling the L-ArGeNOS pathway hence limiting NO synthesis and leading to superoxide anion production. The latter results in nitro-oxidative stress, as on the one hand it causes lipid peroxidation, a characteristic feature of inflammatory vascular diseases, and on the other it reacts with NO forming peroxynitrite (ONOO⁻) that may disturb cellular function and reduce further NO bioactivity.

In the present study, a marked increase in MDA, a marker of lipid peroxidation, and NT, a marker of ONOO⁻ and nitrosative stress, was observed in hypercholesterolaemic rabbits. Both pravastatin and simvastatin attenuated myocardial MDA (free, total, and protein-bound MDA) and NT during reperfusion in normocholesterolaemic and hypercholesterolaemic animals. It seems that the attenuation of nitro-oxidative stress at reperfusion, although it may partly restore NO bioavailability in hypercholesterolemia, may not be a crucial mechanism in direct cardioprotection. Thus, the latter may be related to the varying degrees of activation or phosphorylation of the intracellular mediators.

Previous studies have shown that the cardioprotective effects of statins are derived through the upregulation of eNOS. Indeed, the infarct size-limiting effect of pravastatin in cholesterol-fed rabbits was abolished by L-NAME, a NOS inhibitor. The time of stimulation of intracellular mediators also seems to be important and it has been reported that statins can stimulate endothelial NO release in an acute time frame of seconds to minutes. Several different laboratories have reported that eNOS phosphorylation at Ser-1177 by Akt protein kinase in endothelial cells results in a two-fold increase in eNOS catalytic activity. In fact, the Akt/Pi3 kinase pathway and eNOS are involved in the mechanism of postconditioning. It has been shown that L-NAME aborts the protection afforded by postconditioning in isolated rabbit and rat hearts, and we first show herein that this also occurs in anesthetized rabbits in vivo. Postconditioning increases tissue eNOS activity in mini-swines hence improving endothelial function under normocholesterolaemia but not under hypercholesterolaemia. In the present study, we confirmed the above findings in another experimental model, rabbits. Furthermore, it is well demonstrated that eNOS phosphorylation at Ser-1177 through the Akt/Pi3-kinase pathways is one potential mechanism of acute statin-stimulated NO release. In our study, we showed that both statins phosphorylate eNOS and Akt in normocholesterolaemic animals, although to a different degree, in parallel to their infarct size limiting effect. However, in hypercholesterolaemic rabbits, only pravastatin was able to activate both Akt and eNOS, and the administration of L-NAME abolished the infarct size-limiting effect of both POC and pravastatin, indicating that the protection involves NO production. This may be attributed to the different properties of the two statins.

Pravastatin and simvastatin bear different chemical structure and pharmacokinetic properties. Pravastatin has an open acid structure,
is the most hydrophilic statin, cannot easily cross the cell membrane, and, thus, it is taken up selectively by hepatocytes via active transport. Simvastatin, has a lactone ring structure, is lipophilic and can easily cross the cell membrane by passive diffusion and, thus, has a non-specific tissue distribution. The enzyme activity assay of HMG-CoA reductase substrate catalytic fragments indicates that simvastatin and pravastatin have a high affinity for HMGCoA, suggesting that they are potent inhibitors of this enzyme in the liver, although they are transported differently into the hepatocytes. This may be the reason for the similar effects of both statins on cholesterol levels in our experimental protocol. Moreover, the unbound plasma concentration of pravastatin available for distribution to the peripheral circulation is approximately 100 times greater than that of simvastatin, which is administered as a prodrug. The protein-binding capacity of pravastatin sodium is reported to be 55% with a 45% of the drug being in an unbound plasma form, while the protein-binding capacity of simvastatin is more than 95% with a free plasma form less than 5%. Kaesemeyer et al. showed that simvastatin was only 25–30% as effective as pravastatin in inducing vasorelaxation in isolated aortic rings and stimulating NO production by vascular endothelial cells independently of its effect on hepatic cholesterol metabolism. In a subsequent study, the same group showed that pravastatin resulted in rapid and direct eNOS activation through signalling via the SR-B1 endothelial receptor. Therefore, the differences we observed between the two statins on direct cardioprotection in hypercholesterolaemia in our study may be due to an increased concentration of active pravastatin in comparison to simvastatin at the site of action. Thus, a greater dose of simvastatin may exert similar effects with pravastatin.

Our study seems to translate the findings by Kaesemeyer et al. in vivo, showing for the first time that short-term pravastatin and not simvastatin is able to phosphorylate eNOS under hypercholesterolaemia, and provides the ground for a further investigation of the mechanisms by which pravastatin increases eNOS activity. It seems that pravastatin unlike simvastatin and the other statins has the potential to interact with the endothelium with a unique manner. This may partly be related to the high 45% unbound fraction of pravastatin in plasma, which may interact actively with the endothelium and activate the eNOS/Akt signalling cascade. However, there is little information regarding the molecular mechanisms underlying the effect of NO in postconditioning. In general, it is believed that postconditioning acts by inhibition of the mitochondrial permeability transition pore (mPTP) and it has been shown that NO prevents the opening of mPTP and inhibits the cardiac mitochondrial voltage-dependent anion channel (VDAC).

No-reflow phenomenon, a disorder that blocks the microcirculation during reperfusion, is also involved in reperfusion injury. Previous studies have shown that both postconditioning and statins may contribute to the prevention of this phenomenon and this may be the case in the present study, although we did not assess the effects of our interventions on no-reflow.

In contrast to preconditioning that requires ischaemia application before the occurrence of the index ischaemic event, postconditioning is a more clinically relevant and promising means of myocardial protection against ischaemia/reperfusion injury. However, there are several issues that need to be addressed, such as the time window of protection or the interaction with common risk factors or comorbidities. This is an animal study and data on animals show thus far that postconditioning may not be effective in hypercholesterolaemia. On the other hand, in the human trials by Staat et al. and Hausenloy et al., postconditioning was effective although most of the patients studied had dyslipidaemia. However, the majority of those patients might have been on lipid lowering therapy. Moreover, there was no subanalysis on the effects of conditioning (post- or remote) according to cholesterol status in either of the two studies. On the other hand, as in all animal trials, the direct translation of beneficial interventions to humans is not guaranteed.

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