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Original Article

Atorvastatin does not Affect Ischaemia-Induced Phosphatidylserine Exposition in Humans *in-vivo*

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Aim: Statins can induce pharmacologic preconditioning and thereby reduce infarct size. Cellular phosphatidylserine (PS) exposition occurs in the course of ischaemia and reperfusion and has been associated with injury. In this experiment we studied the effect of atorvastatin on PS exposition after a standardised ischaemia and reperfusion challenge.

Methods: In a double-blind randomised cross-over trial 30 healthy volunteers were allocated to 3 day treatment with atorvastatin (80 mg/day) and placebo ($n=24$), or placebo treatment twice ($n=6$). At the end of each treatment period, volunteers underwent 10 minutes of forearm ischaemic exercise. At reperfusion radiolabeled annexin A5 was administered intravenously and Gamma camera imaging of both hands was performed 1 and 4 hours after reperfusion.

Results: Annexin A5 targeting was not different between atorvastatin treatment ($26.1 \pm 9.8\%$ and $24.0 \pm 9.5\%$ respectively at 1 and 4 hours after reperfusion) and placebo treatment ($25.6 \pm 11.0\%$ and $24.5 \pm 10.7\%$) ($p=0.99$). Our time control experiment did not reveal a carry-over effect.

Conclusions: Our results show that treatment with atorvastatin 80 mg does not reduce forearm PS exposition after ischaemic exercise. This suggests that the role of PS exposure in the prevention of ischemia and reperfusion injury by short term treatment with atorvastatin is limited.

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Key words; Atorvastatin, Ischaemia-reperfusion injury, Phosphatidylserines, Annexin A5 scintigraphy, Pharmacologic preconditioning

Introduction

The cholesterol lowering action of statins (HMG-coenzyme A reductase inhibitors) and its associated reduction in cardiovascular events have been well documented in numerous clinical trials¹⁻⁴. Traditionally, this beneficial effect of statins has been explained by lowering of plasma cholesterol and sub-

sequent reduced progression of atherosclerosis. Apart from its cholesterol lowering properties, statins have other ("pleiotropic") actions, such as up-regulation of ecto-5'-nucleotidase⁵⁻⁷, activation of the phosphatidylinositol 3-kinase-Akt pathway⁸ and up-regulation of NO synthase^{9, 10} which all may increase tolerance against ischaemia and reperfusion (IR)¹¹⁻¹³. Several animal studies have shown reduction of IR-injury as a result of statin treatment in both the heart and the kidney¹⁴⁻¹⁷. This putative protection may explain the clinical observation that early initiation of statin treatment in patients with an acute cardiovascular event improves long-term prognosis as compared with a delayed start of this treatment¹⁸⁻²⁰. Furthermore in two randomised clinical trials one day treatment with

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atorvastatin prior to percutaneous coronary intervention reduced myocardial injury and improved clinical outcomes in patients with acute coronary syndromes^{21, 22}).

Ischaemia induced phosphatidylserine (PS) exposure on cardiomyocytes has been associated with reversible cellular damage and apoptosis^{23, 24}. Treatment to shield exposed PS with diannexin, a homodimer of human annexin A5 (with high affinity for exposed phosphatidylserines) significantly reduces IR-injury in the liver and kidney^{25, 26}. Likewise, diannexin prevents no-reflow in a rabbit cardiac ischemia-reperfusion model²⁷. These observations suggest a critical role for exposed PS in the development of IR-injury. We have developed an experimental model to study PS exposure after IR in healthy volunteers. Our model uses radiolabeled recombinant annexin A5 to visualise PS-exposition that occurs after 10 minutes of forearm ischemia combined with isometric exercise²⁸. Interventions that have been shown in preclinical and clinical research to modulate myocardial infarct size (ischaemic preconditioning, and administration of adenosine, dipyridamole, and caffeine) similarly modulate annexin A5 targeting in our forearm model, further supporting a role for PS-exposure in IR-injury²⁸⁻³⁰).

Using annexin A5 scintigraphy after voluntary ischaemic exercise in healthy males, we have recently shown that annexin A5 targeting after a short pretreatment with rosuvastatin 20 mg once daily was significantly reduced ($16 \pm 1\%$ and $18 \pm 2\%$ respectively at 1 and 4 hours after reperfusion) compared to placebo treatment ($21 \pm 3\%$ and $25 \pm 3\%$) ($p < 0.05$)³¹. As previously reported for other statins in preclinical research with infarct size as endpoint, this effect of rosuvastatin was inhibited by caffeine, an adenosine receptor antagonist³¹).

There is ample evidence from animal and clinical research that treatment for three days with high dose atorvastatin protects against IR-injury^{15, 21, 32-34}. However, we do not know whether PS exposure relates to this clinical benefit of atorvastatin, as differences have been observed in the efficacy of various statins to inhibit HMG-CoA-reductase³⁵ and to induce pleiotropic effects³⁶.

Aim

We aim to assess the role of PS-modulation in the beneficial effect of atorvastatin on IR-injury. We tested the hypothesis that a short pretreatment with atorvastatin 80 mg reduces annexin A5 targeting in our forearm IR-model.

Methods

After approval of the protocol (NCT00441597) by the Institutional Review Board of the Radboud University Nijmegen Medical Centre and in accordance with the declaration of Helsinki (2008), 30 healthy male volunteers (age 18-54) signed informed consent. All participants underwent medical screening to exclude cardiovascular disease, hypercholesterolemia, hypertension, diabetes mellitus, and impaired renal function. Furthermore serum values of creatinin kinase (CK) and alanine aminotransferase (ALT) were measured to exclude participants possibly at risk for adverse events due to atorvastatin use (rhabdomyolysis and toxic hepatitis).

In this double blind randomised cross-over trial (**Fig. 1**) participants were allocated to treatment with either atorvastatin and placebo ($n=24$), or placebo treatment twice ($n=6$). The latter group served as a time-control. Furthermore, it allowed us to verify our assumptions on within subject variability and test-to-test correlation of our annexin A5 scans. Placebo or atorvastatin (80 mg) was administered for three days, and at least 4 weeks were allowed between the two treatment periods as a wash-out. Atorvastatin 80mg (Pfizer, Capelle a/d IJssel, The Netherlands) was capsulated to match placebo by the department of Clinical Pharmacy (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands according to GMP standards). In both treatment periods the first tablet was administered under supervision of the investigator, after venous blood sampling to determine fasting lipid profile. The participant was instructed to take the second capsule in the morning and to abstain from caffeine during the last 24 hours prior to ischaemic exercise. On day 3 after an overnight fast the last tablet was administered under supervision, again after assessment of fasting lipid profile and serum caffeine level to verify protocol compliance.

Sixty minutes after administration of the study medication in all participants maximal voluntary contraction force of the non-dominant forearm was measured with an isometric handgrip dynamometer. Subsequently, the circulation of the non-dominant forearm was occluded for 10 minutes with an upper arm cuff inflated to 200 mmHg. Directly after occlusion volunteers were asked to perform isometric contractions of the finger flexors at 50% of their maximum contraction force. These contractions were performed rhythmically with 5 seconds of contraction followed by 5 seconds of relaxation until exhaustion. The total duration of ischaemia was 10 minutes, independent of the duration of contractions.

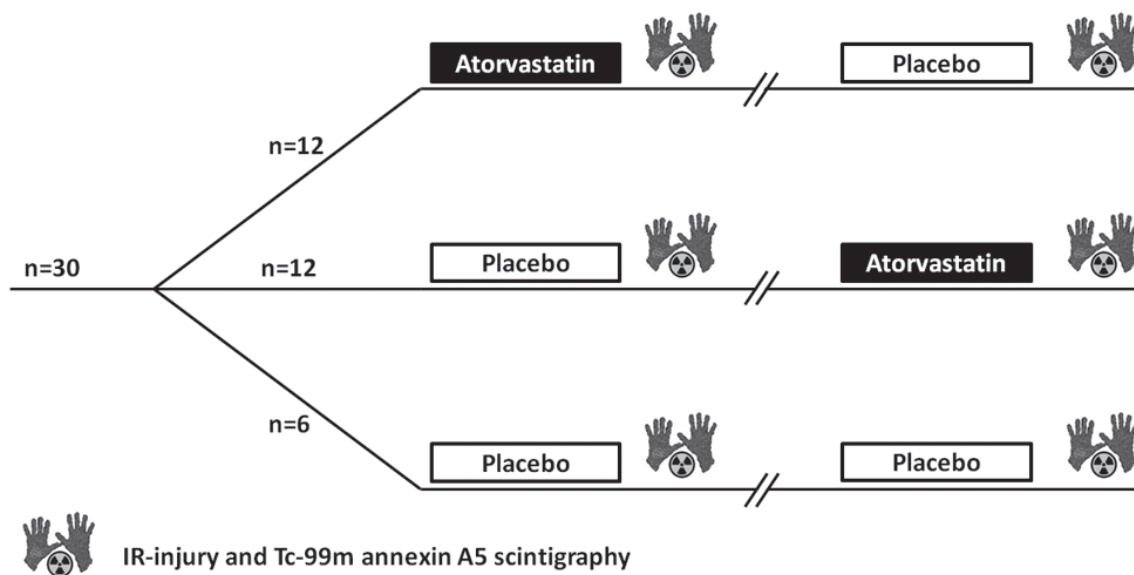


Fig. 1. Study protocol. From the 30 participants 12 were randomised to 3 days of atorvastatin treatment followed by three days of placebo treatment. 12 Participants were randomised to first 3 days of placebo treatment and next 3 days atorvastatin treatment, and 6 participants were randomised to placebo treatment twice. At day three of treatment participants performed 10 minutes of ischemic forearm exercise. Upon reperfusion Tc-99m labelled annexin A5 was administered and scintigraphy was performed 1 and 4 hours after reperfusion. Between both treatment periods a 4 week interval was observed.

Immediately upon reperfusion 100 microgram recombinant human annexin A5, radiolabeled with 450 MBq Technetium-99m was administered intravenously into the dominant arm. One and four hours after this administration both hands were scanned simultaneously by use of a gamma camera (Orbiter camera equipped with low-energy high-resolution collimators; Siemens, Hoffman Estates, Ill, USA) as previously described²⁸.

Caffeine plasma concentrations were determined using reversed-phase HPLC with UV detection set at 273 nm³⁷. Serum lipid profile (total-, HDL- and LDL-cholesterol and triglycerides), creatinine kinase (CK) and alanine amino transferase (ALAT) were determined with a commercially available kit (Aeroset, Abbott, Santa Clara, Cal., USA).

Data Analysis

All the digitised gamma camera images were analysed offline by the same investigator (W.J.O.) using Hermes software (Nuclear Diagnostics, Stockholm, Sweden). A predefined region of interest was placed over the thenar muscle of both hands. Annexin A5 targeting was expressed as the percentage difference in counts/pixel between the experimental (non-dominant) hand and the control hand as previously described²⁸.

Statistical Analysis

Values are expressed as mean \pm standard error (SE) unless indicated otherwise.

This study is the first to use our forearm IR-model in a paired set-up in healthy volunteers. Based on previous studies, a between subject SD of 10% was predicted²⁸⁻³⁰. Conservatively assuming a correlation coefficient of 0.5 between first and second paired observation, we calculated that with an alpha of 0.05 and a power of 90%, using 24 subjects allows us to find a difference in annexin A5 targeting of 7%. This is well in the range of our previous observation after treatment with rosuvastatin³¹. Furthermore 6 additional participants were randomised (in a double-blind fashion) to receive placebo treatment twice to serve as time control experiment. This time-control allows us to verify our assumptions regarding this power analysis afterwards.

The effect of atorvastatin treatment on annexin A5 targeting and lipid profile was tested using ANOVA for repeated measurements. All statistical analyses were performed using SPSS 16.0 for Windows.

Results

Baseline characteristics of all participants are presented in **Table 1**. Caffeine levels were all under 0.85

Table 1. Baseline characteristics of all participants ($n=30$)

	Mean \pm S.D.
Age (years)	23.3 \pm 6.6
BMI (kg/m ²)	22.4 \pm 2.4
Systolic blood pressure (mmHg)	126 \pm 8
Diastolic blood pressure (mmHg)	71 \pm 10
Heart rate (bpm)	62 \pm 8
Glucose (mmol/L)	4.6 \pm 0.7
Total cholesterol (mmol/L)	4.0 \pm 0.6
Triglycerides (mmol/L)	1.19 \pm 0.94
HDL-C (mmol/L)	1.33 \pm 0.26
LDL-C (mmol/L)	2.19 \pm 0.65

mg/L, indicating satisfactory compliance to the 24 hours caffeine abstinence. The annexin A5 targeting was not different between atorvastatin treatment ($26.1 \pm 2.0\%$ and $24.0 \pm 1.9\%$ respectively at 1 and 4 hours after reperfusion) and placebo treatment ($25.6 \pm 2.4\%$ and $24.5 \pm 2.2\%$) ($p=0.999$) (**Fig. 2**).

Three day treatment with atorvastatin significantly reduced total cholesterol (from 4.15 ± 0.10 mmol/L to 3.44 ± 0.10 mmol/L) and LDL cholesterol (from 2.31 ± 0.10 mmol/L to 1.84 ± 0.10 mmol/L as compared to placebo treatment; $p < 0.001$ for both comparisons). HDL cholesterol and triglyceride levels were unaffected by atorvastatin (from 1.22 ± 0.04 to 1.25 ± 0.05 for HDL-C, and from 0.93 ± 0.08 to 0.80 ± 0.09 for triglycerides).

Workload (50% of the maximal strength * duration of ischaemic exercise) did not significantly differ between both study days (4260 ± 284 kg*sec and 4253 ± 219 kg*sec after atorvastatin and placebo respectively ($p=0.96$), indicating that potential differences in ischaemic challenge did not confound the observed lack of effect on annexin A5 targeting.

In the time control group ($n=6$) the annexin A5 targeting after the first visit was $23.5 \pm 5.5\%$ and $23.2 \pm 6.5\%$ (respectively at 1 and 4 hours after reperfusion) and $30.2 \pm 6.1\%$ and $20.3 \pm 7.8\%$ after the second visit (**Fig. 3**). The between-subject standard deviations in annexin A5 targeting were 13.5%, 15.8%, 15.0% and 9.3% at 1 and 4 hours for visit 1 and 2 respectively. Within subject correlation was 0.93 and 0.79 at 1 and 4 hours respectively. This reproducibility should have allowed us to detect a difference in targeting between placebo and atorvastatin of at least 7%, closely resembling our initial power calculation.

Discussion

The novel finding of this study is the lack of

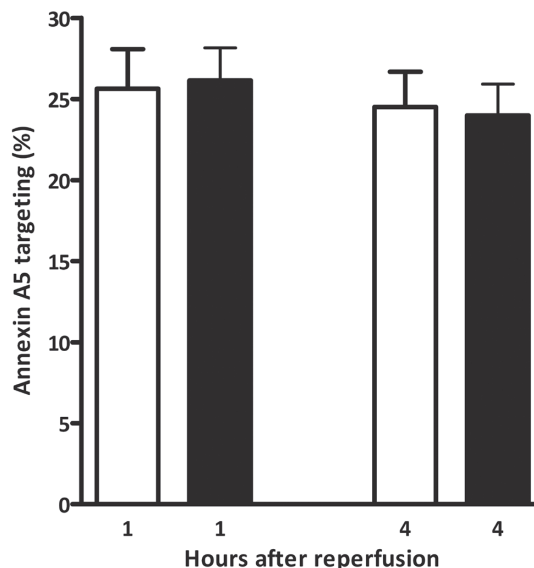


Fig. 2. Annexin A5 targeting 1 and 4 hours after reperfusion, after placebo (open bars) and atorvastatin (closed bars) treatment. Bars represent mean \pm SE.

effect of atorvastatin on annexin A5 targeting after voluntary ischaemic exercise. This indicates that in our forearm model treatment with atorvastatin, in a dose and duration as previously used in clinical trials with patients who present with coronary syndromes^{21, 22, 32}, does not modulate PS exposition after IR, in contrast to treatment with rosuvastatin, as reported recently³¹. As the protective action of atorvastatin in a setting of ischemia and reperfusion has been convincingly shown in multiple animal experiments^{33, 34} and even in several clinical trials^{21, 22, 38}, we conclude that PS modulation is not likely involved in the protective mechanism of atorvastatin.

During reperfusion after an ischaemic event, multiple detrimental processes take place simultaneously including formation of radical oxygen species (ROS), cellular and mitochondrial calcium overload, and a rapid increase (restoration) in pH³⁹. These actions result in mitochondrial damage caused by opening of the mitochondrial permeability transition pore (MPTP), activation of caspases and subsequent cell damage and possible cell death (by either necrosis or apoptosis)⁴⁰.

PS exposition is recognised to be part of the signalling cascade in the course of apoptosis⁴¹. It attracts phagocytes which remove the dying cell before its remnants evoke an inflammatory response⁴². The importance of PS exposition in developing tissue damage after IR injury was clearly demonstrated in several experiments where IR-injury could be reduced by

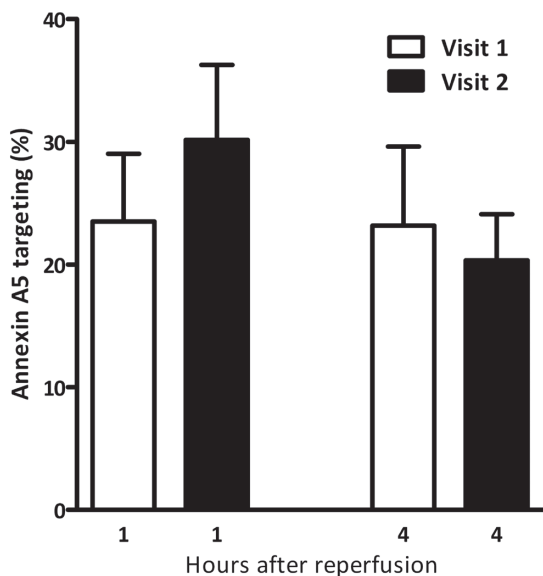


Fig. 3. Annexin A5 targeting 1 and 4 hours after reperfusion in time-control group (placebo treatment twice), after visit 1 (open bars) and visit 2 (closed bars). Bars represent mean \pm SE.

shielding of PS by diannexin treatment^{25, 26, 43}).

The time-control experiment excluded a carry-over effect as a potential reason for the lack of observed effect of atorvastatin on annexin A5 targeting. In addition, interindividual SD and within subject correlation resulted in a power to detect differences between placebo and atorvastatin that resembles the results of our initial power calculation. In fact, based on these measurements we could calculate a minimal detectable difference of less than 10%, which is well below the previously observed effect of rosuvastatin on annexin A5 targeting. Thus, our sample size was sufficient to detect the effect size as expected from our hypothesis and previous observation with rosuvastatin.

The (cardio)protective mechanism of statins is well investigated, though its mechanism has not been fully elucidated⁴⁴. Adenosine appears to play an important role in initiating the cascade (adenosine dependent phosphorylation of ERK1/2, Akt and eNOS) leading to a protective phenotype after statin therapy⁴⁵. Upregulation of ecto-5'-nucleotidase has been shown *in vitro* and in animals *in-vivo* for various statins⁴⁶. In humans *in-vivo* however, this effect has only been explored for rosuvastatin, which increases ecto-5'-nucleotidase activity with 50%⁴⁷.

In our rosuvastatin trial participants were treated 7 days³¹, whereas in this atorvastatin trial participants were treated for 3 days. However we doubt that treat-

ment duration could account for the difference in annexin A5 targeting in these experiments. We have previously shown in humans that activation of ecto-5'-nucleotidase mediates prevention of PS exposure by rosuvastatin after IR. The effect of statins on ecto-5'-nucleotidase appears to occur rapidly^{6, 7}. For example Sanada *et al.* found a significant increase of ecto-5'-nucleotidase activity in dogs for different statins (pravastatin, pitavastatin and cerivastatin) already 10 minutes after treatment⁷. Thus, our discrepant results between rosuvastatin and atorvastatin are not likely explained by differences in treatment duration but rather reflect a difference in pharmacodynamics between these two statins. There are significant differences between several statins in their efficacy to lower LDL cholesterol⁴⁸. Moreover, pleiotropic effects have been shown to differ between lipophilic statins (e.g. atorvastatin) and hydrophilic statins (e.g. rosuvastatin). Lipophilic statins have been reported to render endothelial and vascular smooth muscle cells more susceptible to apoptosis than hydrophilic statins⁴⁹. In humans rosuvastatin inhibits Rho/Rho kinase activity to a greater extent than atorvastatin at a dose that equally reduced LDL cholesterol³⁶. Moreover hydrophilic statins have been suggested to be superior in reducing event rate after a primary acute coronary syndrome in normocholesterolemic patients⁵⁰.

Conclusion

Our results show that treatment with atorvastatin 80 mg does not reduce forearm PS exposition after ischaemic exercise. This suggests that the role of PS exposure in the prevention of ischemia and reperfusion injury by short term treatment with atorvastatin is limited.

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