

Rosuvastatin Increases Extracellular Adenosine Formation in Humans In Vivo

A New Perspective on Cardiovascular Protection

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Objective—Statins may increase extracellular adenosine formation from adenosine monophosphate by enhancing ecto-5'-nucleotidase activity. This theory was tested in humans using dipyridamole-induced vasodilation as a read-out for local adenosine formation. Dipyridamole inhibits the transport of extracellular adenosine into the cytosol resulting in increased extracellular adenosine and subsequent vasodilation. In addition, we studied the effect of statin therapy in a forearm model of ischemia-reperfusion injury.

Methods and Results—Volunteers randomly received rosuvastatin or placebo in a double-blind parallel design (n=21). The forearm vasodilator response to intraarterial dipyridamole was determined in the absence and presence of the adenosine antagonist caffeine. During a separate visit the vasodilator response to nitroprusside and adenosine was established. In addition, healthy men were randomly divided in 3 groups to receive either placebo (n=10), rosuvastatin (n=22), or rosuvastatin combined with intravenous caffeine (n=12). Subsequently, volunteers performed forearm ischemic exercise. At reperfusion, Tc-99m-labeled annexin A5 was infused intravenously and scintigraphic images were acquired, providing an early marker of cell injury. Rosuvastatin treatment significantly increased the vasodilator response to dipyridamole, which was prevented by caffeine. Rosuvastatin did not influence the response to either sodium nitroprusside or adenosine indicating a specific interaction between rosuvastatin and dipyridamole, which does not result from an effect of rosuvastatin on adenosine clearance nor adenosine-receptor affinity or efficacy. Rosuvastatin increased tolerance to ischemia-reperfusion injury, which was attenuated by caffeine.

Conclusions—Rosuvastatin increases extracellular adenosine formation, which provides protection against ischemia-reperfusion injury in humans in vivo. Therefore, statins and dipyridamole may interact synergistically. (*Arterioscler Thromb Vasc Biol.* 2009;29:963-968.)

Key Words: adenosine ■ human ■ ischemia ■ rosuvastatin ■ annexin A5

Numerous clinical trials have documented protection by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors (statins) against cardiovascular events.¹⁻³ This benefit of statins has been attributed to the lowering of plasma cholesterol. However, preclinical research indicates that HMG-CoA reductase inhibition has additional effects, including the activation of ecto-5'-nucleotidase which converses extracellular adenosine monophosphate into adenosine.⁴⁻⁶ Increased adenosine formation favorably influences cardiovascular disease by reducing platelet aggregation,⁷ atherosclerosis formation,⁸ and ischemia-reperfusion injury.⁹ As statins possess similar properties, adenosine is a possible candidate to mediate these effects.^{3,10-12} To our knowledge, the role of extracellular

adenosine formation in the benefit of statins has not been explored in humans.

Here, we report on the results of 3 studies which addressed the effect of rosuvastatin on adenosine formation and its potential relevance in humans in vivo. For this purpose, we used the adenosine receptor antagonist caffeine and the nucleoside transport inhibitor dipyridamole, as pharmacological tools to assess the involvement of endogenous adenosine in statin-induced effects. Dipyridamole reduces clearance of extracellular adenosine and therefore increases extracellular adenosine concentration at sites of adenosine formation. The subsequent increase in adenosine receptor stimulation is responsible for dipyridamole-induced forearm vasodilation as demonstrated previously.¹³ We reasoned that an increase in

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extracellular adenosine should result in an increased vasodilator response to dipyridamole.

Forearm vascular tone and forearm injury in response to ischemic exercise were used as end points. The following hypotheses were tested:

1. A 1-week treatment with rosuvastatin augments the forearm vasodilator response to dipyridamole without affecting adenosine- or nitroprusside-induced vasodilation. This potentiation is abolished by caffeine (study 1, NCT00554138).
2. A 1-week treatment with rosuvastatin reduces injury after forearm ischemic exercise as assessed with annexin A5 scintigraphy (study 2, NCT00315510).
3. Caffeine inhibits the protective action of rosuvastatin against injury after forearm ischemic exercise (study 3, NCT00457652).

Methods

Subjects

After the study protocols were approved by the Institutional Review Board of the Radboud University Nijmegen Medical Centre, a total of 60 healthy volunteers with a normal medical history, physical examination, blood pressure, body mass index, fasting plasma lipid profile, and glucose concentration gave written informed consent before entering the studies. Eight volunteers participated in 2 different studies. Participants of study 2 were not allowed to participate in study 3. Only male volunteers were allowed in the studies with radiolabeled tracer. All studies were performed according to institutional and Good Clinical Practice guidelines.

Study Design

Study 1: Rosuvastatin and Vasodilator Response to Dipyridamole

Twenty-four volunteers were randomly allocated to receive either an 8-day treatment with rosuvastatin (Astra Zeneca SA, Destelbergen, Belgium; 20 mg per day) or fully mimicking placebo (Pharmacy Department HAGA hospital, The Hague, The Netherlands) in a double blind design. Before (day 1) and during (day 7) treatment, the vasodilator response to 4 increasing concentrations of intraarterial dipyridamole was evaluated as previously described¹³ in the absence and presence of an intraarterial infusion of caffeine (90 $\mu\text{g}/\text{min}/\text{dL}$). During a separate visit (day 8) vasodilator responses to intraarterially infused sodium nitroprusside (SNP; Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre), adenosine (Sanofi Aventis), and 3 increasing periods of arterial occlusion were determined as described previously.¹⁴

All experiments were performed after at least 24 hours of caffeine abstinence, in the morning after an overnight fast, with exception of the last experiment (day 8) which was performed in the afternoon after a minimum fasting period of 6 hours. Volunteers were studied in supine position in a temperature-controlled room ($24\pm 1^\circ\text{C}$). At the start of each experiment a 27-gauge arterial needle (kindly supplied by B. Braun Medical BV, Oss, The Netherlands) was inserted into the brachial artery of the nondominant arm for intraarterial drug administration. In both arms forearm blood flow (FBF) was measured simultaneously with venous occlusion plethysmography using mercury-in-silastic-strain gauges and occluded hand circulation as previously described.¹⁴ Total intraarterial infused volume was kept at a constant rate of 150 $\mu\text{L}/\text{min}/\text{dL}$ forearm volume. Each dose of vasodilator was infused into the brachial artery for 5 minutes.

Study 2: Rosuvastatin and Ischemia-Reperfusion Injury

Twenty volunteers randomly received either rosuvastatin (20 mg per day for 7 days) or placebo (Pharmacy Department HAGA hospital, The Hague, The Netherlands) in a double blind parallel design. One hour after ingestion of the last trial medication, injury in response to

ischemic hand gripping was measured using previously described experimental procedures.¹⁵ In brief, circulation to the nondominant forearm was occluded for 10 minutes by inflating an upper arm cuff to 200 mm Hg. From the start of occlusion, volunteers performed rhythmic isometric hand contractions at 50% of their maximal voluntary force for 5 seconds every 10-second period until exhaustion. Immediately on reperfusion recombinant human annexin A5 labeled with Tc-99m (0.1 mg protein; 400 MBq) was administered intravenously. Both hands were imaged at 1 and 4 hours of reperfusion by using a gamma camera (Siemens Orbiter) connected to a Hermes Gold image processing system (Nuclear Diagnostics). Recombinant human annexin A5 was obtained from Theseus Imaging Corporation and conjugated with HYNIC under GMP conditions (Department of Clinical Pharmacy, Radboud University Nijmegen Medical Centre). Labeling of this conjugated annexin with Tc-99m was performed in the morning of administration (Department of Nuclear Medicine, Radboud University Nijmegen Medical Centre) as previously described.¹⁶

This forearm ischemia-reperfusion model detects the loss of membrane asymmetry which results from phosphatidylserine (PS) exposure on the outer membrane leaflet.¹⁵ Phosphatidylserine exposure occurs shortly after an ischemic insult as an early, reversible, sign of cell injury.^{17,18} Sustained exposure of phosphatidylserines is involved in cell death.^{19,20} By labeling recombinant annexin A5 with the γ -emitter Tc-99m, it is possible to visualize phosphatidylserine exposure by gamma camera imaging. Annexin targeting after forearm ischemia mimics infarct size in animals as it behaves similarly in response to various interventions.^{15,16}

Study 3: The Effect of Caffeine on Rosuvastatin-Induced Forearm Protection Against Ischemia-Reperfusion Injury

Twenty-four volunteers received 20-mg rosuvastatin per day for 7 days and were randomly divided in 2 groups to receive either intravenous normal saline or caffeine (4 mg/kg in ten minutes; Genfarma) in a double-blind parallel design. Intravenous caffeine or normal saline were administered 45 minutes before ischemic exercise. One hour after the last intake of rosuvastatin, ischemic exercise was performed and injury quantified as described for study 2.

Analytic Procedures

In all volunteers, blood was collected before and at the end of oral treatment to determine fasting serum lipid profile (total-, HDL-, and LDL-cholesterol and triglycerides), creatinine kinase (CK), and alanine amino transferase (ALAT) with a commercially available kit (Aeroset, Abbott). Compliance to caffeine abstinence was monitored by determination of plasma caffeine concentration before each experiment. Plasma caffeine concentrations were determined by use of reversed-phase HPLC with UV detection set at 273 nm according to Schreiber-Detumeny and Bruguerolle.²¹

Statistical Analysis

Study 1

Computer-assisted forearm blood flow analyses were performed before unblinding. To correct for random changes in FBF unrelated to the intervention the ratio of simultaneously measured FBF in intervention and control arm was calculated (FBF-ratio). The FBF-ratios of the last 4 minutes of reference measurements (during intraarterial saline or saline plus caffeine as appropriate) and last 2 minutes of each dipyridamole, SNP, or adenosine dose-step were averaged to 1 value. Peak FBF-ratio during postocclusive reperfusion periods was used to determine the effect of rosuvastatin treatment on postocclusive reactive hyperemia. Results are expressed as percentage increase in FBF-ratio from reference measurements. An ANOVA for repeated measures was used to determine treatment related changes in the vasodilator response to dipyridamole with and without caffeine (primary end point) and differences in the vasodilator response to SNP, adenosine, and arterial occlusion (secondary end points) between groups.

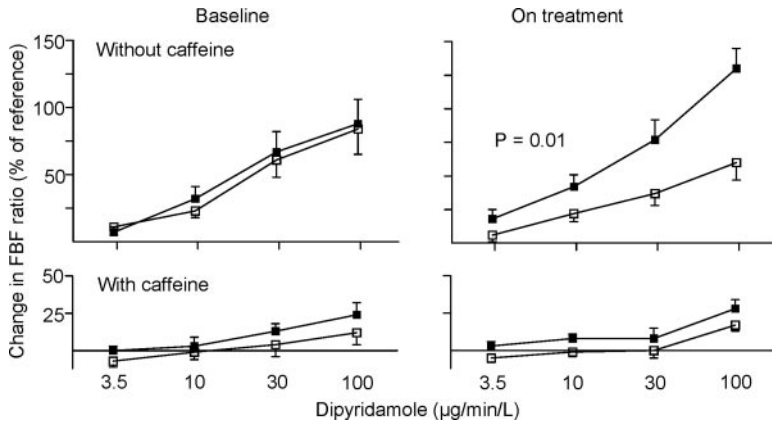


Figure 1. Dipyridamole-induced forearm vasodilation in the absence and presence of caffeine before (baseline) and on treatment in placebo arm (open squares) and rosuvastatin arm (closed squares). Data are expressed as mean±SE. Probability value indicates significant difference between placebo and rosuvastatin arm. Caffeine significantly reduced the vasodilator response to dipyridamole in both treatment arms ($P<0.01$). FBF indicates forearm blood flow; FBF-ratio, FBF experimental arm/FBF control arm.

Study 2 and 3

A predefined region of interest was identified for each hand representing the thenar muscle. Within this region of interest radioactivity was expressed as counts per pixel. Annexin A5 targeting after ischemic exercise was calculated as the percentage difference in radioactivity between the experimental and control hand. The effect of rosuvastatin on annexin A5 targeting and the interaction between rosuvastatin and caffeine was analyzed with an ANCOVA for repeated measures with rosuvastatin and caffeine treatment as between subject factors and workload as a covariate. Workload was defined as the product of 50% of the maximal voluntary force and duration of ischemic exercise. For this analysis, we combined both studies by pooling 2 study arms (10 placebo, 22 rosuvastatin, and 12 rosuvastatin/caffeine treated individuals).

Paired Student *t* tests were used to detect treatment-related changes in lipid profile within groups (secondary end point). All results are expressed as mean±SE, and a 2-sided probability value <0.05 was considered statistically significant.

Results

Study 1: Rosuvastatin and Vasodilator Response to Dipyridamole

Before unblinding, 3 volunteers were excluded (2 in the rosuvastatin group, 1 in the placebo group), leaving 11 and 10 evaluable subjects in the placebo and rosuvastatin-treated arms, respectively. Reasons for exclusion were a defective temperature control in the climate room ($n=1$), development of a common cold with a fever during the treatment period ($n=1$), and elevated plasma caffeine concentrations during all visits ($n=1$; ≥ 2.0 mg/L). In 1 volunteer, on treatment with rosuvastatin, placement of the arterial needle failed on day 8. Therefore, responses to intraarterial SNP and adenosine were only available for 9 volunteers in the rosuvastatin-treated arm.

The baseline vasodilator response to dipyridamole did not differ between the 2 treatment arms (Figure 1). Rosuvastatin significantly reduced fasting plasma total- and LDL-cholesterol (supplemental Table I, available online at <http://atvb>.

ahajournals.org). Rosuvastatin treatment did not influence reference FBF-ratios nor the course of FBF in the control arm between groups (data not shown). Rosuvastatin treatment significantly increased the vasodilator response to dipyridamole compared to placebo (Figure 1; $P=0.01$). When analyzing both groups separately, rosuvastatin augmented dipyridamole-induced vasodilation as compared to baseline ($P=0.01$), whereas placebo treatment did not significantly alter this response. In the presence of caffeine, the vasodilator response to dipyridamole was significantly reduced and not significantly influenced by treatment with rosuvastatin (Figure 1).

Treatment with rosuvastatin did not significantly affect the vasodilator response to either SNP or adenosine. Rosuvastatin significantly increased peak reactive hyperemia after 2, 5, and 13 minutes of arterial occlusion compared to placebo (Figure 2).

Study 2 and 3: The effect of Rosuvastatin and its Interaction With Caffeine on Forearm Ischemia-Reperfusion Injury

Treatment with rosuvastatin significantly reduced fasting total cholesterol and LDL-cholesterol in both caffeine-treated and -untreated study arms (supplemental Table I). Rosuvastatin significantly reduced annexin A5 targeting: 21 ± 3 and $25\pm 3\%$ in the placebo group versus 16 ± 1 and $18\pm 2\%$ in the rosuvastatin group. In rosuvastatin-treated volunteers who received caffeine intravenously the reached plasma concentration just before the ischemia-reperfusion protocol was 7.5 ± 0.3 mg/L. In these volunteers annexin A5 targeting tended to be higher as compared to rosuvastatin-treated individuals who did not receive caffeine: 22 ± 2 and $22\pm 1\%$ ($P<0.07$), thus reducing the effect of rosuvastatin (Figure 3).

Adverse Events

None of the volunteers reported side effects of treatment with rosuvastatin or placebo. In 3 subjects (1 in study 2, 2 in study

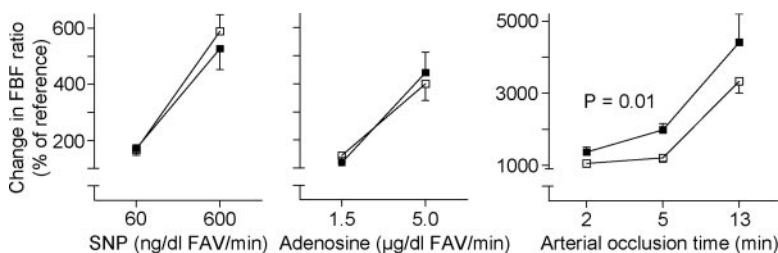


Figure 2. The forearm vasodilator response after 8 days of treatment with either placebo (open squares) or rosuvastatin (closed squares), to SNP (sodium nitroprusside), adenosine, and arterial occlusion. Data are expressed as mean±SE. Probability value indicates significant difference between placebo and rosuvastatin arm. FBF indicates forearm blood flow; FBF-ratio, FBF experimental arm/FBF control arm.

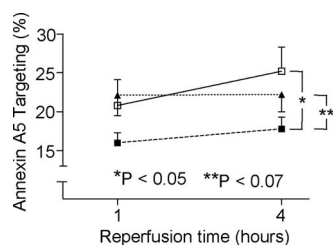


Figure 3. Annexin A5 targeting after ischemic exercise. Ischemic exercise was performed after a 24-hour abstinence from caffeine containing beverages. Subjects had been randomly allocated to a 1-week treatment with placebo (open squares), rosuvastatin (closed squares), or rosuvastatin in combination with a caffeine infusion 45 minutes before the ischemic exercise (closed triangles). Data are expressed as mean \pm SE. Rosuvastatin significantly reduced annexin targeting. This action was inhibited by intravenous caffeine.

3), all treated with rosuvastatin, a moderate (more than twice the upper limit of normal) increase in CK was observed which remained below 4000 U/L and returned to normal after the study.

Discussion

This study indicates that rosuvastatin increases extracellular adenosine formation and that this action may be involved in the protection by rosuvastatin against ischemia-reperfusion injury in humans *in vivo*. This conclusion is based on 2 key-findings: (1) rosuvastatin increases dipyridamole-induced vasodilation without affecting adenosine-induced vasodilation, and (2) rosuvastatin reduces targeting of annexin A5 after ischemic exercise in the absence but not in the presence of caffeine.

Rosuvastatin Increases Dipyridamole-Induced Vasodilation

During normal physiological circumstances the transmembrane concentration gradient drives extracellular adenosine into the cytosol.^{22,23} Dipyridamole inhibits this facilitated diffusion in humans *in vivo* and thereby increases extracellular adenosine concentration, which subsequently activates adenosine receptors at sites of adenosine formation.^{13,14,24–26} These observations justify the use of dipyridamole-induced vasodilation as a read-out of extracellular adenosine formation. Thus, if statins increase extracellular adenosine formation, this should result in a caffeine-sensitive augmentation of dipyridamole-induced vasodilation. Our results proved this hypothesis to be correct. Rosuvastatin did not significantly affect the vasodilator response to adenosine in this group of volunteers. This excludes an effect of rosuvastatin on adenosine clearance, adenosine receptors, or postreceptor signaling as a potential explanation for our observations. Furthermore, the lack of effect of rosuvastatin on SNP-induced vasodilation excludes nonspecific actions of rosuvastatin on the vasodilator capacity of forearm vascular resistance vessels. Therefore, the augmentation of dipyridamole-induced vasodilation is explained by an increase in extracellular formation of adenosine by rosuvastatin. Rosuvastatin enhanced postocclusive hyperemia, and this effect may be implicated in statin-mediated protection against ischemia-reperfusion injury. Further studies are needed to confirm the

involvement of adenosine receptors in this action of rosuvastatin.

Rosuvastatin Reduces Targeting of Annexin A5 After Ischemic Exercise in the Absence but not in the Presence of Caffeine

In this project, we used annexin A5 scintigraphy to investigate the effect of rosuvastatin therapy on ischemia-reperfusion injury. In addition, caffeine was used to reveal a role of adenosine receptor stimulation in the observed benefit.

Like previously demonstrated in the heart,²⁷ treatment with a statin reduced ischemia-reperfusion injury in our forearm model. In addition, our study reveals a potential role for endogenous adenosine in this protective action of statins. We have previously reported that caffeine does not affect annexin A5 targeting after ischemic exercise in the absence of rosuvastatin-pretreatment.¹⁶ Therefore, any effect of caffeine on annexin targeting in the current study indicates a specific interaction with rosuvastatin-pretreatment. Because plasma lipids did not differ between the subjects treated with rosuvastatin + caffeine as compared with those treated with rosuvastatin alone, the partial return of annexin targeting after rosuvastatin treatment in response to caffeine suggests that rosuvastatin-induced protection against ischemia-reperfusion injury is not explained by its effect on plasma lipids alone. Because we powered our study presuming caffeine to completely neutralize the effect of rosuvastatin, the marginal probability value is probably caused by an insufficient sample size.

Implications of Our Observations

Several trials have shown an additional benefit of aggressive lipid lowering as a consequence of more effective inhibition of HMG-CoA reductase, resulting in reduced progression or even retardation of atherosclerosis.²⁸ In contrast, the recently published ENHANCE-trial failed to show such an effect on IMT for the cholesterol absorption inhibitor on top of a statin, despite additional reduction of LDL-C.²⁹ This discrepancy suggests that it may not be the additional reduction of LDL-C, but other so called pleiotropic actions of statins that are responsible for the observed incremental benefit of intensified statin treatment. Upregulation of ecto-5'-nucleotidase and subsequent increases in extracellular adenosine formation may be one of these. Indeed, studies in ecto-5'-nucleotidase-deficient mice indicate an important role for this enzyme in the prevention of vascular inflammation and subsequent atherosclerosis formation in wild-type mice.⁸

Besides reducing atherosclerosis, statins also limit ischemia-reperfusion injury. The ARMYDA-ACS trial showed that this effect occurs within 12 hours in patients undergoing percutaneous coronary intervention,²⁷ and further disproves LDL-C as the sole target of statin therapy. Blockade of mevalonate formation, the principle action of HMG-CoA reductase inhibitors, not only reduces cholesterol synthesis in the liver but also reduces levels of isoprenoid derivatives which play an important role in isoprenylation of small GTPases of the Rho/Rac/Cdc42 family such as RhoA. This action of statins has recently been implicated in their protection against ischemia-reperfusion injury in kidney, heart, and

brain,^{30–32} possibly by activating 5'-ecto-nucleotidase.⁶ In concurrence, animal experiments have shown an acute effect of statins on ischemia-reperfusion injury by increased adenosine receptor stimulation.⁵ Increased availability of adenosine enlarges the reflow area after an ischemic event, which reduces the area with ongoing ischemia during reperfusion. Moreover, release of adenosine and subsequent stimulation of adenosine receptors are essential in mediating ischemic preconditioning,^{16,33,34} which increases cellular resilience to ischemia-reperfusion injury.³⁵ In the present study, caffeine, in a concentration reached after 2 to 3 cups of coffee,^{36,37} attenuated protection by rosuvastatin against sequelae of ischemic exercise. This is in line with previous studies in the rat and dog heart.^{5,38} Therefore, we claim that adenosine plays a key role in statin-induced increased tolerance to ischemia-reperfusion injury in humans in vivo.

The effect of statins on adenosine formation opens a new window for optimizing their clinical effect. Indeed this has been demonstrated in animals in which the combination of a low dose of atorvastatin and dipyridamole reduced ischemia-reperfusion injury where the individual agents did not.³⁹ Although the influence of daily caffeine consumption on statin induced protection has not been investigated in a clinical setting, the combined use of caffeine and statins will be regularly encountered. Despite lingering controversy about the effect of coffee consumption on cardiovascular disease in the general population, specific subgroups of patients may be prone to the adverse effect of adenosine receptor blockade.⁴⁰ In particular, our results predict that in cardiovascular patients the short-term use of caffeine will reduce the therapeutic action of statins.

In conclusion, rosuvastatin increases extracellular adenosine formation. Increased adenosine formation as a result of statin treatment likely contributes to its protection against ischemia-reperfusion injury. Preclinical evidence from the literature suggests that this action may also be involved in the prevention of atherosclerosis by statins. Future studies will have to focus on the optimal exploitation of this property of statins in patient care. This may ultimately lead to improved tailoring of drug therapy to patients at risk for cardiovascular events in which the combination of rosuvastatin and dipyridamole may provide synergistic benefits.

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Disclosures

None.

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