Rosuvastatin Reduces Neointima Formation in a Rat Model of Balloon Injury

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Abstract

Background: Processes of restenosis, following arterial injury, are complex involving different cell types producing various cytokines and enzymes. Among those enzymes, smooth muscle cell-derived matrix metalloproteinases (MMPs) are thought to take part in cell migration, degrading of extracellular matrix, and neointima formation. MMP-9, also known as gelatinase B, is expressed immediately after vascular injury and its expression and activity can be inhibited by statins. Using an established in vivo model of vascular injury, we investigated the effect of the HMG-CoA reductase inhibitor rosuvastatin on MMP-9 expression and neointima formation.

Materials and Methods: 14-week old male Sprague Dawley rats underwent balloon injury of the common carotid artery. Half of the animals received rosuvastatin (20 mg/kg body weight/day) via oral gavage, beginning 3 days prior to injury. Gelatinase activity and neointima formation were analyzed 3 days and 14 days after balloon injury, respectively. 14 days after vascular injury, proliferative activity was assessed by staining for Ki67.

Results: After 14 days, animals in the rosuvastatin group showed a decrease in total neointima formation (0.194 ± 0.01 mm^2 versus 0.124 ± 0.02 mm^2, $p<0.05$) as well as a reduced intima/media ratio (1.26 ± 0.1 versus 0.75 ± 0.09, $p<0.05$). Balloon injury resulted in increased activity of MMP-9 3 days after intervention for both rosuvastatin treated animals and controls with no significant difference observed between the groups. There was a trend towards a reduction in the number of Ki67-positive cells 14 days after injury.

Conclusions: Rosuvastatin attenuates neointima formation without affecting early MMP-9 activity in a rat model of vascular injury.

Introduction

Arterial restenosis following balloon angioplasty remains a common clinical limitation even after stent implantation [1]. This effect is mainly caused by accelerated neointima formation due to smooth muscle cell migration and proliferation after endothelial damage [2]. Several pathophysiological mechanisms have been discussed. Some studies suggest profuse vascular wound healing due to adjacent endothelial cells and endothelial progenitors [3, 4]. Others have demonstrated a TGF-β-dependent mechanism of constrictive arterial remodeling and formation of intimal thickening composed of smooth muscle cells [5]. In part, vascular remodeling is also induced by the release of SMC matrix degrading enzymes, such as matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases (TIMP) [6, 7]. In particular, MMP-9 is upregulated following vessel injury [8]. Inhibition of MMPs resulted in reduced SMC proliferation and inhibition of neointimal hyperplasia [9, 10]. Since it is known that hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are beneficial in primary and secondary prevention of cardiovascular disease, and that their pleiotropic effects are independent of their lipid-lowering potential, some studies have focused on the effect of statin therapy on prevention of restenosis [11-17]. Several in vitro and in vivo studies demonstrated reduction of MMP-9 expression following statin treatment [19, 20].

Recently, it has been demonstrated that the potent HMG-CoA reductase inhibitor rosuvastatin reduces neointima formation in a hypertensive rat model [16]. Furthermore rosuvastatin promotes bone-marrow dependent re-endothelialization and reduces neointima formation in mice [21].

Our study evaluated the effect of rosuvastatin on MMP-9 expression and neointima formation in a rat model of vascular injury.

Materials and Methods

Animals and Drug Treatment

Twenty two male Sprague Dawley rats (380 – 420g; Charles River Laboratories, Sulzfeld, Germany) at the age of 14 weeks were kept within the animal care facility of the University of Heidelberg. Animals received free water and food (standard chow diet) ad li-

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bitum in a temperature controlled room with a 12-hour light/dark cycle. Animals were randomly divided into rosuvastatin treated group and controls. Starting three days prior to balloon injury, half the rats received rosuvastatin (20mg/kg bodyweight / day) via oral gavage. A number of the rats were euthanized 3 days after balloon injury for immunoblot and gelatinolytic assay. For the evaluation of neointima formation, additional rats were euthanized 14 days after injury. Housing and care of animals, and procedures carried out in this study were performed in accordance with the guidelines and regulations composed by the Animal Care Committee of the University of Heidelberg.

Balloon Injury

Rats were anesthetized by intraperitoneal injection of ketamine (100mg/kg body weight) and xylazine (15mg/kg bodyweight). After midline incision of the neck, the left external carotid artery was exposed and a 2F balloon catheter (Baxter Healthcare) was introduced up to the aortic outlet of the common carotid artery. The balloon was then inflated with saline solution until a slight resistance was felt. While rotating, the balloon was pulled back through the common carotid artery to denude the endothelium. This procedure was repeated twice. Afterwards, the catheter was removed, the external carotid artery ligated and the wound was closed. Contralateral right arteries served as uninjured controls. Three and 14 days after surgery rats were anaesthetized as described previously, blood was collected from the inferior vena cava, stored for further use, and animals were euthanized by exsanguination. Rats were perfused with 20 ml phosphate-buffered saline solution at physiological pressure via left ventricle. Left and right common carotid arteries were excised, adhering connective tissue was removed and stored at -80°C.

Determination of Plasma Lipid Levels

Total serum cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were determined enzymatically in heparinized plasma.

Morphometric Analysis of Neointima Formation / Immunohistochemistry

Common carotid arteries were serially sectioned (5 μm) and stained with hematoxylin-eosin (HE). Sections were evaluated blind using computer-assisted morphometry (Olympus Mikroskopie Hamburg, Germany and Image Pro, Media Cybernetics, Silver Spring, USA). The size of the neo-intimal layer was determined as the area between internal elastic lamina and the luminal circumference (data expressed in mm^2). Furthermore, the total number of cells was evaluated by counting nuclei. In addition, neo-intima/media ratios were calculated by a blinded observer.

To determine proliferation and endothelial integrity, serial sections were stained for Ki67 with a rabbit polyclonal antibody (abcam, Cambridge, UK), a rabbit von Willebrand factor antibody (Dako, Carinteria, CA, USA) and a directly FITC conjugated α-smooth muscle actin antibody (Sigma-Aldrich, St. Luis MO, USA). Proliferation index was calculated as number of Ki67-positive cells / total amount of cells.

Protein Extraction for Western Blot and Gelatinolytic Assay

For gelatinolytic assay, frozen arteries were homogenized with lysis buffer (50 mmol/l Tris-HCL, pH 7,6; 150 mmol/l NaCl; 5 mmol/l CaCl$_2$; 0,05% Brij-35; 0,02% NaN3; 1% Triton X-100). Lysates were sheared by ultrasonic disruption, and centrifuged at 14.000 r.p.m at 4°C for 10 min. Supernatants were used as whole cell lysates and protein concentration was determined using the Bradford method (Bio-Rad Protein Assay).

Gelatinolytic Assay

Ten μg protein were used to analyze vessel tissue gelatinase activity. Gelatinase activity was detected in a 10% gelatine gel (Invitrogen, Carlsbad, USA) using SDS running buffer (all chemicals Invitrogen, Carlsbad, USA). After electrophoresis, gels were incubated with zymogram renaturing buffer for 30 min and developed in zymogram developing buffer for 12 hours. Gels were stained with 0.125% Coomassie blue R-250 for 30 min in 10% acetic acid and 50% methanol. Finally, gels were destained with a solution containing 10% acetic acid and 10% methanol until clear bands of gelatinolysis appeared on a dark blue background. The molecular weight of the gelatinolytic bands was estimated by comparison with prestained molecular weight markers (Bio-Rad, Munich, Germany). Gels were dried and scanned by densitometry.

Western Blotting

Ten μg of protein were seperated by 10% SDS-PAGE Gel (Invitrogen, Carlsbad, USA) and transferred to nitrocellulose membranes (Schleicher und Schuell, Germany). After blocking (5% nonfat milk powder dissolved in TBST) the membrane was incubated with primary antibody (polyclonal goat anti rat MMP-9, dilution 1:2000) overnight at 4°C, washed three times with TBST, followed by incubation with a secondary antibody for 1 hour at room temperature. After washing, bands were visualized with ECL (Amersham Biosience, USA), recorded on hyperfilm (Amersham) and quantified by densitometry (Bio-Rad). Antibodies, used in this study, were purchased from Santa Cruz Biotechnology, Santa Cruz, USA.

Statistical Analysis

All data were expressed as mean ± SEM or as mean ± SD, as indicated. Significant differences between means in plasma lipid profiles were determined with the two-tailed unpaired student`s t-test, differences in neointima size using the Mann-Whitney u-test. A p-value <0.05 was considered statistically significant.

RESULTS

EFFECT ON PLASMA LIPID LEVELS

At the time of euthanasia, animals showed no significant differences in total cholesterol, HDL and LDL cholesterol. Furthermore, there were no differences in bodyweight (Table 1).

MORPHOMETRICAL ANALYSIS

Compared to the controls, treatment with rosuvastatin lead to a decrease of neointima area (0.194 ± 0.01 mm^2 versus 0.124 ± 0.02 mm^2, $p<0.05$) and intima/media ratio (1.26 ± 0.1 versus 0.75 ± 0.09, $p<0.05$) 14 days after injury (Fig. 1). Total number of cell nuclei within the neointima was also significantly reduced in the arteries of rosuvastatin-treated rats, as compared to control animals (3950 ± 263 versus 5262 ± 248, $p<0.01$).

PROLIFERATION / INTEGRITY OF ENDOTHELIAL LAYER

Ki67 staining was used to determine proliferation. There was a trend towards a reduction in the number of Ki67-positive cells in rosuvastatin-treated animals (2.1 ± 0.17 versus 1.6 ± 0.13; $p = 0.058$). However, the difference did not reach statistical significance (Fig. 2 AB).

Two weeks after injury, arteries still showed incomplete re-endothelialisation, as evaluated by staining with an immunofluorescence-antibody against von Willebrand factor and α-smooth muscle actin. No significant differences between treatment groups could be detected (data not shown, Fig. 2 C).

GELATINOLYTIC ACTIVITY

Balloon injury of rat arteries upregulated expression of a 92-kD band in the gelatin zymography 3 days after injury. No signal could be detected in the non-denudated contralateral vessels (right CCA), which suggests that these 92-kD proteins are induced by injury. This band corresponds to a form of rat 95-kD type IV collagenase/gelatinase (MMP-9) [21]. However no difference could be detected between rosuvastatin treated and control rats (3754 ± 1074 versus 3488 ± 1816; Fig. 3A).

WESTERN BLOTTING

MMP-9 protein expression in CCA was determined by Western blot in rosuvastatin treated rats and controls. Balloon injury resulted in an induction of MMP-9 ex-pression in arteries 3 days after de-endothelialization. Contralateral vessels in the same rats showed no induction of MMP-9. Again, there was no significant difference between rosuvastatin treated and control animals (Fig. 3B).

DISCUSSION

Percutaneous coronary interventions (PCI) are widely used for treatment of coronary heart disease. However, long-term benefit is often limited by restenosis, occurring in about 30% of patients following bare metal stent implantation [23]. Introduction of drug eluting stents has decreased the incidence of restenosis significantly but is associated with several problems such as stent thrombosis [24].

Remodeling processes of the extracellular matrix by MMPs are essential for neointima formation in postangioplasty restenosis as well as in aortocoronary vein graft vasculopathy [8, 25]. HMG-CoA reductase inhibitors are known to suppress MMP-expression and are therefore a potential treatment option in restenosis prevention [19, 20, 26].

Using a standard animal model of vascular injury we were able to demonstrate a significant reduction of neointima formation in rats treated with rosuvastatin. These data are in accordance with other studies in rats and mice, whereas Kappert et al. (2006) only observed an increase in luminal circumference but no difference in total neointima size, nor in intima/media ratio [16, 17, 26]. Prevention of restenosis by HMG-CoA reductase inhibitors might be dose-dependent. Kappert et al. (2006) used rosuvastatin in a dose of only 1 mg/kg/d whereas we and van de Harst (2006) administered rosuvastatin in a dose of 20 mg/kg/d, a dose previously demonstrated to inhibit hepatic HMG-CoA reductase by 90% in rats [16, 17, 18].

It has been demonstrated that vascular injury causes early upregulation of matrix metalloproteinase-2 (MMP-2, gelatinase A) and matrixmetalloproteinase-9 (MMP-9, gelatinase B) and therefore might play an important role in SMC migration [8]. Using balloon de-endothelialization, an effective method for causing severe vascular damage, we showed that gelatinase B activity was upregulated 3 days after intervention. However, rosuvastatin treatment had no effect on expression or gelatinolytic activity of gelatinase B after balloon injury. Therefore, prevention of restenosis by rosuvastatin must act via an MMP-independent mechanism.

Consistent with other studies, we found Ki67-positive cells within the neointima of all animals 14 days after injury. There was a trend towards reduced

Table 1. Body weight and lipid profile. No significant difference in levels of total cholesterol, HDL and LDL as well as body weight could be detected.

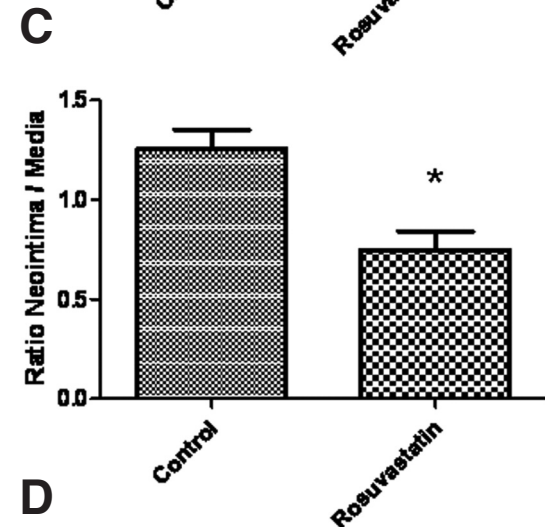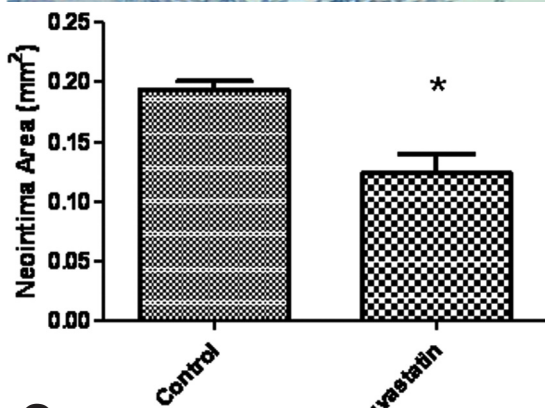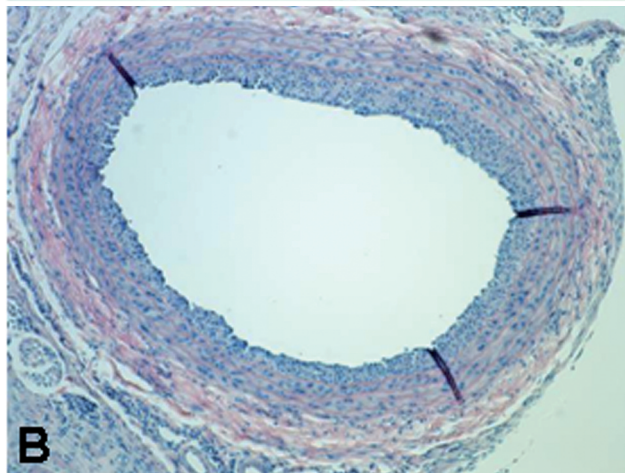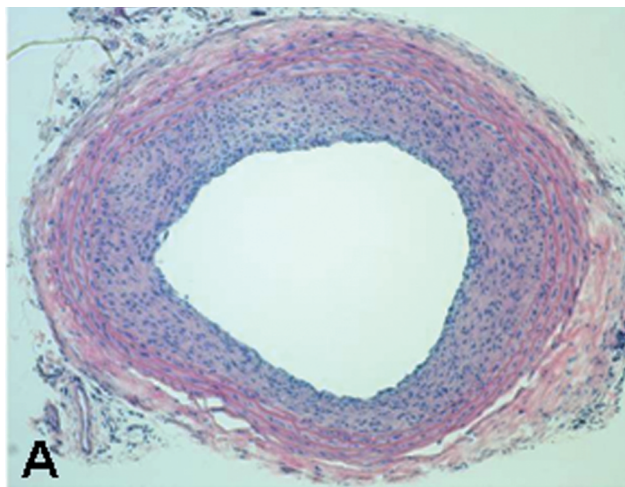|	n	Body Weight (gram)	Total Cholesterol (mg/dl)	LDL Cholesterol (mg/dl)	HDL Cholesterol (mg/dl)	Triglycerides (mg/dl)
Rosuvastatin	10	416 ± 23	49 ± 14	23 ± 9	15 ± 5	52 ± 25
Control	10	409 ± 18	49 ± 4	23 ± 3	14 ± 3	71 ± 23

Mean $\pm$ SD
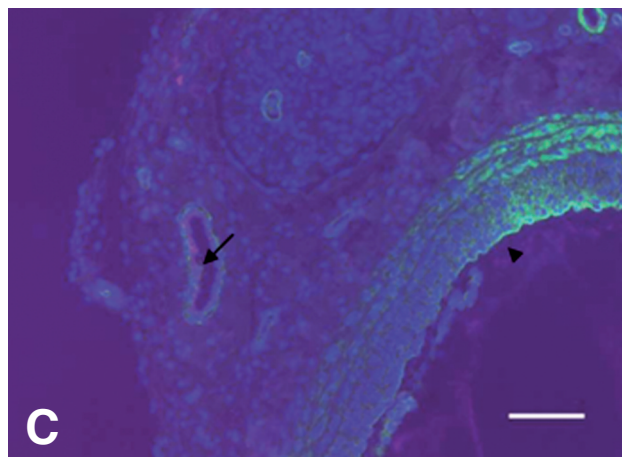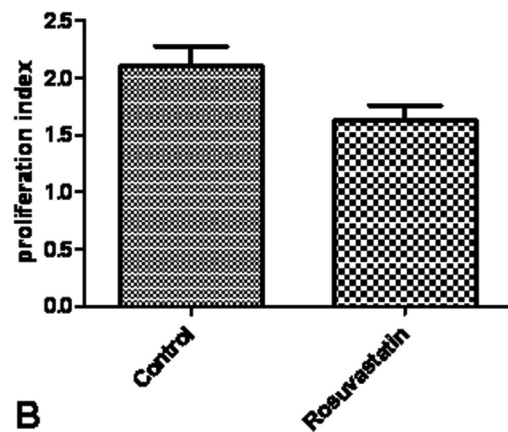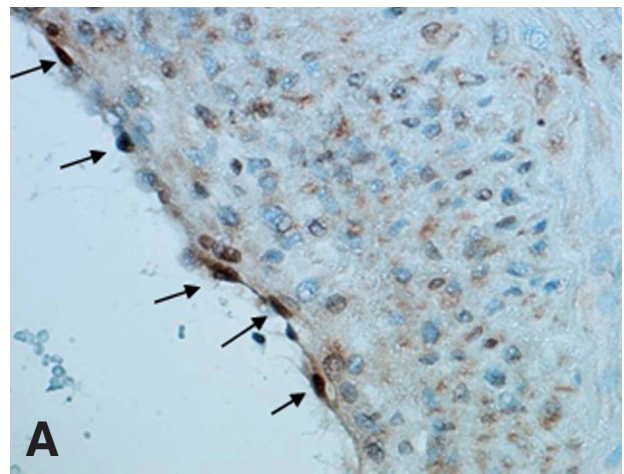
Fig. 2. Ki67-positive cells (arrows) within the neointima at 14 days after injury of a control animal. (A) Rosuvastatin treatment tended to reduce the proliferation index within the neointima (number of Ki67-positive cells / total amount of cells). However at day 14 no significant difference could be detected (p=0.058). (B) Staining with immunofluorescence-labeled antibody against von Willebrand factor (secondary antibody alexa 594 in red) and _-smooth muscle actin in FITC (green) demonstrated that reendotheliazation was not completed 14 days after injury (arrow head) (_-smooth muscle actin in FITC, green). An adventitial artery is shown as a positive control (arrow). (C) Scale Bar = 100 μm.

Fig. 1. Neonintima formation of the common carotid artery (hematoxylin / eosin staining) 14 days after balloon injury in a control animal (A) and an animal treated with rosuvastatin. (B) Treatment with rosuvastatin leads to a reduction of neointima size, as well as a reduction of neointima / media ratio. (C, D).
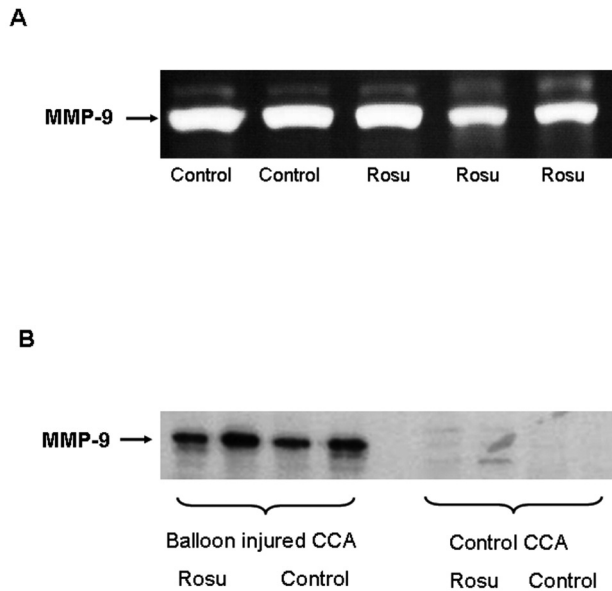
A

B

Fig 3. Gelatinolytic activity assay demonstrated an induction of 92-kD gelatinase B three days after balloon injury in rat common carotid artery. No difference between rosuvastatin treated and controls could be detected (A). Western blot confirmed MMP-9 expression in rat common carotid arteries three days after balloon injury. No significant expression could be detected in non-injured contralateral arteries, which served as controls (B).

expression in the rosuvastatin-treated group, compared to controls. Proliferation after arterial injury is commonly described as an early event which reaches a maximum within the intimal layer between day four and seven [27]. It is possible that the observed trend in our study would reach statistical significance at an earlier time point, as evaluated in the present study. Other studies also demonstrated a strong association between severe arterial injury with disruption of the internal elastic lamina (IEL) and expression of Ki67. Thus, variability in severity of injury between different studies may also contribute to differences in time course of proliferation [28]. Consistent with other studies, proliferating cells are nearly exclusively located in the luminal surface. Recently published data demonstrated antiproliferative effects of Rosuvastatin in an ex-vivo model of balloon injury via urokinase-type plasminogen activator receptor (uPAR) [29]. Furthermore, data acquired by our group and others showed inhibitory effects of statins on the activity of the transcription factors Egr-1, NF-kappaB, and AP-1 [30, 31].

The adjacent cells of the intact vascular wall are mainly responsible for wound repair after injury [3]. Recent data support the possibility that bone marrow-derived cells, circulating in the blood stream contribute to neoangiogenesis and accelerated re-endothelialisation leading to a reduction of neointima formation [32, 33]. Werner et al. (2002) demonstrated the beneficial effects of endothelial progenitor cells in a mouse model of carotid injury: rosuvastatin treatment accelerated re-endothelialisation and significantly reduced neointimal formation [21]. However, it remains unclear whether this is due to a direct effect of statin

therapy or due to a statin-mediated increase in the number of circulating, immature, endothelial cells [34]. We observed a lack of re-endothelialisation at 14 days after injury that was not affected by statin treatment. Differences in endothelial recovery between studies can be explained by variable distances between untraumatized endothelium and denuded areas, since regeneration, at least in part, starts from the adjacent non-injured endothelial layer [35]. It has been reported that even as late as six weeks after injury, some areas of the artery still remained without endothelium [36].

New data furthermore have demonstrated an association between statin treatment and the reduced risk of venous thrombosis which implies an effect of HMG-CoA reductase inhibition and the coagulation system [31, 32]. Studies demonstrated a statin-induced inhibition of the procoagulatory tissue factor and even proposed tissue factor activity as a risk predictor of restenosis after PCI and stent implantation [37, 38, 39, 40].

Clinical studies have suggested that lipid-lowering by statins protects from restenosis after coronary stenting [41, 42]. It has been shown that rosuvastatin significantly decreased LDL, cholesterol, and triglyceride levels and that high-dose rosuvastatin treatment was more effective than similar doses of atorvastatin in lowering direct LDL cholesterol [43]. In contrast, our model, at the time of necropsy, showed no differences in total cholesterol, LDL, or HDL between the groups which also confirms a cholesterol-independent effect on neointima formation in our in vivo-model. The lack of a cholesterol-lowering effect of statin treatment in rodents has been investigated in several studies and seems to be very common, even at higher doses [44, 45]. The mechanisms how statin treatment of apoE-/- mice even causes an increase in serum cholesterol, as observed in some studies, is still unclear [46]. One possible explanation is that continuous administration of statins via chow even stimulates expression and/or activity of HMG-Co A reductase in the liver of treated mice.

In conclusion, our study demonstrates that rosuvastatin inhibits neointima formation after balloon injury and it supports the hypothesis that this effect is MMP-9 independent.

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