Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model
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CHAPTER 5

Rosuvastatin attenuates angiotensin II induced neointimal formation after stent implantation in the rat


ABSTRACT

Aim: Drawbacks of current drug-eluting stents include inhibition of reendothelialization, induction of abnormal coronary endothelial function, and, most important, late in-stent thrombosis. Statin treatment might be a more subtle approach, with known beneficial vascular effects. We investigated the efficacy of oral rosuvastatin treatment to reduce in-stent neointimal formation, both in the absence and presence of high levels of the pro-proliferative substance Angiotensin II (Ang II). Methods: Wistar rats were allocated to 4 treatment groups by two consecutive randomization steps: one to allocate rosuvastatin 0.047% (wt/wt) supplemented rat chow, and one to implant an osmotic minipump releasing Ang II (200ng/kg). Stents were implanted in the abdominal aorta in all groups. After 4 weeks, in-stent neointimal formation and vascular function in the thoracic aorta was determined. Results: In the absence of Ang II, rosuvastatin reduced neointimal formation by 23% as compared to control (0.66±0.06 versus 0.51±0.02 mm²; P<0.05). The presence of Ang II enhanced neointimal area by 30%. This was inhibited to the same extent by rosuvastatin (0.88±0.06 mm² versus 0.67±0.03; P<0.05). In parallel, rosuvastatin improved endothelial dependent vasodilatation, both in the presence and absence of high levels of Ang II. Conclusions: Ang II infusion increases in-stent neointimal formation and decreases endothelial function. We now provide evidence that rosuvastatin effectively inhibits in-stent neointimal formation and in parallel improves endothelial dilator function, both in the presence and absence of high Ang II levels.
INTRODUCTION

Restenosis after stent implantation is mainly caused by neointimal formation: a pronounced hyperplasia of vascular smooth muscle cells that renarrows the vessel. This process is prompted and propagated by endothelial denudation, thrombus formation, and inflammation.(1) Stents coated with the cytostatic agents such as sirolimus and paclitaxel inhibit neointimal formation and reduce clinical restenosis.(2;3) Unfortunately, drawbacks with such stents include inhibition of reendothelialization(4;5), induction of abnormal coronary endothelial function distal to the site of sirolimus-eluting stents(6;7) and, most important, late in-stent thrombosis.(8;9) Now that the initial studies prove that drug-coated stents are promising, the search for more subtle pharmacotherapy has begun. To this end, the ubiquitously used group of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)-reductase inhibitors, or statins, might be an interesting drug class to study more specifically in a stent restenosis model. Although originally developed to reduce plasma cholesterol levels, the benefit of statins is additionally ascribed to pleiotropic effects, including improvement of endothelial function and reduction of tissue activation. The positive effect of statins on endothelial function in patients with coronary artery disease is well established.(10) (11)

With respect to tissue activation, statins might intervene in the pathophysiological processes of in-stent restenosis by several other mechanisms: statins reduce platelet activation, accelerate reendothelialization, and consequently reduce thrombus formation. Furthermore, statins attenuate inflammatory responses, reduce oxidative stress, and inhibit vascular smooth muscle cell migration and proliferation.(12) Within the plethora of activating processes, the inhibitory effects of statins on angiotensin II (Ang II) signalling, e.g. by downregulation of the Ang II type 1 receptor(13), represents a potentially beneficial pharmacological platform to intervene in-stent restenosis and endothelial dilator function. However, they have not been investigated in this context. We hypothesize that statin therapy inhibits in-stent restenosis and improves endothelial dilator function. Additionally, we hypothesize that statin therapy is effective in the presence of high levels of Ang II.

METHODS

Animal Protocol

40 male Wistar rats (Charles-River) each weighing 450 to 520 g received regular rat chow during a run-in period of 2-3 weeks. Thereafter, animals were randomly divided into 2 groups, one group continued to receive regular rat chow, the other group received the same chow, but supplemented with 0.047% (wt/wt) rosuvastatin, selected to deliver 20 mg/kg per day based on food intake, as described previously.(14) Rosuvastatin was provided by AstraZeneca. After one week of pre-treatment all rats were anesthetized with O₂, N₂O, and isoflurane (Abbot B.V.) and a premounted 2.5 x 8 mm bare metal stent (lekton motion petite, Biotronik) was implanted in the abdominal aorta as described previously.(15;16) In addition, both groups were divided into 2 subgroups of which one received an osmotic minipump subcutaneously with a pumping rate of 0.25 µL per hour lasting for 4 weeks (Model 2004; Alzet) to receive Angiotensin II (500 ng/kg per minute). Systolic blood pressures and heart rates were measured under anesthesia with an electrophysgmomanometer after rats were prewarmed for 20 minutes. Animals had free access to water and food. Food intake and body weight were monitored throughout the study.

After 4 weeks, animals were anesthetized and heparinized with 500 IU intravenously (Leo Pharma B.V.), euthanased without recovery, and abdominal aortas harvested, fixed, embedded in methylmetacrylate, sectioned, and stained for histological analysis. The endothelial function was tested in isolated thoracic aortic rings.(17) This study was approved by the animal care and use committee of
the University of Groningen and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

**Histology**
Histomorphometrical analysis was performed on Lawson stained (modified Elastica von Giesson) sections by measurements of the proximal, middle, and distal part of each stent. To assess neointimal formation, areas within the external elastic lamina, internal elastic lamina, and lumen were measured using digital morphometry. The neointimal area, media area, lumen area, and the percentage of stenosis were calculated.(18) A semiquantitative injury score was determined according to the method described by Schwartz et al.(18) Surface adherent leucocytes were counted at x400 magnification and expressed as cells/field. To assess a single measurement for each stent, the mean values of the proximal, middle, and distal sections were calculated.

**Organ Bath Studies With Isolated Aortic Rings**
Vascular measurements were performed as described previously.(17) In brief, periaortic tissue was removed from the aorta, and rings of 2 mm were cut. Rings were connected to an isotonic displacement transducer at a preload of 14 nmol/L in an organ bath containing Krebs solution, pH 7.5, containing (in mmol/L): 120.4 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 11.5 glucose, and 25.0 NaHCO₃ at 37°C and continuously gassed with 95% O₂ and 5% CO₂. After stabilization, during which regular washing was performed, rings were checked for viability by stimulation with phenylephrine (1 mmol/L).
Rings were washed and restabilized. Sets of rings were precontracted with phenylephrine (1 mmol/L). The endothelium-dependent vasodilation was assessed by a cumulative dose of methacholine (10⁻⁹ to 10⁻⁵ mmol/L). In parallel rings, endothelium-independent vasodilatation was assessed by a cumulative dose of nitroglycerine (10⁻⁹ to 10⁻⁵ mmol/L). Drugs were purchased from Sigma-Aldrich.

**N-terminal Atrial Natriuretic Peptide**
Concentrations of N-terminal atrial natriuretic peptide (N-ANP) in plasma were measured with commercially available radioimmunoassays from Biotop (Oulu, Finland) as described previously.(19)

**Statistical Methods**
Data are expressed as means ± SEM. Statistical analysis between groups was performed by a Student’s t test. Differences in dose-response curves between groups were tested by ANOVA for repeated measurements using Greenhouse–Geisser correction for asphericity. All P-values were two-tailed, and a P-value of <0.05 was considered statistically significant. All analyses were performed using SPSS version 12.0 software (SPSS, Chicago, IL, USA).

**RESULTS**
Determined from food consumption the average rosuvastatin intake was 25.9±2.24 mg/kg/day per day. Consistent with previous studies(20) chronic Ang II infusion significantly increased systolic blood pressure at 1, 2 and 4 weeks (1 week 133±4, 2 weeks 140±6, 4 weeks 165±9 mm Hg) compared to animals not receiving Ang II (1 week 104±3, 101±3, 98±4, all P<0.001 versus Ang II infusion). In addition, 4 weeks of Ang II infusion resulted in a significantly higher plasma level of the cardiac load marker N-ANP (1.69±0.18 in Ang II versus 1.19±0.08 in controls; P<0.05). We did not observe an influence of rosuvastatin treatment on either blood pressure or N-ANP levels.
Effect of rosuvastatin and Ang II on neointimal formation
A neointima was present in all stented animals after 28 days. Representative photomicrographs of stented abdominal aortas of the four groups are shown in figure 1. Injury score and stent expansion, expressed as the area within the internal elastic lamina (IEL), were equal among the four groups. Histomorphometrical data are presented in table 1.

Figure 1

Figure 1. Photomicrographs of Lawson–stained sections of rat abdominal aortas. A and B, aorta from control rat. C and D, from Ang II infused rat, G and H, aorta from an Ang II infused rat treated with rosuvastatin. (40 and 400×, respectively).

Furthermore, we did not observe differences in vascular media areas. Neointimal area was significantly decreased by 23% in rosuvastatin treated animals compared to controls (figure 2). In addition, neointimal thickness was significantly reduced by 19% in rosuvastatin treated animals (table 1). In the presence of Ang II, rosuvastatin treatment significantly decreased neointimal area by 24% (figure 2)
and neointimal thickness by 24% (table 1), both to a relatively similar degree as in the absence of Ang II. There was a non-significant reduction in the number of surface adherent leukocytes in rosuvastatin treated animals compared to controls and the number of surface adherent leukocytes was significantly decreased in animals treated with rosuvastatin-treated animals which were exposed to Ang II treatment compared to Ang II controls (figure 3).

The effects of Ang II infusion on vascular function with or without rosuvastatin treatment were examined in thoracic aortic rings. Precontractions to phenylephrine did not differ among groups. Rosuvastatin treatment resulted in significant improvement of endothelium-dependent vasodilatation both in the presence and in the absence of Ang II infusion (P<0.05; figure 4a). The relaxation after administration of endothelium-independent vasodilator nitroglycerine was equal in all groups (figure 4b).

**Table 1.** Histomorphometrical data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Stent (N=8)</th>
<th>Stent+ Rosuvastatin (N=8)</th>
<th>P</th>
<th>Stent+AngII (N=9)</th>
<th>Stent+AngII+ Rosuvastatin (N=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEL Area (mm²)</td>
<td>3.79±0.14</td>
<td>3.51±0.07</td>
<td>NS</td>
<td>3.59±0.07</td>
<td>3.52±0.09</td>
<td>NS</td>
</tr>
<tr>
<td>Media Area (mm²)</td>
<td>0.29±0.02</td>
<td>0.27±0.01</td>
<td>NS</td>
<td>0.30±0.02</td>
<td>0.32±0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Neointimal Thickness (um)</td>
<td>119.9±9.0</td>
<td>97.3±4.2</td>
<td>&lt;0.05</td>
<td>171.3±14.5</td>
<td>129.9±4.5</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The present study demonstrates that rosuvastatin reduces the development of neointimal formation after stent implantation in rats. Furthermore, Ang II infusion enhanced neointimal formation, which was also reduced by rosuvastatin treatment to a similar extent as in the absence of Ang II infusion. In parallel, Ang II increased surface adherent leucocytes and decreased endothelium dependent vasodilatation. Rosuvastatin decreased these surface adherent leukocytes and improved endothelium dependent vasodilatation. These results show that reduction of neointimal formation by rosuvastatin treatment is related to improvement of endothelial function. Although numerous studies on neointimal formation after balloon injury have been published, only limited information is available on the efficacy of statin treatment in reducing neointimal formation after stent implantation. Two previous reports have suggested that statin treatment reduces neointimal formation after stent deployment.(21;22) We used a different statin in a different animal model, but have obtained similar results. In addition, we studied statin treatment in the presence of high levels of Ang II, and assessed whether improvement of neointimal formation is paralleled by improvement of systemic endothelial functioning. The exact molecular mechanisms that underlie these associations cannot be deduced from the current or previous studies. It is conceivable that an improvement in endothelial function may have mediated the decreased neointimal formation.
Endothelial dysfunction is considered a systemic process and has been described as the next target in restenosis prevention. Both endothelial dysfunction and in-stent restenosis are pathophysiological processes involving an abnormal vascular response to injury. The integrity of the vascular wall, and especially the functioning of the endothelium, plays a key role in the response to injury and might provide a common pathway for endothelial dysfunction and in-stent restenosis. A recent study demonstrated that impaired systemic endothelial function of the brachial artery 30 days after PCI, independently predicted the occurrence of in-stent restenosis in patients. Restenosis after balloon injury has been associated with endothelial dysfunction and improvement of endothelial function by local administration of L-arginine, resulted in reduced neointimal thickening. Indeed, statins are also well known to improve endothelial functioning. This effect on endothelial function might be largely independent of LDL cholesterol lowering. Although we did not determine plasma lipid levels in the current study, 20 mg/kg rosuvastatin treatment (and other statins) do not affect plasma levels of total cholesterol, high-density lipoprotein, low-density lipoprotein, or triglycerides in rats. The link between endothelial function and restenosis might also be found at a systemic level. The concept of bone marrow-derived endothelial progenitor cells as a continuous source of endothelial cells to repair vascular injury is emerging. In mice, systemically applied endothelial progenitor cells home in to the site of vascular injury, resulting in the enhanced reendothelialization associated with decreased neointimal formation after balloon injury. Ang II is critically involved in the pathophysiology of multiple cardiovascular diseases, including hypertension and left ventricular hypertrophy. We found

Figure 2. Neointimal area (mm²) in the four groups. Rosuvastatin reduces neointimal formation and also reduces Ang II induced neointimal formation.

Figure 3. Surface adherent leukocytes in the four groups.
increased blood pressure and N-ANP levels in rats receiving Ang II thereby confirming the biological efficacy of Ang II in our model.

Figure 4. Vascular function of the four groups. Effects of Angiotensin II infusion and treatment with rosuvastatin on endothelium-dependent (figure above) methacholine (ME) and endothelium-independent vasodilation to nitroglycerine (NTG).

The Ang II type 1 receptor is the principal mediator of the detrimental effects of Ang II and is involved in the release of reactive oxygen species elicited by Ang II induced activation of NAD(P)H oxidases of the vasculature and inflammatory cells. Ang II and oxidative stress stimulate smooth muscle cell proliferation and vascular hypertrophy. Ang II aggravated neointimal formation in our model. However, we did observe a similar reduction of neointimal formation by rosuvastatin treatment in the presence or absence of Ang II infusion (24 and 23% reduction, respectively). Again, the reduction of neointimal formation by statin treatment was paralleled by improvement of endothelial function. Downregulation of the Ang II type 1 receptors in vascular smooth muscle and attenuation of Ang II induced vascular responses by statin treatment has been reported. However, when Ang II receptor downregulation is of importance, we would have expected to see more effects of rosuvastatin in the Ang II infused rats. Even when Ang II type 1 receptor was indeed downregulated, the abundance of
infused Ang II might still have resulted in maximal intracellular signaling. A limitation of our study is that we did not measure Ang II type 1 receptor expression. On the other hand, as statin treatment has been reported to influence an array of potentially relevant mechanisms, including reduction of platelet activation, thrombus formation, inflammation, oxidative stress and inhibition of vascular smooth muscle cell migration and proliferation proliferation it is as likely that statins interact with signaling peptides other than Ang II in the process of in-stent restenosis.(12) In addition, AT1-receptor blockade does not reduce neointimal formation in rats without supraphysiological angiotensin II levels.(30) Our study has limitations, which need consideration. We used a relatively high dose of statin. However, local vascular drug delivery on coated stents will make it feasible to deliver a safe statin dose in an anti-restenotic concentration range and might provide a new area of research. Preliminary results with statin-coated stents in animals are promising. However, the release profile of this coated stent was only 3 hours, and re-endothelialization of the vasculature was not affected as assessed semiquantitatively.(31) In conclusion, this study demonstrates that systemic treatment with rosuvastatin after stent implantation in the rat abdominal aorta results in attenuation of neointimal formation, and improvement in endothelial dilator function. Excessive tissue activation and endothelial dysfunction brought about by high Ang II levels is effectively countered by rosuvastatin. It seems likely that this beneficial effect was mediated by the improvement of endothelial function, although other relevant mechanisms are possible. Therefore, statin coated stents without the potential side-effects of late stent thrombosis and endothelial dysfunction seen with paclitaxel and sirolimus eluting stents may be a possible alternative.

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