Rapamycin modulates the eNOS vs. shear stress relationship

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Aims Studies in animals and patients indicate that rapamycin affects vasodilatation differently in outer and inner curvatures of blood vessels. We evaluated in this study whether rapamycin affects endothelial nitric oxide synthase (eNOS) responsiveness to shear stress under normo- and hypercholesteremic conditions to explain these findings.

Methods and results Shear stress levels were varied over a large range of values in carotid arteries of transgenic mice expressing human eNOS fused to enhanced green fluorescence protein. The mice were divided into control, low-dose rapamycin (3 mg/kg/day), and high-dose rapamycin (3 mg/kg/day) groups and into normcholesteremic and hypercholesteremic (ApoE−/− on high cholesterol diet for 3–4 weeks) groups. The effect of rapamycin treatment on eNOS was evaluated by quantification of eNOS expression and of intracellular protein levels by en face confocal microscopy. A sigmoid curve fit was used to describe these data. The efficacy of treatment was confirmed by measurement of rapamycin serum levels (2.0 ± 0.5 ng/mL), and of p27kip1 expression in vascular tissue (increased by 2.4 ± 0.5-fold). In control carotid arteries, eNOS expression increased by 1.8 ± 0.3-fold in response to rapamycin. In the treated vessels, rapamycin reduced maximal eNOS expression at high shear stress levels (\(P < 0.05\)) in a dose-dependent way and shifted the sigmoid curve to the right. Hypercholesteremia had a tendency to increase the leftward shift and the reduction in maximal eNOS expression (\(P = 0.07\)).

Conclusion Rapamycin is associated with high eNOS in low shear regions, i.e. in atherogenic regions, protecting these regions against atherosclerosis, and is associated with a reduction of eNOS at high shear stress affecting vasomotion in these regions.

KEYWORDS
Shear stress; Rapamycin; Atherosclerosis; C57BL6

1. Introduction
Rapamycin- or Sirolimus eluting stents (SESs) are widely used for percutaneous coronary interventions because of their positive results on clinical and angiographic outcome. Several studies have shown significant reduction in rates of angiographic restenosis and associated clinical events. However, long-term effects of SES implantation on vascular integrity and coronary endothelial function are largely unknown. In a clinical study in which endothelial dependent vasomotion of vessels after 6 months of SES implantation was assessed, infusion of acetylcholine induced paradoxical vasoconstriction in the vessel segments situated 2 mm distally from the SES.1 In addition, Togni et al.2 also reported

deterioration of endothelial function after 6 months of SES implantation. They found that exercise induces paradoxical coronary vasoconstriction in vessel segments adjacent of the SES.2 While other DES and polymeric stents also show endothelial dysfunction, these findings indicate that the endothelial dysfunction associated with Rapamycin might be related to the regulation of the enzyme endothelial nitric oxide synthase (eNOS).

The ability of eNOS to produce nitric oxide is essential for maintenance of vascular homeostasis. The reduction in the availability of NO response is a major contributor to the initiation of atherosclerotic disease, in-stent restenosis, and endothelial dysfunction. Nitric oxide production is determined by many factors, among which shear stress may be considered one of the most important. Shear stress is the in-plane frictional force induced by the movement of the blood. In a series of animal and human studies by

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others and by us, it was observed that the distribution of neointimal thickness in stents is related to the shear stress pattern. A recent study in human coronaries indicated that shear stress modulated the rapamycin effect in stents.3

Considering the above arguments, we postulated that rapamycin modulates the eNOS expression in response to shear stress. In order to test this hypothesis, we changed shear stress by an in house developed method that creates pre-defined patterns of shear stress in mouse carotid arteries in vivo.4 We used a transgenic mouse model that we developed previously in which the endothelial cells express the human eNOS gene fused to eGFP.5 The GFP signal allowed us to monitor the effect of rapamycin on eNOS expression in response to shear stress in normocholesterolaemic mice. As it is widely accepted that hypercholesterolaemia modulates eNOS expression in a shear stress independent way, we evaluated the effect of rapamycin on eNOS in the presence of hypercholesterolemia.

2. Methods

2.1 Animals and experimental design

For this study, hemizygous eNOS-eGFP transgenic (tg) and eNOS-eGFP/ApoE−/− male and female mice older than 25 weeks of age were used. ENOS-eGFP tg mice were generated with a DNA-construct encoding human endothelial nitric oxide synthase fused to enhanced green fluorescent protein as described previously.5 ENOS-GFP/ApoE−/− mice were generated by cross-breeding eNOS-GFP tg mice with mice deficient in apolipoprotein E (ApoE), which were obtained from Jackson Laboratory. All animals were in C57BL/6J background. Rapamycin was mixed with the standard chow or Western diet (see in what follows) for a period of 11 days. The eNOS-GFP tg and the eNOS-GFP/ApoE−/− mice consisted of a control group (n = 6), a group treated with a low dose of rapamycin (3.0 μg/kg/day; n = 6) and a group treated with a high dose of rapamycin (3.0 mg/kg/day; n = 6). In addition, a series of eNOS-GFP tg mice were studied in which the carotid artery was ligated without rapamycin and with a high dose of rapamycin treatment (n = 4). Animals were housed under standard conditions, with unlimited access to water and food. ENOS-GFP tg mice were fed with standard rodent chow, whereas eNOS-GFP/ApoE−/− mice were fed a high-fat (Western) diet containing 15% (w/w) cacao butter and 0.25% (w/w) cholesterol (Hope Farms, Woerden, The Netherlands) 3 weeks before starting oral administration of rapamycin. A control group (n = 6), a low dose (n = 6), and a high dose (n = 6) group was studied. All groups consisted of equal numbers of males and females. The Erasmus MC review board on animal experiments approved procedures, and animal care and experiments were carried out in compliance with the institutional and national guidelines. The investigation confirms with the Guide for the care and use of laboratory animals published by the US national institute of health (NIH publication No. 85-23, revised 1996).

2.2 Modification of shear stress

In order to create predefined patterns of shear stress, a perivascular shear stress modifier was used as described previously.4 In short, the shear stress modifier, referred to as cast, was surgically positioned around the carotid artery of a mouse and immobilized with a 6/0 suture. The cast gradually decreased the vessel lumen from 500 to 250 μm over a length of 2 mm. Analyses were performed after 4 days of cast placement. This period was chosen as previous measurements showed a steady state in eNOS expression after 4 days. In rapamycin-treated animals, the cast was placed at day 7 after starting the oral administration of rapamycin.

2.3 Confocal microscopy

The carotid arteries from cast instrumented animals were carefully isolated over their entire length and dissected. The casts were carefully removed from the vessel and the vessel was placed between two glass slides. These tissue samples were examined with a confocal laser scanning microscope system (LSM-510, Carl Zeiss Inc., Thornwood, NY, USA), equipped with an Argon laser (458 and 488 nm) and two HeNe lasers (543 and 633 nm). The emission, which resulted from excitation of the tissue with a 488 nm Argon-laser, was passed through a 560 nm long pass filter to optimize detection of the green fluorescence of eGFP. After appropriate settings of the optics (20 ×/0.5 Plan-neofluar objective), of the resolution (8 bits), of the pinhole (1000 μm), and of the scan mode (plane, multitrack), images were captured. Adjacent optical images from one specimen were combined to form a tile in order to provide a complete picture of the vessel segment of interest.

2.4 Computational fluid dynamics

In order to estimate the changes in wall shear stress distribution after cast placement, we applied a well-validated 3D computational fluid package (CFD).6–8 Boundary conditions, material properties, and 3D vessel geometry are essential for CFD to generate a solution. The boundary conditions for the calculations were: no slip conditions at the wall, stationary parabolic inflow at the entrance with a maximal velocity equivalent to measurements obtained in a separate group of mice (see Supplemental Material for details) and zero stress outflow. Three-dimensional vessel geometry was obtained from the contours of the en face eGFP images, assuming a circular geometry and after correction for longitudinal shrinkage of the intact vessel. On basis of this vessel geometry, a mesh was generated, which consisted of ~25 000 nodes. Because viscosity of the blood is dependent on shear rate, we used a non-Newtonian fluid model (‘Carreau model’) to estimate viscosity.7 The accuracy of the calculations was set at 10−4 m/s, which resulted in a numerical error of wall shear stress of ~1%.

2.5 Measurement of total cholesterol and rapamycin levels

Total cholesterol levels in plasma of eNOS-GFP/ApoE−/− mice were determined enzymatically with the Free Cholesterol C kit (WAKO, Neuss, Germany) after hydrolysis of cholesterol esters with cholesterol esterase from Candida cylindracea (Roche, Mannheim, Germany). Rapamycin levels in whole blood were obtained from a commercially available immunoassay (Abbott Laboratories, Abbot Park, USA).

2.6 Gene expression analysis

Carotid arteries were isolated from the eNOS-GFP tg animals untreated and treated with a high concentration of rapamycin (n = 5 per group). Total RNA from the samples was obtained using the RNAeasy kit (Qiagen, Cologne, Germany) and directly reversed transcribed into cDNA. PCR reactions were performed using a real-time fluorescent determination in the iCycler IQ Detection System (Biorad, Veenendaal, The Netherlands). Gene expression levels of p27kip1 and eNOS were assessed. Target gene mRNA levels were expressed relatively to hypoxanthine guanine phosphoribosyl transferase. p27kip1 was determined as it is known that the expression of this gene is specifically affected by rapamycin.

2.7 Data analysis and statistics

The method to describe the coupling between shear stress and eNOS-GFP expression has been described before and is available in the Supplemental Material. Briefly, the eNOS-GFP vessel segments treated with a cast were traced manually and the contours...
were used to reconstruct the blood vessel in three dimensions. This reconstruction was used to generate a 3D mesh, and to perform CFD. From the same segment eNOS-GFP levels was quantified by averaging pixels located on 100 lines perpendicular to the vessel centre line at location where shear stress was obtained with CFD. As a final step, eNOS-GFP values (y-axis) were related to local shear stress values (x-axis), and a non-linear curve fitting routine consisting of sigmoid relationship with an extra intercept was applied (see Supplemental Material for details). This analysis was performed on eNOS-GFP mouse carotids without rapamycin treatment (control), with rapamycin treatment and after crossing the eNOS-GFP mice with ApoE mice to induce hypercholesteremia.

Student’s t-tests were used to evaluate rapamycin serum levels, and the eNOS-GFP and P27kip1 expression levels in the control and the rapamycin-treated group. The estimated parameters of the sigmoid curve fit (eNOS_{50}, EC_{50} and eNOS_{max}; see Supplemental Material for details) were tested with a two-way ANOVA. *Δ eNOS was calculated as eNOS_{max}—eNOS_{50}. For display purposes all eNOS and shear stress data were sorted and averaged for every 10 consecutive data-points and the resulting curve was presented with a standard deviation. Statistical significance was considered at \( P < 0.05 \). All values are reported as mean ± SEM.

3. Results

3.1 Animal characteristics and validation of treatment adequacy

The rapamycin plasma levels were 2.0 ± 0.5 ng/mL after treatment with the highest dosage of rapamycin (3 mg/kg/day) in the eNOS-GFP tg and the eNOS-GFP/ApoE − /− groups. These plasma levels were associated with a two-fold and three-fold increase of p27kip1 gene expression levels in the vascular wall of the carotid arteries from eNOS-GFP tg and eNOS-GFP/ApoE − /− mice, respectively, as compared with the controls (Figure 1A). Total cholesterol levels remained unchanged in the ApoE mice before and after rapamycin treatment (32.7 ± 4.5 and 28.5 ± 4.4 mM, respectively).

3.2 Rapamycin affects eNOS expression differently in high and low shear stress regions

The expression of the human eNOS in the contra-lateral control carotid arteries increased by 1.8 ± 0.3-fold after treatment with the highest dose of rapamycin (Figure 1B). In accordance with the mRNA levels, rapamycin treatment increased intracellular eNOS protein levels in low shear region (Figure 2, lower panel). In contrast, intracellular eNOS protein levels decreased in high shear regions with incremental dosages of rapamycin (Figure 2, upper panel). These observations were further studied by relating eNOS protein levels to shear stress levels obtained by CFD (Figures 3 and 4). The sigmoidal relationship between shear stress and eNOS levels showed a rapamycin-dose dependent decline in eNOS response [Figure 3C; control, rapamycin low dose (LD), rapamycin high dose (HD)]. The subsequent curve fit analysis revealed two different components: a dose-dependent decrease in eNOS_{max} from 67.4 ± 14.6 (AU) to 34.1 ± 7.7 (AU); \( P < 0.05 \), and a decrease in EC_{50} from 10.7 ± 2.6 to 4.8 ± 0.5 N/m² (Figure 3A; \( P < 0.05 \)). The slope and intercept (see Supplemental Material) remained unaffected.

As it was difficult to obtain accurate measurements of eNOS protein at the lowest levels of the curve fit in a reliable way (Figure 3C), we also studied an extra series of mice, in which the carotid artery was ligated (i.e. zero shear stress) and eNOS protein levels were investigated (see Supplemental Material). Rapamycin significantly increased eNOS expression in the ligated group confirming the above-mentioned measurements.

3.3 Rapamycin affects shear stress-induced eNOS response in dysfunctional endothelium

In order to evaluate the additional effect of endothelial dysfunction, we also studied the effect of rapamycin on the shear stress eNOS relationship in ApoE − /− mice after a high cholesterol diet of 4 weeks. The relationship between shear stress and eNOS expression could be described by a
sigmoidal relationship similar as for the non-hypercholesterolaemia conditions (Figure 4C). Further analysis revealed that maximal intracellular eNOS max protein levels decreased from 63.7 ± 7.3 (Arbitrary Units or AU) to 22.4 ± 7.2 (AU), and the EC50 was decreased from 8.2 ± 1.4 to 4.8 ± 1.4 N/m² (Figure 4A and B).

3.4 Rapamycin and hypercholesterolaemic both affect the shear stress response independently, and to a similar extent

By comparison of Figures 3C and 4C it is clear that hypercholesterolaemia has additional effect on eNOS expression, on
Rapamycin and eNOS

Table 1  Effect of rapamycin and hypercholesterolaemia on eNOS shear stress sensitivity and maximal eNOS protein levels

<table>
<thead>
<tr>
<th></th>
<th>Normocholesterolaemic mice</th>
<th>Hypercholesterolaemic mice</th>
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<tr>
<td></td>
<td>Control</td>
<td>Rapa 0.3 µg/kg</td>
</tr>
<tr>
<td>EC50</td>
<td>11.7 ± 5.6</td>
<td>3.8 ± 1.0*</td>
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<tr>
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<td>Control</td>
<td>Rapa 0.3 µg/kg</td>
</tr>
<tr>
<td>EC50</td>
<td>8.1 ± 4.5**</td>
<td>5.6 ± 1.6**</td>
</tr>
<tr>
<td>ENOs max</td>
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<tr>
<td>Constant</td>
<td>5.0 ± 4.8**</td>
<td>8.2 ± 4.5**</td>
</tr>
</tbody>
</table>

EC50, shear stress (Pa) at 50% saturation of the eNOS expression; ENOs max (AU), maximal eNOS expression obtained at high shear stress values. Angle (AU/Pa), tangent of the curve at EC50; Constant, eNOS expression (AU) at zero shear stress; i.e. the constitutive expression.

*P < 0.05 Rapa vs. control.
**P < 0.07 normocholesterolaemic vs. hypercholesterolaemic.
***P < 0.05 normocholesterolaemic vs. hypercholesterolaemic.

top of those of shear stress. In order to quantify these effects, the parameters of the curve fit were subjected to a two-way ANOVA (Table 1). This analysis showed that both hypercholesterolaemia and shear stress similarly affected the reduced sensitivity (i.e. EC50) of eNOS to shear stress (Table 1). Likewise, no major differences were noted for the maximal eNOS protein levels in the endothelial cell (Table 1).

4. Discussion

The main findings of the present study are that rapamycin treatment in aged and gender matched animals with and without hypercholesterolaemia: (i) increases rapamycin plasma levels and tissue p27kip1 expression, indicating efficacy of the treatment; (ii) is associated with increased eNOS mRNA and protein levels in carotid arteries of normocholesterolaemic mice at low and physiological shear stress levels, which is in accordance with earlier studies; (iii) is associated with decreases in eNOS protein levels at high shear stress levels in normocholesterolaemic animals, both by a decreased sensitivity and an increased reduction of the maximal shear stress response, and (iv) has a more pronounced effect in ApoE–/– mice, implying a sensitizing effect of hypercholesterolaemia.

Rapamycin has been successfully used for treating in-stent restenosis due to its anti-proliferative and anti-inflammatory properties.9–12 However, knowledge of the effects of the drug on vasomotion is limited. Hofma et al.1 recently assessed the endothelium-dependent vasomotion in the coronary vessel segments of patients implanted with an SES. As the intracoronary stent scaffolds completely inhibit vasomotion, measurements were performed in 17 mm vessel segments, 2 mm downstream from the SES. They observed a paradoxical vasoconstriction in response to acetylcholine stimulation. Acetylcholine is a potent agonist of eNOS, and should trigger NO production by this enzyme, resulting in vasorelaxation. The unexpected response to acetylcholine might imply that there is a deleterious effect of Sirolimus on eNOS expression or activation.

However, variable effects of the drug on the eNOS expression have been reported. Rapamycin did not effect eNOS protein levels in the thoracic aorta in rats,13 but did increase eNOS expression in the arterial wall of hypercholesterolaemic ApoE–/– mice.14 As the eNOS promoter is shear stress responsive,15 and we previously found that rapamycin possibly modulates the shear stress responsiveness of the vessel wall,3 we argued that rapamycin might affect the response of eNOS to shear stress. Our study show that rapamycin is associated with increments in eNOS levels and its expression patterns within the low and physiological shear stress range that is normally found in straight vessel segments (0–3.0 Pa; Figure 2), in normocholesterolaemic and hypercholesterolaemic animals. This finding is consistent with previous data, showing elevated eNOS levels in straight murine aortas after administration of rapamycin.14 These findings might imply that systemic administration of rapamycin will provide protection against the onset of atherosclerosis by increasing eNOS availability at these predilection sites. Indeed, it was shown that rapamycin could reduce atherosclerosis in low shear regions via a p27kip1 independent mechanism,16 facilitated by a reduction in pro-atherogenic cytokines and an increase of anti-atherogenic cytokines in ApoE deficient mice treated with rapamycine.17 Remarkably, eNOS levels are reduced in the high shear stress range (>3.0 Pa) in both normocholesterolaemic and hypercholesterolaemic animals.

Hypercholesterolaemia by itself also affected the eNOS vs. shear stress relationship, a finding that was not reported before. In a recent study, the coronary vessels of patients implanted with SESs show impaired vasomotion during exercise in the vessel segments up- and downstream from the stented area, leading to vasoconstriction.2 While large differences exists between animal and patient studies, and the present study do not employ a drug-eluting stent approach, our data may offer an explanation for these findings, as rapamycin reduces eNOS expression during high shear stress (>3 Pa) conditions, a situation that may be applicable to the up- and downstream regions of the SESs.2 Diffusion of rapamycin into the peri-stent vascular regions could therefore induce endothelial dysfunction by affecting the expression of eNOS, resulting in the observed paradoxical vasoconstriction.

While we did not perform a detailed analysis into the mechanism it might imply that the effect of rapamycin on protein synthesis is shear stress-dependent, i.e. it inhibits protein synthesis in high shear stress regions, while it stimulates protein synthesis in low shear stress regions. It implies therefore that shear stress regions exist where rapamycin does not exert any effect (i.e. the cross-over of the control and the rapamycin modulated curve). An estimate from our data shows these regions are associated with a
shear stress value of \(~ 3\) Pa both for control and hypercholesterolaemic conditions.

### 4.1 Limitation of the study

For the calculations of the shear stress field, we performed a 3D reconstruction of the blood vessel from the flat specimen assuming a cylindrical geometry. We have tested this assumption by measuring diameter of individual mice before sacrifice and compared these measurements with 3D reconstruction diameters. The difference between prediction and measurements never exceeded the 5%. Another assumption underlying our CFD calculations was that entrance shear stress was 1.5 N/m². Comparing Doppler velocities obtained from a separate group of animals with entrance velocities based upon this assumption. Doppler measurements were 2.0 ± 0.6 mL/min \((n = 9)\) and entrance velocities varied between the 1.8–2.2 mL/min \((n = 10)\).

Four days of cast placement was used throughout the experiments. This period was chosen as eNOS expression changes induced by shear stress were in a steady state and the period was too short for fibrotic tissue to interfere with the measurements.

Over-expression of eNOS-GFP may result in a negative feedback by the increased NO production inhibiting transcription of the gene. Thus, the effect of shear stress on eNOS expression might have been underestimated in the present study. However, the expression of murine eNOS in wild-type controls shows a similar response to the shear stress alterations induced by the cast. The observed effect of shear stress on eNOS-GFP is therefore not restricted to the human eNOS transgenic construct.

The GFP reporter might interfere with eNOS localization. eNOS myristoylation and palmitoylation, which are both important for the localization, occur at the N-terminus. It is unlikely that these processes are affected, because the GFP part is fused at the C-terminus of the eNOS protein. Although GFP could induce changes in protein folding, our experiments show normal localization of eNOS-GFP in the Golgi complex and plasma membrane. Previous studies extensively studied the location and function of the eNOS-GFP fusion protein in vitro. These studies clearly indicate that the GFP moiety does not interfere with the localization and the function of eNOS.

The change in vessel geometry by cast placement may compress fibro-elastin layers of the vessel wall and thereby increase background auto-fluorescence. This does not affect our data, however, because the auto-fluorescent signal was subtracted from the total GFP signal. The tapering effect of the cast could also compress the endothelial cells to make them fit into a smaller area, increasing the number of cells per area. Co-localization studies of the endothelium in the different shear stress regions do not show an increase in cell number per area, or a decrease in cell size. In addition, the up-regulation of eNOS-GFP in single endothelial cells is observed, providing further evidence that the eNOS-GFP signal in the high shear vessel segment is not an artefact created by the tapered shape of the cast.

Comparison between data from the present study and SEs should be done with caution: in the present study rapamycin was given orally, while in DES it is presented locally. Furthermore, while we tested for biological effect of the rapamycin we did not perform a detailed analysis of the concentration profile in the wall, making quantitative comparison between DES and this study difficult.

In conclusion, our results indicate that systemic administration of rapamycin protects the low shear stress regions from plaque initiation via an eNOS/shear stress-dependent mechanism. In contrast, rapamycin decreases eNOS responsiveness to high shear stress, rendering a loss of protection via eNOS/shear stress mechanism in these regions. Hypercholesterolaemia has a tendency to increase these effects of rapamycin.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

### Conflict of interest: none declared.

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