Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model
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CHAPTER 4
Effects of angiotensin II and angiotensin II type 1 receptor blockade on neointimal formation after stent implantation.


ABSTRACT

Aim: To evaluate the effect of supraphysiological levels of angiotensin II and selective angiotensin II type 1 receptor (AT1-receptor) blockade on neointimal formation and systemic endothelial function after stent implantation in the rat abdominal aorta.

Methods: Male Wistar rats were randomized to one of three groups; control (n=8), angiotensin II infusion (n=9, 200ng/kg/min), or candesartan cilexetil (n=8, AT1-receptor blocker: rats received 14.4 mg/kg-1*day-1). Stents were implanted in the abdominal aorta. Histological analyses were performed at 4 weeks. Endothelial function was determined in isolated thoracic aortic rings. Results: Neointimal area was increased in the angiotensin II treated group versus the control group, 0.88 mm²±0.21 versus 0.66 mm²±0.16 (p<0.05). Neointimal thickness was 171µm±44 in angiotensin II treated animals and 120µm±25 in the control group (p<0.05). In addition, endothelial function was attenuated in angiotensin II treated animals (P=0.01). Candesartan cilexetil treatment did not result in reduction of neointimal area and did not reduce neointimal thickness compared to the control group. Candesartan had no effect on endothelial function. Conclusions: Supraphysiological levels of angiotensin II aggravates neointimal formation in the stented rat abdominal aorta, and in parallel decreases endothelial function. AT1-receptor blockade does not reduce neointimal formation in rats without supraphysiological angiotensin II levels.
INTRODUCTION

The renin-angiotensin system has been implicated in the pathophysiology of in-stent restenosis (1). Angiotensin II (Ang II) Type 1 (AT1) receptors are found abundantly on smooth muscle cells derived from human in-stent restenotic lesions and Ang II induces vascular smooth muscle proliferation through the activation of mitogen-activated protein kinase (2;3), and elevated Ang II levels aggravate neointimal formation after vascular injury (4). Several large trials investigated the effect of Angiotensin Converting Enzyme (ACE)-inhibitors on restenosis after angioplasty and stenting, but found no reduction in restenosis (5;6). This inefficacy of ACE-inhibitors to reduce restenosis has been attributed to inadequate tissue ACE inhibition and the existence of alternative pathways of Ang II formation, such as chymase (7-9). Alternative approaches, using direct inhibition of Ang II using AT1-receptor blockers reduced restenosis after vascular injury in the rat carotid model (10;11). However, clinical trials with AT1-receptors blockers this far have shown no effect (12;13). Considering the discrepancy between animal research and clinical trials we hypothesized that supraphysiological Ang II levels are needed for Ang II mediated neointimal formation. Accordingly, the effect of candesartan cilexetil (a selective AT1-receptor blocker) in the setting of in-stent restenosis in rats with physiological levels of Ang II was determined. Restenosis after balloon injury is explained by negative arterial remodeling (shrinkage) and also by neointimal formation, while restenosis after stenting depends only on neointimal formation (14-16). To confirm the role of the renin-angiotensin system in the setting of in-stent restenosis, we also determined the effect of Ang II infusion. We assessed the role of Ang II infusion and candesartan treatment on endothelial function in relation to neointimal formation.

METHODS

All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Stent implantation was performed in thirty rats. Overall mortality was approximately 17%. Most animals died either of perforation or thrombosis of the aorta immediately following stenting.

Animal Protocol

This study was approved by the animal care and use committee of the University of Groningen. Male Wistar WU rats (Charles Rivers) weighing 450 to 520 g were anesthetized with O2, N2O, and isoflurane 2% (Abbot B.V.). Premounted, 2.5 x 8 mm, bare metal stents (Lekton Motion Petite, Biotronik) were implanted in the abdominal aorta as described previously (17). There were 3 groups: control group, Ang II infusion group and candesartan-treated group. In candesartan-treated animals normal rat chow was mixed with candesartan(10 mg·kg−1·day−1) and administrated ad libitum starting one week before stent implantation. The concentration of candesartan in the diet was 0.25mg·g−1. Baseline weight, weight at 4 weeks and daily oral intake were measured in the candesartan-treated group. Rats in the Ang II infusion group received an osmotic minipump (Model 2004; Alzet) subcutaneously for Ang II delivery (200 ng/kg per minute) after the stent implantation. Blood pressure was measured serially (0,1,7,14 and 28 days after stent implantation) under general anesthesia using an electrosphygmomanometer in the tail of the rat. After 28 days, animals were anesthetized and heparinized with 500 IU intravenously (Leo Pharma B.V.). Abdominal aortas were harvested, fixed, embedded in methylmetacrylate, sectioned, and stained for histological analysis. The thoracic aortas were quickly excised and mounted in organ baths to assess endothelial function.
Histology
Histomorphometrical analysis was performed on Lawson-stained sections by measurements of the proximal, middle, and distal parts of each stent. To assess neointimal formation, areas within the external elastic lamina, internal elastic lamina, and lumen were measured using digital morphometry by means of an Olympus BX-50F4 microscope, an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). The neointimal area, media area, lumen area, and the percentage of stenosis were calculated as described previously (18). The injury scores were assessed as described by Schwartz et al and Kornowski et al (19;20).

N-terminal Atrial Natriuretic Peptide
Concentrations of N-terminal atrial natriuretic peptide (N-ANP) in plasma were measured with a commercially available radioimmunoassay from Biotop (Oulu, Finland) as described previously (21).

Organ Bath Studies With Isolated Aortic Rings
Organ bath studies were performed as described previously(22;23). In brief, periaortic tissue was removed and rings of 2 mm in length were cut and rings were connected to an isotonic displacement transducer. After stabilization, during which regular washing was performed, rings were checked for viability by stimulation with phenylephrine (10 µmol/L). Rings were washed and restabilized. Rings were precontracted with phenylephrine (10 µmol/L). The endothelium-dependent vasodilation was assessed by a cumulative dose of metacholine (10nmol/L to 10 µmol/L). Endothelium independent vasodilation was assessed by a cumulative dose of nitroglycerine in parallel rings. Vascular responsiveness to Ang II (0.1 nmol/L to 1 µmol/L) was assessed in parallel rings as described previously(24).

Statistical methods
Data are expressed as mean ±SD unless specifically stated otherwise. Comparison of means was determined by ANOVA with Bonferonni correction for multiple comparisons. All P-values were two-tailed, and a P-value of <0.05 was considered statistically significant. Analyses were performed using SPSS software (SPSS version 12.0, Chicago, IL, USA).

RESULTS
Candesartan dose
Baseline weight (mean±SD) was 474 gram ±30, weight at 4 weeks was 510±34 and daily oral intake was 28±0.7. The mean candesartan dose received over the 4 week period was calculated to be 14.4 mg·kg⁻¹·day⁻¹.

Histological analysis
Histomorphometric measurements are presented in Table 1. Stent expansion, expressed as the internal elastic lamina area and injury score was similar among groups. In Ang II infused animals, neointimal area (Figure 1) and neointimal thickness were significantly increased. Candesartan treatment did not change neointimal area or neointimal thickness compared to the control group. Representative photomicrographs of stented abdominal aortas of the three groups are shown in Figure 2.
Table 1. Neointimal formation.

<table>
<thead>
<tr>
<th>Variable</th>
<th>A (N=8)</th>
<th>B (N=9)</th>
<th>C (N=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean injury score</td>
<td>0.06±0.08</td>
<td>0.11±0.13</td>
<td>0.08±0.19</td>
<td>P=1.00/P=1.00</td>
</tr>
<tr>
<td>Neointimal area</td>
<td>0.66±0.16</td>
<td>0.88±0.21</td>
<td>0.71±0.14</td>
<td>P=0.048/P=1.00</td>
</tr>
<tr>
<td>Neointimal thickness(µm)</td>
<td>120±25</td>
<td>171±44</td>
<td>132±32</td>
<td>P=0.019/P=1.00</td>
</tr>
<tr>
<td>Media area(mm²)</td>
<td>0.29±0.06</td>
<td>0.30±0.05</td>
<td>0.30±0.04</td>
<td>P=0.86/P=1.00</td>
</tr>
<tr>
<td>Internal elastic lamina area(mm²)</td>
<td>3.79±0.39</td>
<td>3.59±0.22</td>
<td>3.68±0.38</td>
<td>P=1.00/P=1.00</td>
</tr>
</tbody>
</table>

Endothelial Function
The effects of Ang II infusion and candesartan treatment were examined in thoracic aorta rings. Endothelium-dependent relaxation was decreased in the Ang II group compared to the control group (Figure 3). Candesartan did not change endothelial-dependent vasodilatory response to metacholine. Endothelial-independent vasodilatory response to nitroglycerine did not differ among groups and are shown in Figure 4.

Effectiveness of Ang II delivery and Ang II type 1 receptor blockade.
Blood pressure was increased in the Ang II group and decreased in the candesartan-treated group (Table 2). N-ANP levels were raised (p=0.05) in the Ang II group as compared to the control group (1.71±0.22, versus 1.21±0.14). Candesartan did not change N-ANP levels (1.10±0.21, p=1.00) compared to the control group. Ang II was notably blocked in the candesartan group (Table 2).

Table 2. Effect of Angiotensin II (Ang II) and candesartan on vascular responsiveness to Ang II and blood pressure.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of candesartan started 7 days</th>
<th>A (N=8)</th>
<th>B (N=9)</th>
<th>C (N=8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline blood pressure (mmHg)</td>
<td>97±8.0</td>
<td>93±14</td>
<td>74±11 #</td>
<td>P=1.00/P=0.002</td>
<td></td>
</tr>
<tr>
<td>Mean blood pressure (mmHg)</td>
<td>99±11</td>
<td>148±20</td>
<td>76±11</td>
<td>P=0.001/P=0.001</td>
<td></td>
</tr>
<tr>
<td>Maximum Ang II response (%PE)</td>
<td>37±10%</td>
<td>38±8%</td>
<td>1.0±2%</td>
<td>P=1.00/P=0.001</td>
<td></td>
</tr>
</tbody>
</table>

A=Control  B=Ang II  C=Candesartan  (A:B/A:C)
Figure 1: Neointimal area is significantly higher in the Ang II group (grey bar) compared to the control group (black bar), but neointimal area in the candesartan treated group (dark grey bar) is not significantly lower compared to the control group.

Figure 2: Photomicrographs of stented abdominal aortas showing the neointima, internal elastic lamina, external elastic lamina, stent struts (blank spaces in vessel wall) and media: A and B (control), C and D (Ang II infused) E and F (candesartan-treated, x40 and x400).

DISCUSSION

In the present study, we examined the effect of high serum Ang II levels and Ang II type 1 receptor blockade on neointimal formation in a rat abdominal aorta stenting model. Increased Ang II levels increased neointimal area and thickness. Ang II also increased blood pressure. In contrast, Ang II type 1 receptor blockade did not reduce the neointimal area or thickness. Endothelial function decreased in the Ang II group, but did not change in the candesartan group.

Effect of candesartan on in-stent restenosis

The effect of candesartan on neointimal formation in this study seemingly conflicts with earlier studies performed in the rat carotid balloon injury model where a reduction in neointimal formation after treatment with AT1-receptor blockers was observed (25;26). However, balloon injury and stenting have different mechanism of restenosis as demonstrated in different animal models and in humans. Restenosis after balloon injury is explained by negative arterial remodeling (shrinkage) and also by neointimal formation, while restenosis after stenting depends only on neointimal formation(27-29). Sustained chemokine expression and leukocyte recruitment indicate that inflammation is more prominent after stenting (30). Anti-inflammatory properties of AT1-receptor blockers after balloon injury in the femoral artery have been reported, but are still unknown after stenting(31).
Only two previous studies examined the effect of AT1-receptor blockade on neointimal formation after stent implantation; their results were conflicting. Huckle et al. found no reduction of neointimal formation in the coronary artery of pigs, while Ohtani et al. found a reduction of neointimal formation in the iliac arteries of cynomolgus monkeys, and in the common carotid arteries of rabbits(32;33). An important limitation of the study of Othani et al. was the omission of injury scores, an important factor influencing neointimal formation. The groups in the study of Huckle et al. had equal injury scores, making the groups well comparable(34). So the question should be raised whether the effect of systemic AT1-receptor blockage on restenosis in the balloon injury model can be extrapolated to the different process of in-stent restenosis, because most preclinical studies done in an angioplasty setting are successful in reducing neointimal formation while results in studies evaluating the effect on in-stent restenosis are not so clear. This question is justifiable by the fact that most trials with AT1-receptor blockers have shown no effect of AT1-receptor blockade on in-stent restenosis(35;36). Only Yoshida et al. found a reduction of neointimal formation (using intravascular ultrasound) after candesartan compared to placebo treatment in clinically stable patients(37). However, this study suffered some methodological difficulties. In the candesartan group up to 47% of the patients received a Multi-Link stent, whereas only 28% of the control group did. This bias is important because Multi-Link stent designs are known to be associated with decreased neointimal formation(38). In addition, in the study of Yoshida et al. the clinical variables of restenosis rate and target lesion revascularization did not differ between the two groups. There could be several other explanations for the lack of effect of the AT1-receptor blocker on in-stent restenosis in this study. Inadequate dosing would be the most
important one. The rats in this study however received a higher dose of candesartan than in the balloon injury models (39;40). A second explanation could be inadequate tissue levels of candesartan. However the aortic rings in the candesartan treated rat showed no response on Ang II indicating high tissue levels of candesartan (Table 2). Furthermore the rats were pretreated before stent implantation to increase local tissue levels at an early stage after the stenting injury. Thirdly mechanical stretch can stimulate AT1-receptors without the involvement of Ang II. However, candesartan is an inverse agonist which has been shown to inhibit AT1-receptor stimulation by mechanical stretch (41). Therefore, AT1-receptor stimulation by mechanical stress is likely to have been blocked by candesartan in this study. Fourthly, we only used a limited number of rats per group, leaving the possibility that we missed small sized effects on our primary endpoint. Considering the standard deviation, post-hoc calculation of the detectable alternative is approximately 0.22 mm2 (based on a power of 0.8 and an alpha of 0.05). Considering the observed non-significant difference of 0.05 mm2 between candesartan treated animals versus controls, we will need to study at least 142 animals per group to detect such a small difference (42).

**Effect of high levels of Ang II on in-stent restenosis**

We showed that high levels of Ang II can induce neointimal formation. We found also a diminished endothelial function in rats treated with Ang II. The positive correlation between endothelial dysfunction and increased neointimal formation found in this study after Ang II infusion is known (43). One link which could explain the parallel effects of Ang II on endothelial function and neointimal formation is oxidative stress. Ang II can induce oxidative stress by the NAD(P)H oxidase system and this Ang II induced oxidative stress has been linked to endothelial dysfunction (44;45). The endothelial dysfunction however may also be indirectly caused by hypertension which was observed in the Ang II treated rats. We do not think that blood pressure affected neointimal formation since hypertension is not a risk factor for in-stent restenosis (46).

In conclusion, candesartan does not decrease neointimal formation after stent implantation, however high levels of Ang II can increase neointimal formation after stenting and lead to an impairment of endothelial function. It could be that AT1-receptor stimulation by Ang II is not a major contributor to in-stent restenosis and only high doses lead to neointimal formation. This is supported by our finding that in normal rats without any prior activation of the renin-angiotensin system high dose AT1-receptor blockade does not lead to a reduction in neointimal formation. The current study only examined systemic AT1-receptor blockade, limited by its systemic side-effects like hypotension. However, local delivery could allow even higher dosing, and should be a focus of future studies. Data of Wilson et al. and Taguchi et al. has suggested that high local dosing of AT1-receptor blockers might be a more successful approach for reducing neointimal formation compared to systemic delivery (47;48). Wang et al. used drug-eluting stents for high local delivery of valsartan and found reduced neointimal formation. Interestingly they found an increase in AT2-receptor expression (49). It has been suggested that AT2-receptor stimulation has anti-inflammatory and anti-proliferative effects beneficial for the reduction of neointimal formation and that AT1-receptor blockers can stimulate AT2-receptors (50;51).

Besides AT2 receptor stimulation, future studies could also focus on alternative ways of renin-angiotensin modulation such as stimulation of the production of Ang 1-7 or alternatively stimulation of the kinin system (52-54).
Treatments of patients with combination therapy (ACE-inhibition and AT1-receptor blockade) might employ these mechanisms. ACE-inhibitors would stimulate kinin production and ACE-inhibitors and AT1-receptors would work synergistically to increase Ang 1,7(55;56). One study by Kim et al. showed in rabbits that combination therapy of ACE-inhibitors and AT1-receptor blockers was more effective in reducing neointimal formation compared to ACE-inhibitors and AT1-receptor blockers alone (57).

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Reference List


