INTRODUCTION

Atherosclerosis is a progressive inflammatory disease of the large and medium-sized arteries, characterized by the formation of plaques in the vessel wall. During the development of the disease, the stability of the atherosclerotic plaque plays a major role. Features of plaque instability include a large necrotic core, high infiltration of inflammatory macrophages and a thin fibrous cap composed of few smooth muscle cells (SMCs) and collagen fibres. When a plaque develops such an unstable phenotype, it may easily rupture, followed by thrombosis and clinical complications such as myocardial infarction and stroke.

Angiotensin II (Ang II), an important substrate in the renin-angiotensin-aldosterone system (RAAS), can influence atherogenesis via several mechanisms. Firstly, Ang II causes vasoconstriction and the release of aldosterone, leading to an increase in blood pressure which is a well-known risk factor for atherosclerosis. Secondly, Ang II is responsible for a range of blood pressure independent effects, such as upregulation of adhesion molecules on endothelial cells, activation of macrophages and an increased expression of matrix metalloproteinases (MMPs) and inflammatory cytokines. Eventually, these effects will result in the formation of rupture-prone atherosclerotic plaques with an increased risk of cardiovascular events.

In the present study, we used apolipoprotein E deficient mice (ApoE-/-) with a heterozygous mutation in the fibrillin-1 gene (Fbn1 C1039G+/-) to assess the responsiveness of the ApoE-/-Fbn1C1039G+/- mouse model to risk factors of atherosclerosis, such as high blood pressure and inflammation.

METHODS AND RESULTS

Osmotic minipumps filled with phosphate buffered saline or Ang II (1,000 ng/kg/day) were implanted in ApoE-/-Fbn1C1039G+/- mice at week 14 of Western diet (WD). After 4 weeks of therapy, we were not able to detect an increase in blood pressure, but we observed severe aneurysms in both the thoracic and abdominal aorta of Ang II treated mice. Plaque size and composition in the proximal ascending aorta was not altered. Nevertheless, coronary fibrosis, cardiac hypertrophy and a 6-fold increase in the occurrence of myocardial infarction in Ang II-treated mice was observed, which did not result in a higher mortality.

CONCLUSION

The study showed that Ang II treatment in ApoE-/-Fbn1C1039G+/- mice increased cardiac hypertrophy, coronary fibrosis and the incidence of myocardial infarctions, without affecting blood pressure, plaque vulnerability and survival.

Keywords: Atherosclerosis – cardiac remodelling – plaque vulnerability – blood pressure – mouse model – mortality.
the fibrillin-1 gene (Fbn1 C1039G+/-). These mice develop increased arterial stiffness due to fragmentation of the elastin fibres. Recently, we reported that this mouse model shows accelerated plaque progression, spontaneous plaque rupture, myocardial infarction and sudden death. The goal of this study was to evaluate whether the ApoE-/-Fbn1C1039G+/- mouse model responds to risk factors of atherosclerosis, such as high blood pressure and inflammation. Therefore, we administered Ang II for a period of 4 weeks and assessed plaque stability, the presence of myocardial infarctions (MI) and sudden death.

METHODS

Mice

Female ApoE-/-Fbn1C1039G+/- mice (C57BL/6J background) were fed a Western diet (WD; TD88137, Harlan Teklad) starting at an age of 6 weeks. The animals were housed in a temperature-controlled cage with a 12-hour light/dark cycle and had free access to water and food. Cases of sudden death were documented. At the end of the experiment (18 weeks WD), body weight was determined and plasma samples were obtained from the retro-orbital sinus of anaesthetized mice (xenon pentobarbital 75 mg/kg, i.p.). Subsequently, the mice were sacrificed with carbon dioxide (2.5% of the normal concentration). The heart and aortic arch were collected, weighed and analysed. Analysis of total plasma cholesterol was performed via a commercially available kit (Randox, Crumlin, UK). The animal procedures were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and all experiments were approved by the ethics committee of the University of Antwerp.

Angiotensin II infusion

At 14 weeks WD, ApoE-/-Fbn1C1039G+/- mice were anaesthetized with sevoflurane (8% for induction and 4.5% for maintenance, SevoFlo®, Penlon vaporizer) and subcutaneously implanted with osmotic minipumps (Alzet, model 1004) filled with either Ang II (1,000 ng/kg/day, n = 12, Sigma) or phosphate buffered saline (control, n = 11). The minipumps allowed continuous delivery of Ang II over a period of 28 days.

Blood pressure measurements

Peripheral blood pressure was measured at week 14 (baseline), 16 and 18 of WD in conscious mice via a tail cuff. Mice were placed in Plexiglas restrainers in a heating chamber (37°C) and kept in the dark. Ten minutes of acclimatization was allowed before measurements were initiated. At the distal end of the tail, a pulse sensor and occluding cuff controlled by a programmed Electro-Sphygmomanometer (Narco Bio-systems, Austin, TX) were placed. Voltage output from the sensor and cuff were recorded and analyzed by a Powerlab signal transduction unit and associated Chart software (ADInstruments, Colorado Springs, CO). To be accustomed to the procedure, mice were trained in four tutorials before the baseline measurement. At least eight separate pressure measurements were averaged from each animal on each recording day.

Histology

After sudden death or sacrifice of ApoE-/-Fbn1C1039G+/- mice, the proximal ascending aorta and the heart were collected. After determining heart weight, tissues were fixed in 4% formaldehyde (pH 7.4) for 24 hours, dehydrated overnight in 60% isopropanol and embedded in paraffin. Serial cross sections (5 µm) of the proximal ascending aorta and heart were prepared for histological analysis. Atherosclerotic plaque size, the length of the internal elastic lamina (IEL), stenosis and necrotic core (acellular areas with a threshold of 3,000 µm²) of the proximal ascending aorta were analyzed on hematoxylin-eosin (H-E) stained sections. Immunohistochemical staining with a primary antibody against Mac-3 (553322, Pharmingen, San Diego, CA) was used to determine the percentage of macrophages in the plaques. Collagen content was measured in Sirius red stained sections. Fibrous cap thickness was determined as the median value of 10 measurements per atherosclerotic plaque on α-SMC actin (Sigma, St Louis, MO) stained sections. The occurrence of myocardial infarctions, coronary plaques and perivascular fibrosis, measured as the perivascular collagen area divided by the luminal area (PVCA/LA) of 10 coronary arteries (microcirculation) per mouse, was analyzed on Masson’s trichrome stained sections (cut from the middle of the heart to the apex). If plaques were present in the coronary arteries (microcirculation), plaque size and percentage stenosis were measured on Masson’s trichrome stained sections. All images were acquired with Universal Grab 6.1 software using an Olympus BX40 microscope and were quantified with Image J software.

Statistical analysis

All data are expressed as means ± SEM. Statistical analysis was performed using SPSS software (version 20, SPSS Inc., Chicago, IL). Statistical tests are specified in the figure legends. Differences were considered significant at P < 0.05.
Angiotensin II induces myocardial infarctions of macrophages, fibrous cap thickness and collagen content (table 2).

Aneurysms in the thoracic aorta

Treatment with Ang II resulted in severe aneurysm formation in the thoracic aorta of ApoE-/-Fbn1C1039G+/-mice, which impaired atherosclerotic plaque evaluation in this region (figure 1).

In the Ang II treated group, 50% was diagnosed with an aneurysm. In contrast, no aneurysms were seen in control mice (Pearson’s chi-squared test; \( P = 0.006 \)).

Coronary plaques, perivascular fibrosis and heart weight

The number of mice showing plaque in the coronary arteries was slightly, but not significantly increased after Ang II infusion (table 3). Coronary plaque size and the degree of stenosis was not different between both treatment groups (table 3 and figure 2A).

Perivascular fibrosis of the coronary arteries was measured by dividing the perivascular collagen area by the luminal area of the blood vessel. This fibrosis was significantly more pronounced in Ang II treated mice (25 ± 4% versus 15 ± 2% in control mice; figure 2A and B).

As a result of Ang II infusion, heart weight (divided by body weight) of ApoE-/-Fbn1C1039G+/-mice was significantly higher than in control conditions (control: 0.8 ± 0.1% versus Ang II: 1.7 ± 0.4%; figure 2C).

Results

Blood pressure

Baseline measurements before onset of Ang II treatment, did not show differences in systolic blood pressure (the BP), diastolic blood pressure (Dia BP) and pulse pressure (PP) between both groups (table 1). Treatment with Ang II did not influence the BP, the PP and PP over time excepted for trends (table 3).

Table 1

<table>
<thead>
<tr>
<th>Weeks treatment</th>
<th>0</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>Control 134 ± 6</td>
<td>132 ± 4</td>
<td>131 ± 5</td>
</tr>
<tr>
<td>Angiotensin II 132 ± 5</td>
<td>119 ± 2</td>
<td>144 ± 11</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>Control 113 ± 6</td>
<td>109 ± 4</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>Angiotensin II 108 ± 4</td>
<td>97 ± 3</td>
<td>126 ± 11</td>
<td></td>
</tr>
<tr>
<td>Pulse pressure (mm Hg)</td>
<td>Control 21 ± 2</td>
<td>23 ± 3</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Angiotensin II 24 ± 3</td>
<td>22 ± 3</td>
<td>18 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM, BP: blood pressure, Repeated measures ANOVA; \( P > 0.05 \).

Table 2

<table>
<thead>
<tr>
<th>Control</th>
<th>Angiotensin II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque size ( \times 10^3 ) ( \mu )m²</td>
<td>629 ± 88</td>
</tr>
<tr>
<td>Internal elastic lamina ( \mu )m</td>
<td>3,789 ± 212</td>
</tr>
<tr>
<td>Stenosis (%)</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>Necrotic core (%)</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Fibrous cap thickness ( \mu )m</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Collagen (%)</td>
<td>33 ± 3</td>
</tr>
</tbody>
</table>

Data from proximal ascending aorta, mean ± SEM, control n = 9, Ang II n = 8-9, Independent samples t test; \( P > 0.05 \).
Myocardial infarctions and survival

The occurrence of MI was significantly higher in Ang II treated mice as compared to controls (table 3). In the Ang II group 58% of mice developed MI until the end of the experiment, which was not different from control mice where survival was 73%.

DISCUSSION

It is known that Ang II can promote the development of atherosclerotic plaques by increasing systemic blood pressure4. However, in the present study no elevation in blood pressure was observed. Similar studies in mice

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Coronary plaque parameters and occurrence of myocardial infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>Coronary plaque (%)a</td>
<td>27 (3/11)</td>
</tr>
<tr>
<td>Coronary plaque size (µm²)</td>
<td>3,368 ± 1,693</td>
</tr>
<tr>
<td>Coronary stenosis (%)</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>Myocardial infarctions (%)b</td>
<td>9 (1/11)</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM, control n = 3-11, Ang II n = 5-12, Independent samples t test; *P < 0.05.

a Percentage of mice showing coronary plaques, Pearson's chi-squared test; P > 0.05.
b Percentage of mice showing myocardial infarctions, Pearson's chi-squared test; *P < 0.05.

Fig. 2

Coronary perivascular fibrosis and heart weight.

(A) Trichrome Masson staining of the coronary arteries showed the presence of fibrosis and atherosclerotic plaques, with no difference in plaque size and the degree of stenosis between control and Ang II treated mice. Fibrosis was more pronounced after Ang II treatment and the example shows a coronary artery in close proximity to an infarcted area (P = plaque, L = lumen, scale bar = 50 µm).

(B) The percentage of perivascular fibrosis (perivascular collagen area divided by luminal area = PVCA/LA) was significantly increased in Ang II treated mice as compared to controls (control n = 10, Ang II n = 11, Independent samples t test; *P < 0.05).

(C) Heart weight divided by body weight (bw) was higher in mice that received Ang II treatment (control n = 7, Ang II n = 9, Independent samples t test; *P < 0.05).
Angiotensin II induces myocardial infarctions

This might be caused by a vicious cycle, in which Ang II as a compensatory cardiovascular cell. Moreover, under certain conditions, Ang II may promote plaque development and inflammation independent of its haemodynamic function. Furthermore, we cannot exclude that the chronic increase in cardiac fibrosis is partly mediated by direct effects of Ang II on mesenchymal fibroblasts. Therefore, the production of transforming growth factor-β (TGF-β) was induced. TGF-β is known to inhibit platelet aggregation, promote smooth muscle cell proliferation and induce fibrosis.

In conclusion, Ang II treatment in ApoE-/-Fbn1C1039G+/- mice is more severe than in ApoE-/- mice. This is in contrast with previous studies that report accelerated plaque development and enhanced inflammation in ApoE-/- mice when Ang II treatment was started before the onset of atherosclerosis. A limitation of the present study is the lack of echocardiographic data, which could have provided additional information on left ventricular function and cardiac remodelling. Nevertheless, the occurrence of MI, coronary perivascular fibrosis and cardiac hypertrophy was significantly increased. Ang II was also observed to induce abdominal aortic aneurysms in ApoE-/- mice. In contrast, we previously reported that chronic mental stress, another clinically relevant risk factor, did increase plaque vulnerability and atherosclerotic plaque size and composition. However, in this study, a significant increase in heart weight was not observed in the ApoE-/-Fbn1C1039G+/- mice. In contrast, Ang II treatment did not affect blood pressure, atherosclerotic plaque size and composition. Furthermore, the occurrence of MI, coronary perivascular fibrosis and cardiac hypertrophy in ApoE-/-Fbn1C1039G+/- mice was not accompanied by an increased presence of transforming growth factor-β (TGF-β) immunoreactivity, which proves the validity of the ApoE-/-Fbn1C1039G+/- mouse as a model for plaque rupture and supports the use of this model to evaluate potential plaque-stabilizing therapies.

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CONFLICT OF INTEREST

none.
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