Decreased Tissue Levels of Cyclophilin A, a Cyclosporine A Target and Phospho-ERK1/2 in Simvastatin Patients with Abdominal Aortic Aneurysm

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WHAT THIS PAPER ADDS
In this paper we propose a new anti-inflammatory role for simvastatin in human abdominal aortic aneurysm (AAA) wall tissue. Simvastatin interferes with cyclophilin A (CyPA) formation and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) activation. CyPA, a cyclosporine A-binding protein, influences AAA formation and the ERK1/2 signalling pathway in animal and in vitro studies and CyPA-deficient mice are resistant to aneurysm formation. Statins decrease CyPA in smooth muscle cells but their influence on CyPA in human AAA is unknown. Therefore, investigating the statins in cardiovascular disease (CVD), such as AAA, may reveal new therapeutic effects of those drugs.

Background: Cyclophilin A (CyPA), a cyclosporine A-binding protein, influences abdominal aortic aneurysm (AAA) formation and the ERK1/2 signalling pathway in animal and in vitro studies. Statins decrease CyPA in smooth muscle cells although their influence on CyPA in human AAA is unknown.

Material and methods: The study was performed on AAA wall-tissue samples obtained from 30 simvastatin-treated and 15 non-statin patients (2:1 case to control). The patients were matched by age, sex and AAA diameter. We investigated the gene expression of CyPA, its receptor extracellular matrix metalloproteinase inducer (EMMPRIN) by real-time RT-PCR. CyPA and EMMPRIN protein level and phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1/2) were measured by Western blot.

Results: The AAA wall tissue from simvastatin-treated patients had significantly lower CyPA gene expression and protein levels ($P = 0.0018$, $P = 0.0083$, respectively). Furthermore, phosphorylation of ERK1 and ERK2 was markedly suppressed in the simvastatin group ($P = 0.0002$, $P = 0.0027$, respectively). However, simvastatin did not influence EMMPRIN gene and protein expression.

Conclusion: Simvastatin-treated patients with AAA exert lower CyPA messenger RNA (mRNA), as well as CyPA intracellular protein levels and a decreased amount of phospho-ERK1/2. Thus, the interference with signalling pathways leading to CyPA formation and ERK1/2 activation reveals a new anti-inflammatory role of statins in AAA.

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Keywords: Cyclophilin A, Simvastatin, Abdominal aortic aneurysm, ERK1/2, Statins, Inflammation

Abdominal aortic aneurysms (AAAs) are an important health issue in the elderly, affecting approximately 10% of individuals over 65.1 In Western countries AAA may cause as much as 2% of all deaths.2 At our unit 1198 patients with AAAs have been treated within the last 12 years.

From the morphological point of view, the AAA is considered as a dilatative form of atherosclerosis that is critically connected with inflammation, oxidative stress and degradation of the cellular matrix.3,4 Overproduction of reactive oxygen species (ROS) is regarded as an important driver that lies upstream of the inflammatory cascade. Some mediators, which are induced in response to ROS, were named secreted oxidative stress-induced factors (SOXFs). Cyclophilin A (CyPA), a chaperone that binds cyclosporine A, is highlighted as a major SOXF.5–7 Elevated levels of CyPA have been reported in advanced atherosclerosis lesions3 and up-regulation of intracellular and
extracellular CyPA occurs during AAA formation in mice. Furthermore, excessive free radical generation during ischaemia/reperfusion leads to increased CyPA production by leucocytes and macrophages. Further, increased CyPA protein levels are observed in polymorphonuclear neutrophils (PMNs) of AAA patients. Cerebrovascular disease (CVAD) was defined by a history of transient ischaemic attack, stroke, carotid artery stenting or surgery, respectively. Cardiac insufficiency was defined by a global ejection fraction of <50% in echocardiography; all patients were without cardiac symptoms at the time of the surgery. Peripheral artery disease was defined by symptomatic claudication and a corresponding finding in the CTA at the level of the iliac and/or femoro-popliteal vessels.

Hypertension was defined by the intake of antihypertensive and/or a repeatedly elevated blood pressure exceeding 140 over 90 mmHg. Type 2 diabetes was defined by the intake of antidiabetics or requirement of insulin. Dyslipidaemia was defined as recommended in the European Society of Cardiology/European Atherosclerosis Society (ESC/EAS) guidelines for the management of dyslipidaemia. Smoking (y/n) pertains to nicotine consumption within the last 3 years.

The study was approved by the local research Ethics Committee (EC 294/2009).

**Tissue harvesting**

After aortic cross-clamping and longitudinal incision of the aneurysm, the thrombus (present in 39 of 45 patients) was removed and about 3 cm² of the aneurysm sac was excised at the site of its maximum diameter. Aneurysm samples were immediately frozen in liquid nitrogen and stored at −80 °C. For subsequent analysis aneurysm tissue was processed on ice. The aneurysm wall was divided into 50-mg pieces and rinsed with ice-cold saline to eliminate the liquid components, such as blood and residual thrombi.

**Western blot analyses of CyPA, EMMPRIN and ERK1/2**

Equal protein amounts of tissue extracts were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and CyPA, EMMPRIN, p42, phospho-42, p44 and phospho-44 were assessed by Western blotting using the respective rabbit anti-human monoclonal antibodies followed by horseradish peroxidase (HRP)-conjugated donkey secondary antibodies (Abcam, Cambridge, UK and Cell Signalling, Danvers, MA, USA, respectively). Signal intensity was quantified using an Imagine Master VDS (BioRad Laboratories Inc., Hercules, CA, USA) and normalised to β-tubulin. Assays were performed twice with tissue samples from different donors.

**Real-time polymerase chain reaction for CyPA and EMMPRIN**

Frozen tissue was homogenised using a ball mill (Retsch, Haan, Germany), and mRNA was isolated using the High Pure RNA Tissue Kit (Roche, Basel, Switzerland). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time polymerase chain reaction (PCR) was performed using the LightCycler® TaqMan® Master (Roche) according to the manufacturer’s instructions. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5′-AGCCACATCGCTCAGACAC-3′,
Statistical analysis
Continuous demographic and biochemical data are presented as median, minimum and maximum, and demographic categorical data are described with absolute frequencies and percentages. Data are 2:1 matched in the simvastatin and non-statin groups. A generalised linear model (binomial, logit) with an exchangeable correlation matrix was used to analyse matched binary outcome data. A linear mixed model with a compound symmetry variance-covariance matrix was used to analyse matched continuous outcome data. In the case of skew residuals no difference in the tissue gene expression and the protein level of EMMPRIN in the two examined groups (Fig. 1(A)). Similarly, the intracellular CyPA protein level was markedly decreased in the simvastatin group when compared to the non-statin group (P = 0.0083, Fig. 1(B) and (C)). However, there was no difference in the tissue gene expression and the protein level of EMMPRIN in the two examined groups (P = 0.6495 and P = 0.408, respectively; Fig. 2(A) and (B)). Fig. 2(C) shows a representative Western blot including three simvastatin and two non-statin patients.

Simvastatin decreases CyPA gene expression and protein level in human AAA wall explants
The tissue gene expression of CyPA from AAA patients treated with simvastatin was significantly lower than in the non-statin group (P = 0.0018, Fig. 1(A)). Similarly, the intracellular CyPA protein level was markedly decreased in the simvastatin group when compared to the non-statin group (P = 0.0083, Fig. 1(B) and (C)). However, there was no difference in the tissue gene expression and the protein level of EMMPRIN in the two examined groups (P = 0.6495 and P = 0.408, respectively; Fig. 2(A) and (B)). Fig. 2(C) shows a representative Western blot including three simvastatin and two non-statin patients.

Simvastatin reduces pERK1/2 in human AAA wall explants
In Fig. 3(A) the densitometric analysis comparing 30 simvastatin and 15 non-statin patients is shown (2:1 case to control). The amounts of phospho-p42/p44 (pERK1) and phospho-p44/p44 (pERK2) were significantly lower in AAA tissues from simvastatin patients when compared to the non-statin group (P = 0.0002 and P = 0.0027, respectively) (Fig. 3(A) and (B)). Fig. 3(C) shows a representative Western

Table 1. Patient demographics.

<table>
<thead>
<tr>
<th></th>
<th>Non-statin patients (n = 15)</th>
<th>Simvastatin patients (n = 30)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (range)</td>
<td>68 (50–73)</td>
<td>67 (55–80)</td>
<td>0.186</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>12 (80%)</td>
<td>24 (80%)</td>
<td>1.000</td>
</tr>
<tr>
<td>AAA diameter (mm)</td>
<td>57 (48–102)</td>
<td>55 (48–120)</td>
<td>0.439</td>
</tr>
<tr>
<td>Body mass index, mean (range)</td>
<td>27.55 (21.95–37.55)</td>
<td>25.89 (21.56–31.25)</td>
<td>0.025</td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>3 (20%)</td>
<td>9 (30%)</td>
<td>0.479</td>
</tr>
<tr>
<td>Cerebrovascular artery disease</td>
<td>10 (67%)</td>
<td>15 (50%)</td>
<td>0.161</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>4 (27%)</td>
<td>9 (30%)</td>
<td>0.803</td>
</tr>
<tr>
<td>Cardiac insufficiency</td>
<td>1 (7%)</td>
<td>8 (23%)</td>
<td>0.083</td>
</tr>
<tr>
<td>Hypertension</td>
<td>15 (100%)</td>
<td>28 (93%)</td>
<td>0.317</td>
</tr>
<tr>
<td>Type 2 diabetes</td>
<td>4 (27%)</td>
<td>8 (27%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Smoking</td>
<td>12 (80%)</td>
<td>22 (73%)</td>
<td>0.617</td>
</tr>
<tr>
<td>Cholesterol [mg/dl], median (range)</td>
<td>240 (143–323)</td>
<td>199 (110–264)</td>
<td>0.002</td>
</tr>
<tr>
<td>LDL [mg/dl], median (range)</td>
<td>164.4 (79.2–218)</td>
<td>112.5 (47–218)</td>
<td>0.003</td>
</tr>
<tr>
<td>HDL [mg/dl], median (range)</td>
<td>45.0 (36–68)</td>
<td>48.0 (29–75)</td>
<td>0.477</td>
</tr>
<tr>
<td>CRP [mg/dl], median (range)</td>
<td>0.43 (0.03–7.6)</td>
<td>0.43 (0.06–3.0)</td>
<td>0.291</td>
</tr>
<tr>
<td>Fibrinogen [mg/dl], median (range)</td>
<td>407 (280–594)</td>
<td>359 (240–549)</td>
<td>0.102</td>
</tr>
<tr>
<td>Leucocytes [mln/ml], median (range)</td>
<td>8.15 (5.5–12)</td>
<td>8.0 (5.09–13.0)</td>
<td>0.473</td>
</tr>
<tr>
<td>Creatinine [mg/dl], median (range)</td>
<td>1.05 (0.75–1.44)</td>
<td>0.99 (0.76–4.0)</td>
<td>0.907</td>
</tr>
</tbody>
</table>

Data are presented as frequencies or median (minimum–maximum). Statistical significance for binary variables was assessed using generalised linear models, while metric values were analysed using linear mixed regression models.

* The measurements were made in serum samples.
blot including three simvastatin and two non-statin patients.

**DISCUSSION**

In the present study we showed that AAA wall tissue from patients treated with simvastatin has lower gene expression and intracellular concentration of CyPA. Moreover, simvastatin-treated subjects with AAA had lower activity of the ERK1/2 signalling pathway compared to age-, sex- and AAA diameter-matched non-statin patients. However, we observed no difference in the gene expression and the protein level of EMMPRIN between the simvastatin and non-statin AAA wall tissue.

AAA development is a multifactorial process that depends to a great extent on macrophage-derived matrix metalloproteinase-9 (MMP-9) and VSMC-derived MMP-2. Statins were shown to interfere with AAA expansion; however their role remains controversial. Recently, Takagi et al. indicated that statin therapy might be effective in prevention of the growth of small AAA, while Karrow et al. showed no correlation. Further, controversies have arisen around statin influence on VSMC proliferation which number is decreased in the media of human AAA tissue. Here some studies highlight the anti-proliferative role of statins in the neointima while others described their proliferative action under hyperglycaemia. Yet it is not clear whether the induction of apoptosis in VSMC by statins is beneficial or detrimental. Nevertheless, it is apparent that a short course of cyclosporine A, a CyPA-binding drug, stabilises the diameter of formed AAA and increases VSMC content in an animal model.

It was previously demonstrated that AAA tissue had a higher concentration of CyPA than the healthy aorta, increased CyPA levels were found in PMNs in AAA patients. Therefore, drugs that influence CyPA concentration may impact AAA formation. Statins whose
pleiotropic actions include a decrease in the gene expression and protein concentration of MMP-3, MMP-9 and TNF-α and lead to an increase in antioxidant enzyme activity in human AAA tissue may play an important role. Here we present evidence that simvastatin treatment in patients with AAA significantly decreased CyPA in AAA wall on the gene and protein levels. Our results are in line with the study of Suzuki et al., who suggested that simvastatin inhibits vesicular secretion of CyPA in VSMC probably by inhibiting isoprenylation of small guanosine triphosphatases (GTPases). However, further studies are required to investigate the mechanism of simvastatin-induced decrease of CyPA in human AAA wall in detail.

In cells CyPA exerts its action when it binds to the EMMPRIN receptor, which is highly expressed in the diseased aortic wall of AAA patients. So far there are limited data about the influence of statins on EMMPRIN tissue expression. Abe et al. indicated that fluvastatin influences EMMPRIN expression in macrophages probably via its antioxidant properties. However, our study showed no difference in the gene expression and protein level of EMMPRIN in simvastatin and non-statin AAA wall tissue. This may be explained by the occurrence of different cell types such as VSMC, endothelial cells and immune cells in aneurysm tissue.

AAA tissue is characterised by up-regulated activity of different signalling transcription pathways including NF-κB and activator protein-1 (AP-1). Lately Ghosh et al. reported on increased activity of ERK1/2 in human AAA tissue and reduced AAA formation after ERK1/2 inhibition in animal models. Our results show a decreased amount of pERK1/2 in AAA wall tissue in simvastatin-treated patients compared to the non-statin group. ERK-mediated cell signals are essential for cellular proliferation, differentiation and survival, and improper activation of ERK1/2 is associated with immunological disorders. Both ERK isoforms are ubiquitously expressed in almost all mammalian tissues, with ERK-2 levels generally greater than ERK-1 levels. Recently, Bahmed et al. indicated that extracellular CyPA stimulates ERK1/2 phosphorylation in cancer cells and Jin et al. showed that CyPA mediates phosphorylation of ERK1/2 and IκBα of NF-κB in human endothelial cells. Therefore, decreased CyPA levels in the simvastatin group observed in our study may be causative for the blunted activity of ERK1/2. This corresponds to the report by Tristano et al. that the phosphorylation of ERK1/2 is inhibited by simvastatin in VSMC. Simvastatin also reduced c-Raf and Ras expression, the components of mitogen-activated protein kinase (MAPK) pathway, in VSMC during high glucose conditions.

Inhibition of phosphorylation of ERK1/2 by statins may be potentially dangerous regarding preservation of VSMC. It was indicated that simvastatin inhibits angiotensin II-mediated stimulation of ERK1/2 but simvastatin withdrawal escalates angiotensin II-mediated effect, therefore leading to VSMC degradation. Nevertheless, targeting the ERK1/2 pathway with drugs that inhibit either the expression CyPA or phosphorylation of ERK1/2 isoforms could be a potential therapeutic approach to prevent AAA formation as well as progression.

CONCLUSION

Our study demonstrates that simvastatin treatment reduces cyclophilin A gene expression and protein concentration in human AAA wall tissue as compared to the non-statin patients. Moreover, simvastatin inhibits phosphorylation of the ERK1/2 signalling pathway. Thus, decreased CyPA concentration in AAA tissue by simvastatin may influence ERK1/2 inhibition suggesting a yet-unknown anti-inflammatory action of statins in human abdominal aortic aneurysms.
ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

None.

REFERENCES


