

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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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

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extracellular CyPA occurs during AAA formation in mice.⁸ Furthermore, excessive free radical generation during ischaemia/reperfusion⁹ leads to increased CyPA production by leucocytes and macrophages.¹⁰ Further, increased CyPA protein levels are observed in polymorphonuclear neutrophils (PMNs) of AAA patients.¹¹

CyPA wields its action when binding to the extracellular matrix metalloproteinase inducer (EMMPRIN) receptor localised on vascular smooth muscle cells (VSMCs).¹² Recently Chen et al.¹³ demonstrated that EMMPRIN is strongly expressed in human AAA lesions. The overexpression of EMMPRIN leads to activation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2). The cascade of EMMPRIN—ERK—nuclear factor- κ B (NF- κ B) is speculated to be the main signalling pathway for CyPA in monocytes/macrophages.¹⁴

In our recent study we presented decreased activity of the NF- κ B signalling pathway and lowered level of ROS and tumour necrosis factor (TNF)- α in AAA tissue from patients treated with simvastatin.¹⁵ Simvastatin belongs to hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors which, among others, influence aneurysm formation.^{16,17} Suzuki et al.¹⁸ indicate that simvastatin decreases ROS-mediated CyPA release from VSMC. Additionally, simvastatin inhibits angiotensin II-mediated stimulation of ERK1/2 phosphorylation in VSMC.¹⁹ However, the role of statins in intracellular CyPA level and their influence on the ERK1/2 pathway in human AAA tissue remains elusive.

Therefore, the aim of the study was to assess the influence of simvastatin on cyclophilin A levels in AAA wall tissue. Next, we wanted to verify if the ERK1/2 signalling pathway is modulated by simvastatin in AAA wall tissue.

MATERIAL AND METHODS

Patients

The study was performed on 45 patients undergoing open AAA repair between September 2009 and December 2011 at our institution according to our previous study.¹⁵ Briefly, exclusion criteria were the intake of statins other than simvastatin, non-steroidal anti-inflammatory drugs except aspirin in the medication list, chronic diseases such as liver disease, inflammatory disease, malignant disease, recreational drugs' intake and alcohol abuse. After written informed consent, patient data were prospectively collected and aneurysm wall tissue was harvested during aneurysm repair for retrospective analysis. Patients were matched in a 2 (simvastatin) to 1 (non-statin) ratio, respectively, by age, gender and AAA diameter. Fifteen patients without statin medication (12 men, 3 women) were incorporated into the control group and 30 patients who had simvastatin (24 men, 6 women) in their medical history (20–40 mg daily dosage) for a minimum of 6 months were included in the study as the simvastatin group. The AAA diameter was measured with preoperative computed tomography angiography (CTA).

Coronary artery disease (CAD) was defined by a history of angina pectoris or myocardial infarction. 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.²⁰ 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 (Bio-Rad 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',

reverse primer: 5'-GCCCAATACGACCAAATCC-3'), CyPA (forward primer 5'-GTCAACCCACCGTGTCTTC-3', reverse primer: 5'-TTTCTGCTGTCTTTGGACCTTG-3') and EMMPRIN (forward primer: 5'-GGGAGAGTACTCTGCGTCTT-3', reverse primer: 5'-ACTTCACAGCCTTCACTCTGG-3'). The amplification conditions consisted of an initial incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 63 °C for 20 s and 72 °C for 6 s and a final cooling to 40 °C. Data were analysed using LightCycler Software Version 3.5 (Roche).

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 a logarithmic transformation usually led to normally distributed errors.

All *P*-values are two-sided and $P \leq 0.05$ was considered significant. Statistical analyses were performed by the software package SAS (Version 9.3; SAS Institute Inc., Cary, NC, USA) and the software package Statistical Package for the Social Sciences (SPSS) (SPSS 17.0, Chicago, IL, USA) was used for graphics.

RESULTS

Demographic data

In Table 1, the characteristics for non-statin and simvastatin-treated patients are shown. The two groups

were comparable in age, aneurysm diameter, co-morbidities and risk factors. The median aneurysm diameter was 57 mm (48–102 mm) for the non-statin and 55 mm (48–120 mm) for the simvastatin patients. The simvastatin group had markedly lower body mass index ($P = 0.025$) and better lipid profile with significantly decreased total cholesterol and low density lipoprotein (LDL) cholesterol ($P = 0.002$ and $P = 0.003$, respectively). We found no differences in C-reactive protein (CRP), fibrinogen, creatinine and leucocyte levels among the two groups (all $P \geq 0.05$).

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/p42 (pERK1) and phospho-p44/p-44 (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.

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

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.

^a The measurements were made in serum samples.

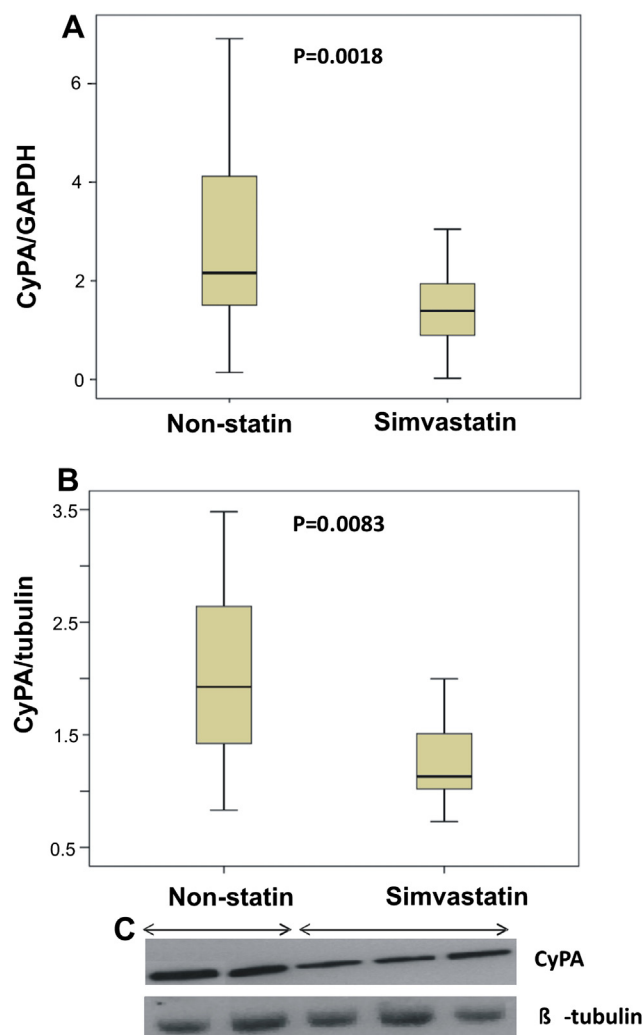


Figure 1. Simvastatin treatment suppressed CyPA mRNA expression and CyPA protein level in human AAA wall tissue. (A) CyPA mRNA expression. (B) Densitometrical quantification of CyPA protein level (mean values $\pm$ SD). (C) Representative Western blot ($n = 2$ and $n = 3$ for AAA tissue wall from the non-statin and simvastatin patients, respectively).

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-2.²¹ Statins were shown to interfere with AAA expansion; however their role remains controversial. Recently, Takagi

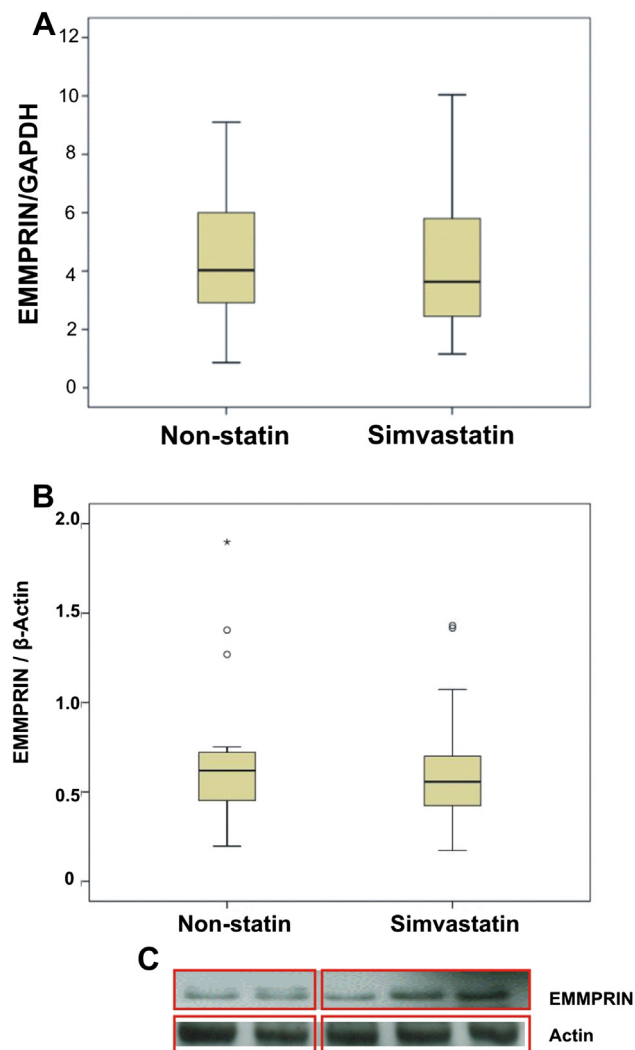


Figure 2. Simvastatin treatment had no influence on EMMPRIN mRNA expression and protein level in human AAA wall tissue. (A) EMMPRIN gene expression. (B) Densitometrical quantification of EMMPRIN protein level (mean values $\pm$ SD). (C) Representative Western blot ($n = 2$ and $n = 3$ for AAA tissue wall from the non-statin and simvastatin patients, respectively).

et al.¹⁷ indicated that statin therapy might be effective in prevention of the growth of small AAA, while Karrowini et al.²² showed no correlation. Further, controversies have arisen around statin influence on VSMC proliferation which number is decreased in the media of human AAA tissue.²³ Here some studies highlight the anti-proliferative role of statins in the neointima while others described their proliferative action under hyperglycaemia.^{24,25} 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.²⁶

It was previously demonstrated that AAA tissue had a higher concentration of CyPA than the healthy aorta;^{3,27} increased CyPA levels were found in PMNs in AAA patients.¹¹ Therefore, drugs that influence CyPA concentration may impact AAA formation. Statins whose

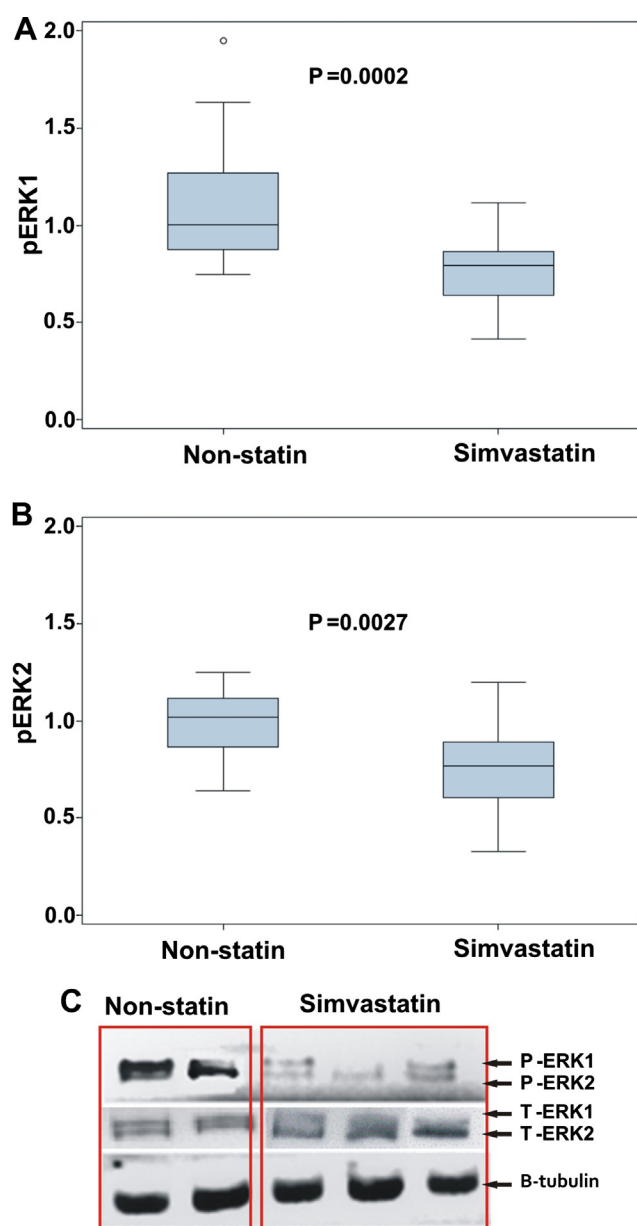


Figure 3. Simvastatin treatment inhibited p-ERK1 (A) and p-ERK2 (B) activation in human AAA wall samples. (C) Representative Western blot ($n = 2$ and $n = 3$ for AAA tissue wall from the non-statin and simvastatin patients, respectively). Data are present as the ratio of phosphorylated form to total form (fold) over tubulin and represents mean values $\pm$ SD.

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.^{15,28,29} 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.,¹⁸ who suggested that simvastatin inhibits vesicular secretion of CyPA in VSMC probably by inhibiting isoprenylation of small guanosine triphosphatases (GTPases).¹⁸ 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,³⁰ which is highly expressed in the diseased aortic wall of AAA patients.¹³ So far there are limited data about the influence of statins on EMMPRIN tissue expression. Abe et al.³¹ 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- κ B and activator protein-1 (AP-1).³² Lately Ghosh et al.³³ 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.³⁴ 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.³⁵ indicated that extracellular CyPA stimulates ERK1/2 phosphorylation in cancer cells and Jin et al.³⁶ showed that CyPA mediates phosphorylation of ERK1/2 and I κ B α of NF- κ B 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.¹⁹ 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.³⁷

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.³⁸ 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.

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CONFLICT OF INTEREST

None.

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