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# Simvastatin Decreases Free Radicals Formation in the Human Abdominal Aortic Aneurysm Wall via NF-κB

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## WHAT THIS PAPER ADDS

- Our work demonstrates that simvastatin exerts profound effects on free radical formation in aortic aneurysms. We propose nuclear factor-kappaB (NF-kB) being involved in the signalling events. To the best of our knowledge, our report shows the first human data in this special field.

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ABSTRACT

*Objectives:* Statins have been reported to suppress the progression of abdominal aortic aneurysm (AAA). However, the effects of statins on inflammatory processes and free radicals generation are poorly understood.
*Methods:* Wall samples from 51 patients (simvastatin patients, $n = 34$; non-statin patients, $n = 17$; matched by sex, age and aneurysm size) subjected to elective open AAA repair were analysed. We examined the effects of simvastatin on lipid peroxidation (4-hydroxy-trans-2-nonenal (4-HNE)), hydrogen peroxide ($H_2O_2$), tumour necrosis factor alpha (TNF-α) concentration, superoxide dismutase (SOD) and catalase (CAT) activity as well as nuclear factor kappa B (NF-κB) pathway activation in human AAA wall samples.
*Results:* Treatment with simvastatin resulted in a decrease in 4-HNE and TNF-α concentration (median 4.18 μg/mg protein vs. 4.75, $p = 0.012$; median 10.33 pg/ml vs. 11.81, $p = 0.026$, respectively). CAT activity was higher in the simvastatin group (median 3.98 U ml vs. 3.19, $p = 0.023$). NF-κB expression was lower ($p = 0.018$) in the simvastatin group. However, simvastatin had little effect on $H_2O_2$ concentration ($p = 0.832$) and SOD activity ($p = 0.401$).
*Conclusion:* Simvastatin inhibits free radicals and TNF-α generation and improves antioxidant capacity of human AAA wall tissue, possibly through the suppression of NF-κB activity. This may be one possible explanation how statins can inhibit AAA oxidative stress.


The abdominal aortic aneurysm (AAA) causes 1–2% of all male deaths over the age of 65 in western countries.[1] As opposed to the occlusive form of atherosclerosis, aneurysm may be considered as a dilatative form of atherosclerosis.[2] In atherosclerotic plaques excessive recruitment of monocytes, macrophages, T cells, B cells and natural killer (NK) cells is observed.[3] Their activation leads to secretion of humoral inflammatory factors and reactive oxygen species (ROS).

ROS play a crucial role in the genesis of vascular pathologies and lipid peroxidation.[4] It has been shown that human aneurysmal aorta is characterised by increased oxidative damage and pro-

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oxidant enzyme overexpression.[5] ROS lead to the activation of intracellular signalling pathways such as c-Jun N-terminal kinase (JNK) or nuclear factor kappa B (NF-κB).[6] NF-κB is a redox-sensitive transcription factor activated by the ROS in arterial diseases.[7] Moreover, activation of NF-κB can be elicited by a wide range of stimuli, including vascular injury, cytokines and bacterial endotoxines.[8,9] Normally, NF-κB is kept inactive in the cytoplasm by binding to inhibitory proteins, namely, the IκB family. Upon activation, phosphorylated NF-κB translocates to the nucleus, where it leads to the transcription of proinflammatory genes.[10] Inhibition of NF-κB, at least in part, could counteract the toxic effects of ROS.

Statins, beyond their effect on lipid profile, also exert pleiotropic effects on endothelial function, vascular inflammation and immunomodulation.[11,12] One of the commonly used statins is simvastatin. It decreases tumour necrosis factor alpha (TNF-α) serum level and suppresses aneurysm expansion in adult rats, as well as reducing matrix metalloproteinases 9 (MMP-9) and NF-κB levels, when used in animal models of hypercholesterolaemia.[13–15] Simvastatin decreases levels of different interleukins (IL-4, -5, -6, -8 and -10), interferon gamma (INF-γ), TNF-β and MMP-8 in AAA. It also inhibits T lymphocytes and macrophages recruitment in the AAA walls.[16]

However, the effects of simvastatin on ROS concentration in AAA tissues have not been characterised yet. Therefore, we have here compared human AAA tissue samples from patients, treated with or without simvastatin, to determine whether simvastatin treatment influences oxidative stress and antioxidant properties of human AAA wall tissue. Moreover, we wanted to verify whether the NF-κB pathway in human AAA wall may be influenced by simvastatin treatment.

## Material and Methods

### *Patients*

Between September 2009 and November 2011, 98 patients underwent elective open AAA repair at our institution. Exclusion criteria for our study were the intake of statins other than simvastatin, chronic diseases such as liver disease, malignant disease, drug intake or alcohol abuse. Of the patients eligible for our study, only 17 patients received no statin therapy (14 men and three women). Following a written informed consent, patient data were prospectively entered into a database. Subsequently, the aneurysm wall tissues were harvested during aneurysm repair procedures, to be used in retrospective analysis. Patients were matched in a 2 (simvastatin) to 1 (non-statin) ratio, respectively, by age, gender and AAA diameter. Accordingly, 34 patients who received simvastatin (29 men and five 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. For one 2:1 match with a female non-statin patient, only one comparable female patient within the simvastatin group was available; and a male simvastatin patient was matched instead. The AAA diameter was determined with preoperative computed tomography angiography (CTA).

Coronary artery disease (CAD) was defined by a history of angina pectoris or myocardial infarction. Cerebrovascular disease (CVAD) is defined by a history of transient ischaemic attack, stroke, carotid artery stenting or surgery, respectively. Patients with asymptomatic high grade (>85%) carotid stenosis, diagnosed by duplex sonography or CTA were subjected to carotid artery surgery prior to aneurysm repair (one patient in each group). Heart insufficiency was defined by a global ejection fraction of <50% in echocardiography. 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 antihypertensives 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 and/or insulin resistance. Nicotine (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*

The aorta was approached transperitoneally. After aortic clamping and longitudinal incision of the aneurysm, thrombus (present in 82 of 98 patients) was removed and about 3 $cm^2$ of the aneurysm sack at the site of its maximum diameter were excised. Aneurysm samples were immediately frozen in liquid nitrogen and stored at −80 °C. For subsequent analysis, aneurysm tissue was processed on ice. Aneurysm wall was divided into 50 mg pieces and rinsed with ice-cold saline to eliminate liquid components, such as blood and residual thrombi.

### *Lipid peroxidation level measured as 4-hydroxy-trans-2-nonenal (4-HNE) level*

We measured concentrations of 4-HNE, a natural bi-product of lipid peroxidation, using an enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA, USA). Briefly, sample or standard 4-HNE protein adducts were probed with the primary antibody, followed by horseradish peroxidase (HRP)-conjugated secondary antibody. The absorption was read at 450 nm (Perkin Elmer, Victor 3 reader, MA, USA). Values were expressed as μg/mg of protein. Assays were performed twice with three different tissue scraps.

### *Hydrogen peroxide measurement*

Tissue samples were homogenised in 1.15% KCl using a tissue raptor (IKA Werke GmbH & Co. KG, Germany). Next, a mixture of phosphate-buffered saline (PBS) (pH 7.0) and HRP (1 U/ml; HRP) containing 400 μM homovanilic acid (HVA) was added to assay HRP ± HVA, while PBS and 1 U/ml HRP was added to the other tube to assay HRP. After 60 min of incubation in 37 °C, PBS and 0.1 M glycine–NaOH buffer (pH 12.0) with 25 mM ethylenediamine tetra acetic acid (EDTA) were added to stop the enzymatic reaction. Fluorescence was measured at excitation wave (312 nm) and emission wave (420 nm) using a fluorescence spectrophotometer F4500 (Hitachi, JPN). Readings were converted into hydrogen peroxide ($H_2O_2$) concentration (μmol/mg protein) using the regression equation and normalised to aortic weight. Assays were performed twice with three different tissue scraps.

### *Measurement of catalase and superoxide dismutase activity*

Frozen aortic wall sample was homogenised and tissue catalase (CAT) or superoxide dismutase (SOD) activity was measured spectrophotometrically using respective assay kits (Cayman Chemical, NC, USA) following the manufacturer's instruction. The absorption was read at 540 nm for CAT and at 450 nm for SOD assay (Perkin Elmer, Victor 3 reader, MA, USA). Values were expressed as pg/ml and normalised to total protein. Assays were performed twice with three different tissue scraps.

### *Immunoenzymatic assay of TNF-α concentration*

Fifty milligrams of frozen aortic wall was gridded in HEPES buffer (20 mM HEPES (pH 7.6), 1.5 mM EDTA, 0.5 mM benzamidine

and enzyme inhibitors). After centrifugation (18 000 r.p.m., 4 °C, 20 min), the supernatant was diluted and the TNF-α levels were quantified according to the manufacturer's instructions using specific ELISA kits (Quantikine TNF-α, R&D Systems, MN, USA). The absorption was read at 450 nm (ELx800 Bio-Tech Instruments Reader, VT, USA). Values were expressed as pg/ml and normalised to total protein. Assays were performed twice, with three different tissue scraps.

*Western blot analyses of p65 and phosphorylated p65 (pp65)*

Equal protein amounts of nuclear extracts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and p65 and pp65 were assessed by Western blotting using the anti-rabbit p65 or pp65 antibody (Santa Cruz Biotechnology, CA, USA and Cell Signaling, MA, USA, respectively). Signal intensity was quantified using an Imagine Master VDS (Bio-Rad, CA, USA) and normalised to β-tubulin. Assays were performed twice with different tissue scraps.

*Statistical analysis*

Continuous demographic and biochemical data are presented as median, minimum and maximum, demographic categorical data are described with absolute frequencies and percentages. Data are 2:1 matched in non-statin and simvastatin 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 case of skew residuals, a logarithmic transformation usually led to normally distributed errors. Only in two variables (creatinine and SOD), it was necessary to exclude an extreme outlier.

All $p$-values are two-sided and $p \leq 0.05$ was considered significant. Statistical analyses were performed by the software package Statistical Analysis Software (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 non-statin patients and simvastatin patients are compared. The two groups are well comparable in age, aneurysm diameter, co-morbidities and risk factors. The median aneurysm diameter was 55 mm (48–120) for the non-statin and 55 mm (48–120) for the simvastatin patients. The level of total cholesterol and low-density lipoprotein (LDL) cholesterol was lower ($p = 0.017$ and 0.057, respectively) in the simvastatin group. C-reactive protein (CRP) level, fibrinogen, creatinine and leukocyte count did not differ among the two groups.

*The influence of simvastatin on tissue oxidative stress and antioxidant status in AAA*

The influence of simvastatin on oxidative stress parameters and tissue antioxidant status is shown in Table 2. In the simvastatin patients, lipid peroxidation level, expressed as the 4-HNE concentration, was significantly lower ($p = 0.012$) compared with the non-statin patients. $H_2O_2$ level was comparable in both groups ($p = 0.832$).

The simvastatin patients had increased activity of CAT ($p = 0.023$), but not SOD activity in AAA wall samples ($p = 0.650$).

**Table 1**
Patient demographics. Data are presented as frequencies or median (minimum–maximum).

| Parameter | Non-statin patients ($n = 17$) | Simvastatin patients ($n = 34$) | $p$ |
|---|---|---|---|
| Age (years), median (range) | 69.83 (50.3–75.3) | 67.29 (55.6–80.3) | 0.786 |
| Sex (male) | 14 (82%) | 29 (85%) | 0.793 |
| AAA diameter ($r$) (mm) | 55 (48–120) | 55 (48–120) | 0.102 |
| Body mass index, mean (range) | 27.2 (23–37.6) | 26.0 (22–34.5) | 0.282 |
| Cholesterol [mg/dl], median (range) | 238 (143–323) | 203.5 (110–300) | 0.017 |
| LDL [mg/dl], median (range) | 144 (60–218) | 117 (56–218) | 0.057 |
| HDL [mg/dl], median (range) | 46.0 (20–68) | 48.0 (29–75) | 0.514 |
| Coronary artery disease | 3 (18%) | 8 (23%) | 0.647 |
| Cerebrovascular artery disease | 0 (0%) | 2 (6%) | 0.547 |
| Peripheral artery disease | 3 (18%) | 12 (35%) | 0.123 |
| Heart insufficiency | 2 (12%) | 7 (21%) | 0.491 |
| Hypertension | 15 (88%) | 31 (91%) | 0.754 |
| Type 2 diabetes | 3 (18%) | 7 (21%) | 0.822 |
| Nicotine | 12 (70%) | 24 (70%) | 1.000 |
| CRP [mg/dl], median (range) | 0.42 (0.03–3.0) | 0.40 (0.06–9.45) | 0.946 |
| Fibrinogen [mg/dl], median (range) | 384 (260–557) | 364.5 (213–650) | 0.733 |
| Leucocytes [mln/ml], median (range) | 8.05 (5.5–11.6) | 8.0 (5.09–13.0) | 0.642 |
| Creatinine [mg/dl], median (range) | 1.21 (0.77–1.48) | 1.05 (0.76–4.0) | 0.638[a] |

Statistical significance for binary variables was assessed using generalized linear models, while metric values were analysed using linear mixed regression models.
[a] An extreme outlier of 4.0 was deleted in simvastatin group for $p$-value comparison.

*The influence of simvastatin on TNF-α concentration and NF-κB pathway activation*

TNF-α concentration in AAA samples of simvastatin patients was significantly decreased compared to non-statin patients ($p = 0.026$) as indicated in Table 2.

In Fig. 1(A), the densitometric analysis comparing 12 non-statin and 12 simvastatin patients is shown (1:1 case to control). The ratio of the phosphorylated p65 form over the total form of p65 was significantly lower ($p = 0.018$) compared to the non-statin patients. Fig. 1(B) shows a representative Western blot including three simvastatin and three non-statin patients.

## Discussion

Prospective clinical studies investigating the effect of simvastatin on AAA are challenging to conduct, because statins and acetyl salicylic acid represent standard therapy for concomitant atherosclerosis.[17] However, it has been increasingly appreciated that statins may interfere with signalling pathways involved in AAA.

**Table 2**
The influence of simvastatin on oxidative stress parameters (lipid peroxidation and hydrogen peroxide level), antioxidant power (antioxidant enzymes activity) and TNF-α concentration in human AAA wall homogenates (median (min–max)).

| | Non-statin ($n = 17$) | Simvastatin ($n = 34$) | $p$ |
|---|---|---|---|
| 4-HNE (μg/mg protein) | 4.75 (2.91–17.16)[b] | 4.18 (1.21–7.83)[b] | 0.012 |
| $H_2O_2$ (μmol/mg protein) | 0.16 (0.03–1.89) | 0.23 (0.04–1.01) | 0.832 |
| SOD activity (U/ml) | 0.17 (0.14–0.26) | 0.18 (0.14–0.55) | 0.650[a] |
| CAT activity (nmol/min/ml) | 3.19 (1.29–5.34) | 3.98 (1.62–7.10) | 0.023 |
| TNF-α (pg/ml) | 11.81 (7.53–19.75) | 10.33 (8.39–13.68) | 0.026 |

4-HNH – 4-hydroxy-trans-2-nonenal; $H_2O_2$ – hydrogen peroxide; SOD – superoxide dismutase; CAT – catalase.
[a] An extreme outlier of 0.55 was deleted in simvastatin group for $p$-value comparison.
[b] $n = 14$ and $n = 30$ in the non-statin and the simvastatin group, respectively.

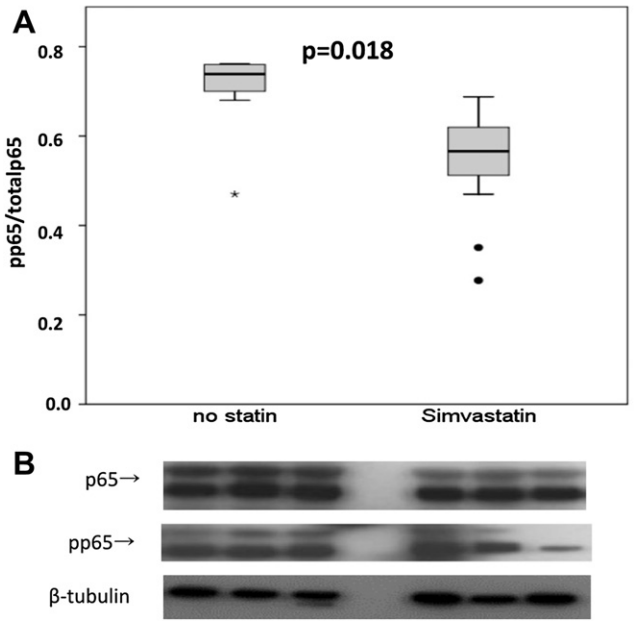


**Figure 1.** Simvastatin treatment counteracts NF-κB activation in abdominal aortic aneurysm wall samples. (A) Densitometrical quantification of p65 shown as the ratio of the phosphorylated form over the total form of the protein ($n = 12$ in the non-statin and $n = 12$ in the simvastatin group, 1:1 case–control). (B) Representative Western blot.

We hence intended to explore in *ex vivo* studies whether simvastatin treatment influences oxidative stress and NF-κB p65 subunit levels in human AAA-tissue.

AAA tissue is characterised by enhanced infiltration of macrophages and lymphocytes, which are a source of ROS such as superoxide anion ($O_2^{\cdot-}$), $H_2O_2$ and nitric oxide.[18] In our present study, the general level of ROS, indicated by lipid peroxidation, in the aneurysm tissue was markedly upregulated in the non-statin group. This effect was reversed by simvastatin. This is in line with Yoon et al.,[19] who evidenced that simvastatin, in a dose-dependent manner, decreases intracellular ROS formation in vascular smooth muscle cells. In addition, some studies performed on rabbits with atherosclerosis have suggested that simvastatin and atorvastatin could act as an antioxidant and inhibit oxidation of LDL by activated monocytes-derived macrophages.[20,21]

In our work, simvastatin significantly increased CAT activity, but had no effect on $H_2O_2$ concentration and SOD activity. In cells, $H_2O_2$ is generated from $O_2^{\cdot-}$ in a reaction catalysed by SOD. Therefore, no increase in SOD activity may be a possible explanation for the unchanged $H_2O_2$ level in our study. Moreover, changes in CAT levels without alteration in SOD concentration were observed previously.[22] Wassmann et al.[20] showed that atorvastatin upregulates CAT activity *in vitro* and *in vivo*, whereas it elicits no influence on the expression of SOD and glutathione peroxidase.

To date, little is known about the influence of statins on $H_2O_2$ generation in aortic tissue, mainly since $H_2O_2$ has been employed as ROS generator in experimental models investigating the antioxidant role of statins.[23] We, thus, speculate that in our study an increase in CAT activity was not accompanied by $H_2O_2$ depletion due to the high heterogeneity of AAA tissue, as well as a randomised localisation of inflammatory cells which are the main source of $H_2O_2$. It is thought that statins may increase CAT activity by activating Akt signalling pathway.[23] However, the exact molecular mechanisms by which statins influence the CAT activity remain to be investigated.

Several studies have shown that circulating and tissue levels of TNF-α were increased in patients with AAA, when compared with individuals without AAA.[24,25] Furthermore, in the mouse AAA models, the blockade of TNF-α attenuated aneurysm formation.[26] In our study, simvastatin treatment significantly decreased TNF-α concentration in human AAA tissue. This may be attributed to the anti-inflammatory action of statins. Simvastatin has recently been shown to decrease monocyte secretion of TNF-α, IL-6 and IL-1β in patients with hypercholesterolaemia.[27] Simvastatin also decreased TNF-α, IL-6 and inhibited NF-κB in a rat model of cardiopulmonary bypass.[28]

Oxidative stress in the healthy vascular wall leads to a profound activation of the NF-κB pathway[29] and a strong activation of NF-κB characterises aneurysmal tissue.[30] A series of animal studies have demonstrated that NF-κB inhibition may result in the prevention of experimental AAA suggesting that NF-κB may be a key transcription factor in aneurysm formation.[31,32] Moreover, NF-κB signalling pathway activation leads to MMP-2 and MMP-9 gene overexpression and destruction of elastic fibres in aortic tissue.[31] So far, only *in vitro*[33] or animal[13] studies have shown that either superoxide anion or $H_2O_2$-induced NF-κB activation can be suppressed by simvastatin. Our study shows that in human AAA segments excessive free radicals concentration is accompanied by NF-κB activation and that simvastatin patients exhibit lower NF-κB p65 subunit levels. Therefore, the mechanism by which simvastatin decreases TNF-α concentration appears to involve NF-κB activation in human AAA wall tissue segments.

Albeit the role of statins as a lipid-lowering drug is not questioned, there is a conflicting body of evidence regarding the use of statins as standard therapy in the treatment of aneurysm patients. The most recent study published by Karrowni et al.[34] indicated a decreased small AAA growth rate in patients receiving statins. Similar results were obtained by Takagi et al.[35] Nevertheless, a recent large growth rate follow-up study showed no correlation between simvastatin and low AAA progression.[34] Hurks et al.[16] similarly found no convincing evidence for use of different statins in causing significant decreases in the levels of proteases or inflammatory compounds.

Our present work demonstrates that simvastatin exerts pleiotropic effects in human AAA tissue and that it has profound impact on the free radical formation in aortic aneurysms. We further propose that NF-kB plays an important role in this signalling cascade.

### *Limitations of the study*

Although our study indicates a plausible positive role of simvastatin on oxidative stress and NF-κB signalling pathway in human AAA wall tissue, it has several limitations. First, the study was conducted on a relatively small group of patients. Therefore, to diminish the risk of false positive results, a 2:1 case:control study was performed. Second, 70% of samples used in our study are obtained from active smokers, whose levels of free oxygen species are higher compared to non-smokers, mainly through the activation of inflammatory cells. Smokers are more likely to have AAA than non-smokers.[36] However, considering that in both the non-statin and simvastatin group the level of nicotine users were similar, we can justify our assumption that the effect of nicotine had only low significance. Third, the spectroscopic and immunoenzymatic methods of detecting oxidative stress markers in AAA samples are less sensitive than determination of proinflammatory gene expression in endothelium, media and adventitia, though further investigations are necessary to confirm the role of simvastatin on oxidative stress markers in AAA segments.

## Conclusion

Our study shows that simvastatin treatment reduces NF-κB activation and decreases TNF-α concentration in human AAA wall

tissue, compared to non-statin patients. Moreover, simvastatin increases CAT level and inhibits lipid peroxidation. Therefore, pharmacological strategies targeting these generic components in AAA may be effective in stopping the course of aortic aneurysm disease.

## Conflict of Interest

None.

## Acknowledgements

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