Aleksandra Piechota-Polanczyk, M. Mittlboeck[1], Ihor Huk
Department of Surgery, Division of Vascular Surgery, Medical University of Vienna, Vienna, Austria
[1] Center for Medical Statistics, Informatics, and Intelligent Systems, Medical University of Vienna, Vienna, Austria

THE INFLUENCE OF SIMVASTATIN ON NADPH OXIDASE IN PATIENTS WITH ABDOMINAL AORTIC ANEURYSYM

Background: Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, have been used clinically as cholesterol-lowering drugs, however in recent years, accumulating evidences indicate the possible beneficial effect of statins on abdominal aortic aneurysm.

Material and methods: The study was performed on AAA tissue wall samples obtained from 13 simvastatin and 13 non-statin patients (1:1 case to control). The patients were matched by sex-, and AAA diameter. Following our previous study we investigated a gene expression of NADPH oxidase 4 (NOX4) by real time RT-PCR and a protein level of NOX4 by Western blot.

Results: The mean age, sex and AAA diameter were comparable between analysed groups ($p>0.05$). The simvastatin group had a tendency towards lower BMI and CRP level ($p<0.05$ and $p<0.07$, respectively). Biochemical analysis of AAA wall tissue indicated that simvastatin patients had slightly lower gene and protein level of NOX4 when compared to non-statin AAA wall tissue ($p>0.05$, respectively).

Conclusion: Simvastatin treatment to patients undergoing AAA open repair has little influence on NADPH oxidase 4 gene expression and protein level.

Keywords: simvastatin, NADPH oxidase 4, abdominal aortic aneurysm.

Abdominal aortic aneurysms (AAA) occur in 8% of men after 60 years of age [1]. The mechanisms of AAA are complex and include increased metalloproteinase activity, inflammation and excessive reactive oxygen species production (ROS) within the vessel wall. Reactive oxygen species production may be induced by sheer stress on the vascular wall or by cytokines produced mainly by leucocytes. NADPH oxidase is the main source of superoxide anion considered as one kind of the most active reactive oxygen species [2]. Superoxide anion is next spontaneously converted to hydrogen peroxide in cells. Up to now seven NOX homologes (NOX1, NOX3, NOX4, NOX5, Duox1, and Duox2) of gp91-phox (NOX2) have been identified in various non-phagocytic cells [3]. From those NOX4 is most highly expressed in endothelial cells [4]. Studies in murine models of AAA demonstrated that NADPH oxidases are also critical for ROS production in aneurysms [5].

Simvastatin belongs to HMG-CoA reductase inhibitors which catalyze the conversion of HMG-coA to mevalonate and influence aneurysm formation [6, 7]. Our previous publication presented a link between statins and ROS in AAA patients. We presented a decreased activity of NF-κB signaling pathway, and lowered level of ROS and TNF-α in AAA tissue from patients prescribed with simvastatin [8]. Furthermore, we highlighted a possible link between statin therapy and decreased level of cyclophiln A, a potent secreted oxidative stress-induced factor, in AAA tissue therefore suggesting a new anti-inflammatory role of statins [9].

Therefore, this study was undertaken to assess whether simvastatin influence NADPH oxidase 4 concentration in AAA wall tissue.

Material and methods

Continuous demographic and biochemical data are presented as median, minimum and maximum, demographic categorical data are described with absolute frequencies and percentages. Data are 1:1 matched in simvastatin and non-statin groups. All p-values are two-sided and $P\leq0.05$ 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 SPSS (SPSS 17.0, Chicago, IL, USA) was used for graphics.

Patients

The study was performed on 26 patients undergoing open AAA repair between Sept. 2009 and Dec. 2011 at our institution. Exclusion criteria for our study were the intake of statins other than simvastatin, chronic diseases such as liver disease, malignant disease, drugs intake, and alcohol abuse. After written informed consent, patient data were prospectively entered into a database. Subsequently the aneurysm wall tissue was har-

vested during aneurysm repair for retrospective analysis. Patients were matched in a 1 (simvastatin) to 1 (non-statin) ratio, respectively, by gender, and AAA diameter. 13 patients without statin medication (11 men, 2 women) were incorporated to the control group and 13 patients who had simvastatin (10 men, 3 women) in their medical history (20–40 mg daily dosage) for a minimum of six months were included in the study as the simvastatin group. The AAA diameter was determined with preoperative computed tomography angiography (CTA).

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 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.

Western blot analyses of NADPH oxidase 4

Equal protein amounts of whole cell extracts were separated by SDS-PAGE, and NOX4 was assessed by Western blotting using the respective rabbit anti-human monoclonal antibodies followed by HRP-conjugated donkey secondary antibodies (Santa Cruz Biotechnology). Signal intensity was quantified using an Imagine Master VDS (Bio-Rad, CA) and normalized to β-tubulin. Assays were performed twice with different tissue scraps.

RealTime Palymerase Chain Reaction for CyPA and EMMPRIN

Frozen tissue was homogenized using a ball mill (Retsch, Haan, Germany), and mRNA was isolated using the High Pure RNA Tissue Kit (Roche). Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche). RealTime-PCR was performed using LightCycler® TaqMan® Master (Roche) according to the manufacturer's instructions. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre. For GAPDH (forward primer: 5'-AGCCACATCGCTCAGACAC-3', reverse primer: 5'- G C C C A ATA C G A C C A A AT C C - 3'), for N O X 4 (forward primer: 5' - GCTGACGTTG-CATGTTTCAG-3', reverse primer: 5'- CGGGAG-GGTGGGTATCTAA-3'). The amplification conditions consisted of an initial incubation at 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 63°C for 20 sec and 72°C for 6 sec and a final cooling to 40°C. Data was analysed using LightCycler Software Version 3.5 (Roche).

Statistic 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 1:1 matched in simvastatin and non-statin groups. 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 SPSS (SPSS 17.0, Chicago, IL, USA) was used for graphics.

Results and Discussion

Our study indicated that the tissue gene expression of NOX4 from AAA patients treated with simvastatin was slightly lower than in the AAA non-statin group (8.33 (0.98-24.88) vs.4.10 (0.54-22.53, p=0.352, Fig.1). Similarly, the intracellular NOX4 level was insignificantly decreased in simvastatin group when compared to the non-statin group (1.17 (0.71-2.20) vs. 0.99 (0.59-1.67), p=0.131, Fig.2).

Although in our study we did not observed an effect of simvastatin on NOX4 concentration in AAA tissue wall it was documented by Guzik et al. [10] that NADPH oxidases are predominant sources of superoxide anion in AAA and that a significant correlation between NADPH-stimulated vascular superoxide anion production in AAA segments and intra-operatively determined aneurysm size occurs.

The mechanisms underlying the pleiotropic effects of statins on vascular function remain largely unknown. Direct vasorelaxation effect of statins correlated with their effect on ROS formation have been reported in rat aortic rings [11]. Tian et al. [12] demonstrated that HMGCo-A reductase, rosuvastatin, reduced the expressions of NADPH oxidase subunits p22 phox (membranous subunit), p67 phox (cytosolic subunit) in diabetic mice. Similarly, Giunti et al. [13] presented reduced accumulation of NOX4, advanced glycation end products, receptor for advanced glycation endproducts, and nitrotyrosine in kidney during diabetes. Also in endothelial cells protein expression of Nox4, ROS generation, and vascular endothelial growth factor (VEGF) level was decreased under lovastatin or atorvastatin treatment during oxidative stress [14, 15]. Furthermore, previous studies indicated that simvastatin may inhibit H_2O_2 -indcued upregulation of NOX4 in osteoblasts [16]. Therefore, a link between statin treatment and NOX4 concentration in vascular wall especially under oxidative stress is observed.

Our study shows that simvastatin treatment has little effect on NOX4 gene expression and protein

Table 1

Patient demographics.
Data are presented as frequencies or median (minimum-maximum)

	Non-statin Patients (n=13)	Simvastatin Patients (n=13)	P
Age (years), median (range)	70 (50-78)	67 (57-80)	
Sex (male)	11 (80%)	10 (80%)	1.000
AAA diameter (mm)	57 (48-102)	56 (48-100)	
Body mass index, mean (range)	27.70 (23.00-37.60)	25.20 (22.70-29.00)	0.05
Coronary artery disease	3 (23%)	0 (0%)	0.500
Cerebrovascular artery disease	3 (23%)	3 (23%)	1.000
Peripheral artery disease	0 (0%)	2 (15%)	0.157
Cardiac insufficiency	3 (23%)	0 (0%)	0.317
Type 2 diabetes	2 (15%)	0 (0%)	0.414
Smoking	3 (23%)	7 (54%)	0.655
Cholesterol [mg/dl], median (range)*	238 (143-323)	207 (144-261)	0.266
LDL [mg/dl], median (range)*	144.0 (79.2-218)	123.2 (75-218)	0.414
HDL [mg/dl], median (range)*	46.0 (43-68)	47.0 (34-61)	0.989
CRP [mg/dl], median (range)*	0.43 (0.03-3.00)	0.20 (0.06-1.02)	0.0681
Fibrinogen [mg/dl], median (range)*	384 (280-594)	338 (240-544)	0.141
Leucocytes [mln/ml], median (range)*	7.96 (5.3-11.6)	8.0 (5.7-11.04)	0.685
Creatinine [mg/dL], median (range)*	1.12 (0.9-1.44)	0.92 (0.76-4.0)	0.211

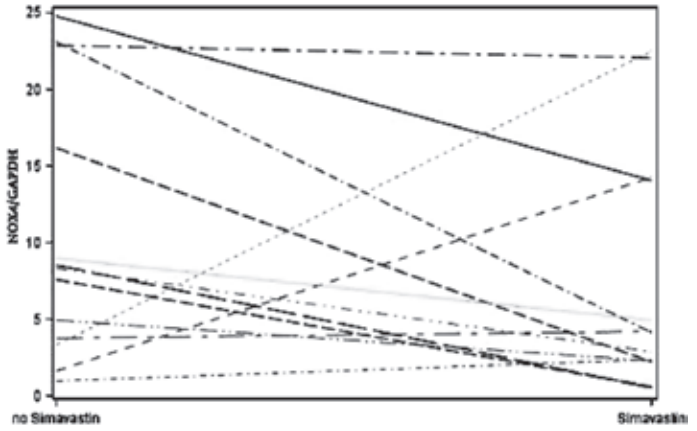

Fig. 1. Simvastatin influence on NOX4 mRNA expression in human AAA wall tissue. N=13 in simvastatin and N=13 in non-statin group. P>0.05

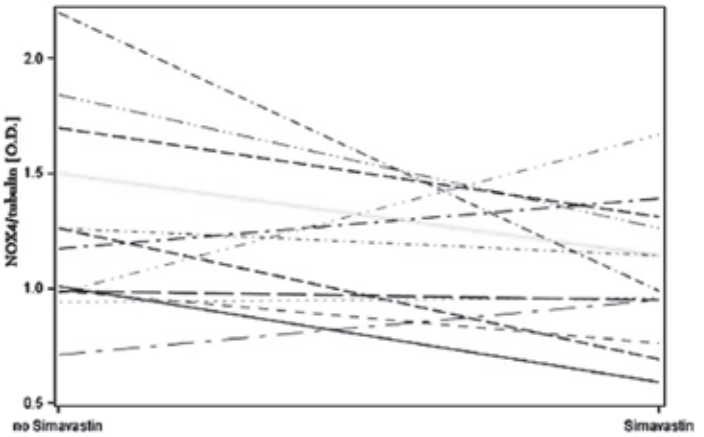

Fig. 2. Densitometrical quantification of NOX4 in AAA tissue samples from patients untreated or treated with simvastatin. N=13 in simvastatin and N=13 in non-statin group. P>0.05

concentration in human AAA wall tissue as compared to the non-statins patients. However, further studies are required to determine the role of simvastatin on NADPH oxidases in human abdominal aortic tissue.

Acknowledgements

The study was supported by grant number 181110 from the Medical University of Vienna, Department of Surgery.

References

1. Weintraub, N.L., Understanding abdominal aortic aneurysm. N Engl J Med, 2009. 361(11): p. 1114-6.
2. Schramm, A., et al., Targeting NADPH oxidases in vascular pharmacology. Vascul Pharmacol, 2012. 56(5-6): p. 216-31.
3. Hohler, B., B. Holzapfel, and W. Kummer, NADPH oxidase subunits and superoxide production in porcine pulmonary artery endothelial cells. Histochem Cell Biol, 2000. 114(1): p. 29-37.
4. Kuroda, J., et al., The superoxide-producing NAD(P) H oxidase Nox4 in the nucleus of human vascular endothelial cells. Genes Cells, 2005. 10(12): p. 1139-51.
5. Xiong, W., et al., Inhibition of reactive oxygen species attenuates aneurysm formation in a murine model. Atherosclerosis, 2009. 202(1): p. 128-34.
6. Takagi, H., M. Matsui, and T. Umemoto, A meta-analysis of clinical studies of statins for prevention of abdominal aortic aneurysm expansion. J Vasc Surg, 2010. 52(6): p. 1675-81.
7. Forsdahl, S.H., et al., Risk factors for abdominal aortic aneurysms: a 7-year prospective study: the Tromso Study, 1994-2001. Circulation, 2009. 119(16): p. 2202-8.
8. Piechota-Polanczyk, A., et al., Simvastatin decreases free radicals formation in the human abdominal aortic aneurysm wall via NF-kappaB. Eur J Vasc Endovasc Surg, 2012. 44(2): p. 133-7.
9. Piechota-Polanczyk, A., et al., Decreased tissue levels of cyclophilin A, a cyclosporine a target and phospho-ERK1/2 in simvastatin patients with abdominal aortic aneurysm. Eur J Vasc Endovasc Surg, 2013. 45(6): p. 682-8.
10. Guzik, B., et al., Mechanisms of oxidative stress in human aortic aneurysms--association with clinical risk factors for atherosclerosis and disease severity. Int J Cardiol, 2013. 168(3): p. 2389-96.
11. Lopez, J., et al., Participation of K+ channels in the endothelium-dependent and endothelium-independent components of the relaxant effect of rosuvastatin in rat aortic rings. J Cardiovasc Pharmacol Ther, 2008. 13(3): p. 207-13.
12. Tian, X.Y., et al., Rosuvastatin improves endothelial function in db/db mice: role of angiotensin II type 1 receptors and oxidative stress. Br J Pharmacol, 2011. 164(2b): p. 598-606.
13. Giunti, S., et al., The pleiotropic actions of rosuvastatin confer renal benefits in the diabetic Apo-E knockout mouse. Am J Physiol Renal Physiol, 2010. 299(3): p. F528-35.
14. Li, J., et al., Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood-retinal barrier breakdown in db/db mice: role of NADPH oxidase 4. Diabetes, 2010. 59(6): p. 1528-38.
15. Bao, X.M., C.F. Wu, and G.P. Lu, Atorvastatin inhibits homocysteine-induced oxidative stress and apoptosis in endothelial progenitor cells involving Nox4 and p38MAPK. Atherosclerosis, 2010. 210(1): p. 114-21.
16. Huang, W., et al., Simvastatin protects osteoblast against H2O2-induced oxidative damage via inhibiting the upregulation of Nox4. Mol Cell Biochem, 2012. 360(1-2): p. 71-7.

Стаття надійшла до редакції 19.02.2014 р.

О. Пєхота-Поляньчик , М. Мітлбек[1], І. Гук
Відділення судинної хірургії, Віденський Медичний Університет, Відень, Австрія
[1]Центр медичної статистики, інформатики та інтелектуальних систем, Віденський Медичний Університет, Відень, Австрія

ВПЛИВ СИМВАСТАТИНУ НА НАДФН-ОКСИДАЗУ У ХВОРИХ З АНЕВРИЗМОЮ ЧЕРЕВНОЇ АОРТИ

Статини (інгібітори 3-гідрокси-3-метилглутарил-коензим А-редуктази) використовуються в клінічній практиці як гіпохолестеринемічні препарати. В останні роки з'являється все більше доказів, що свідчать про можливий сприятливий ефект статинів при аневризмі черевної аорти (АЧА), в патогенезі якої важливу роль відіграє надмірна продукція активних радикалів кисню в судинній стінці. NADPH-оксидаза є основним джерелом супероксидного аніону – одного з найбільш активних радикалів кисню, а в ендотеліальних клітинах переважає NADPH-оксидаза 4-го типу (NOX4) .

Матеріал і методи: Дослідження проводилося на зразках тканини стінки АЧА 13 пацієнтів, які отримували симвастатин і 13 пацієнтів контрольної групи, які не отримували даний препарат. Пацієнти обох груп були порівнянні за статтю та діаметру АЧА. Ми досліджували експресію гена NOX4 за допомогою методики полімеразної ланцюгової реакції зі зворотною транскрипцією (ЗТ-ПЛР) в реальному часі. Рівень білка NOX4 досліджувався методом вестерн-блоттингу.

Результати: Середній вік, стать пацієнтів і діаметр АЧА були співставними між групами порівняння ($p>0,05$). У групі пацієнтів, які отримували симвастатин, відзначалася тенденція до більш

низького індексу маси тіла і рівня С-реактивного білку ($p<0,05$ і $p<0,07$, відповідно). Біохімічний аналіз тканини стінки АЧА показав, що у пацієнтів, які отримували симвастатин, експресія гена і рівень білка NOX4 були недостовірно нижче в порівнянні з контрольною групою ($p>0,05$).

Висновок: Терапія симвастатином у пацієнтів, які перенесли операцію з приводу АЧА , має незначний вплив на експресію гена NOX4 і рівень білка NOX4 в стінці аневризми.

Ключові слова: симвастатин, NADPH-оксидаза 4, аневризма черевної аорти.

А. Пехота-Поляньчик, М. Митлбек[1], И. Гук

Отделение сосудистой хирургии, Венский Медицинский Университет, Вена, Австрия

[1] Центр медицинской статистики, информатики и интеллектуальных систем, Венский Медицинский Университет, Вена, Австрия

ВЛИЯНИЕ СИМВАСТАТИНА НА НАДФН-ОКСИДАЗУ У БОЛЬНЫХ С АНЕВРИЗМОЙ БРЮШНОЙ АОРТЫ

Статины (ингибиторы 3-гидрокси-3-метилглутарил-коэнзим А-редуктазы) используются в клинической практике как гипохолестеринемические препараты. В последние годы появляется все больше доказательств, свидетельствующих о возможном благоприятном эффекте статинов при аневризме брюшной аорты (АБА), в патогенезе которой важную роль играет избыточная продукция активных радикалов кислорода в сосудистой стенке. NADPH-оксидаза является основным источником супероксидного аниона – одного из наиболее активных радикалов кислорода, а в эндотелиальных клетках преобладает NADPH-оксидаза 4-го типа (NOX4).

Материал и методы: Исследование проводилось на образцах ткани стенки АБА 13 пациентов, получавших симвастатин и 13 пациентов контрольной группы, не получавших данный препарат. Пациенты обеих групп были сопоставимы по полу и диаметру АБА. Мы исследовали экспрессию гена NOX4 с помощью методики полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР) в реальном времени. Уровень белка NOX4 исследовался методом вестерн-блоттинга.

Результаты: Средний возраст, пол пациентов и диаметр АБА были сопоставимы между группами сравнения ($p>0,05$). В группе пациентов, получавших симвастатин, отмечалась тенденция к более низкому индексу массы тела и уровню С-реактивного белка ($p<0,05$ и $p<0,07$, соответственно). Биохимический анализ ткани стенки АБА показал, что у пациентов, получавших симвастатин, экспрессия гена и уровень белка NOX4 были недостоверно ниже по сравнению с контрольной группой ($p>0,05$).

Вывод: Терапия симвастатином у пациентов, перенесших операцию по поводу АБА, имеет незначительное влияние на экспрессию гена NOX4 и уровень белка NOX4 в стенке аневризмы.

Ключевые слова: симвастатин , NADPH оксидаза 4 , аневризма брюшной аорты.