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Peroxynitrite Footprint in Circulating Neutrophils of Abdominal Aortic Aneurysm Patients Is Lower in Statin than in Non-statin Users

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WHAT THIS PAPER ADDS

Statins seem to selectively reduce reactive nitrogen species in circulating white blood cells.

Objectives: Extensive reactive oxygen and nitrogen species (also reactive species) production is a mechanism involved in abdominal aortic aneurysm (AAA) development. White blood cells (WBCs) are a known source of reactive species. Their production may be decreased by statins, thereby reducing the AAA growth rate. Reactive species production in circulating WBCs of AAA patients and the effect of statins on their production was investigated.

Methods: This observational study investigated reactive species production in vivo and ex vivo in circulating WBCs of AAA patients, using venous blood from patients prior to elective AAA repair ($n = 34$; 18 statin users) and from healthy volunteers ($n = 10$). Reactive species production was quantified in circulating WBCs using immunofluorescence microscopy: nitrotyrosine (footprint of peroxynitrite, a potent reactive nitrogen species) in snap frozen blood smears; mitochondrial superoxide and cytoplasmic hydrogen peroxide (both reactive oxygen species) by live cell imaging. Neutrophils, lymphocytes, and monocytes were examined individually.

Results: In AAA patients using statins, the median nitrotyrosine level in neutrophils was 646 (range 422–2059), in lymphocytes 125 (range 74–343), and in monocytes 586 (range 291–663). Median levels in AAA patients not using statins were for neutrophils 928 (range 552–2095, $p = .03$), lymphocytes 156 (101–273, NS), and for monocytes 536 (range 535–1635, NS). The statin dose tended to correlate negatively with nitrotyrosine in neutrophils (R_s −0.32, $p = .06$). The median levels in controls were lower for neutrophils 466 (range 340–820, $p < .01$) and for monocytes 191 (range 102–386, $p = .03$), but similar for lymphocytes 99 (range 82–246) when compared to the AAA patients. There were no differences in mitochondrial superoxide and cytoplasmic hydrogen peroxide between statin and non-statin users within AAA patients.

Conclusions: It was found that the peroxynitrite footprint in circulating neutrophils and monocytes of AAA patients is higher than in controls. AAA patients treated with statins had a lower peroxynitrite footprint in neutrophils than non-statin users.

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INTRODUCTION

Extensive production of reactive nitrogen and oxygen species (RNS and ROS), also referred to as reactive species, results in oxidative stress. Circulating white blood cells (WBCs), mainly neutrophils, are capable of RNS and ROS production.[1] In the aortic wall oxidative stress can cause extracellular matrix (ECM) degeneration and smooth muscle cell apoptosis, which are recognised as hallmarks of abdominal aortic aneurysm (AAA) pathogenesis.[1–3]

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Production of ROS, of which superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are typical examples, and production of RNS, mainly peroxynitrite, which is formed by a reaction between superoxide and nitric oxide, are among others facilitated by endothelial cells, smooth muscle cells, and locally infiltrating WBCs.[4–7] Nitrotyrosine is a footprint left after peroxynitrite and other RNS production.[8,9] Nitrotyrosine levels in cells therefore indicate the amount of RNS the cell has encountered. Several factors tend to influence levels of reactive species in the aneurysm wall.[10] Smoking is suggested to increase locally produced oxidative stress in the aortic wall.[10] Statin therapy is thought to decrease local oxidative stress levels in the aneurysm wall.[11,12] An improved antioxidant capacity is likely to be involved, but a decrease in the formation of free radicals, if any, has so far not been shown and remains to be investigated.[13] Statins have even been proposed to decrease the growth rate of small AAAs.[14] However, not much is known about reactive species production in AAA patients and how statins can affect their production systemically. The aim was to determine whether systemically circulating WBCs are also a source of reactive species in AAA patients and whether the production is influenced by statin therapy.

It was hypothesized that in patients treated by statins the production of reactive species in circulating WBCs would be lower than in untreated patients. In this study, individual production of reactive species by circulating neutrophils, lymphocytes, and monocytes in AAA patients was analysed. The RNS via nitrotyrosine levels in the WBCs was measured and ROS quantified by direct measurement of mitochondrial superoxide and cytoplasmic hydrogen peroxide production via live cell imaging. AAA patients with and without statin therapy, were differentiated.

MATERIAL AND METHODS

The study was approved by the Medical Ethics Committee of the VU University Medical Center (VUmc) Amsterdam (reference number: 2010/193). Written informed consent was obtained from all patients and volunteers.

Patient selection and blood sample gathering

Thirty-four consecutive AAA patients were included, of which 30 were males (in accordance with current epidemiology).[3] Venous blood from AAA patients was collected in EDTA containing tubes prior to elective aneurysm repair at the medical centre. Excluded subjects were those with acutely treated AAA, and ruptured or thoraco-abdominal aortic aneurysms, as the latter have a pathogenesis distinct from AAA.[15] As controls, the first 10 consecutive healthy persons (9 males) that voluntarily applied for the study were included. Exclusion criteria for the control group were any comorbidities, use of medications, and age younger than 40 years.

Blood smears were made immediately and then snap frozen and stored in −80 °C until use. Blood samples were cooled at 4 °C and directly centrifuged; finally, the buffy coat was extracted. The buffy coat extract was then immediately re-suspended for immunofluorescence labelling and live cell imaging (see below). Live cell imaging was performed within 2 h of sample procurement (see study design in Fig. 1). Pre-operative creatinine and total leukocyte measurements were performed by the department of Clinical Chemistry in the VUmc. Manual leukocyte differential counts were performed on all blood smears. Aneurysm diameters were measured by computed tomography angiography. If multiple angiograms had been performed ($n = 12$), aneurysm growth is presented in centimetres per month.

Immunofluorescence staining

Measurement of peroxynitrite levels via nitrotyrosine. Blood smears were air-dried, fixed with 4% formalin, and washed in 0.05% Tween (cell-washing solution). Smears were stained at room temperature with anti-nitrotyrosine rabbit immunoglobulin primary antibody for 1 h (1:50; Molecular Probes, Eugene, Oregon, United States) and Alexa 488 anti-rabbit secondary antibody for 30 min (1:100; Molecular Probes, Eugene, Oregon, United States). The cell membrane glycocalyx was labelled with Alexa 555 fluorescent wheatgerm-agglutinin probe (1:50; Molecular Probes, Eugene, Oregon, United States) and then mounted with Vectashield mounting medium-containing DAPI nuclear stain (Vector Laboratories Inc., Burlingame, California, United States). Negative control smears were treated in the same fashion, but without primary antibodies.

Measurement of superoxide and hydrogen peroxide production via live cell imaging. Buffy coat extract was re-suspended in ADS buffer at 37 °C (116 mM NaCl/5.3 mM KCl/1.2 mM $MgSO_4 \cdot 7H_2O$/1.13 mM $NaH_2PO_4 \cdot H_2O$/20 mM Hepes/5 mM glucose/1 mM $CaCl_2$) and labelled with MitoSOX Red superoxide indicator (mitochondrial ROS) and 5-6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCF) acetyl ester C6827, sensitive for hydrogen peroxide (cytoplasmic ROS) according to the manufacturer instructions (Molecular Probes, Eugene, Oregon, United

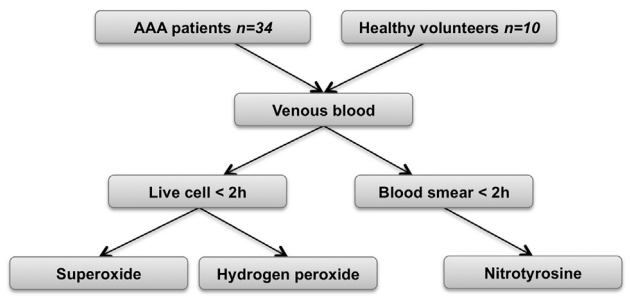

Figure 1. Study design. Diagram of the study design. Venous blood from AAA patients and healthy volunteers was analysed. Superoxide and hydrogen peroxide were measured by live cell imaging and nitrotyrosine was measured by immunofluorescence microscopy.

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