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Effects of atorvastatin and diet interventions on atherosclerotic plaque inflammation and [^{18}F]FDG uptake in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice

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ABSTRACT

Background and aims: Uptake of the positron emission tomography (PET) tracer 2-deoxy-2-[^{18}F]-fluoro-D- glucose ([^{18}F]FDG) into macrophages is a sensitive marker of inflammation in atherosclerosis. To assess the anti-inflammatory effects of statins, we studied whether atorvastatin therapy reduces aortic [^{18}F]FDG uptake in hypercholesterolemic mice deficient in low-density lipoprotein receptor (Ldlr), and expressing only apolipoprotein B-100 (*Ldlr*$^{-/-}$*Apob*$^{100/100}$).

Methods: Thirty-six *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice were fed a high-fat diet (HFD) for 12 weeks and then allocated to receive a HFD (n = 13), chow diet (Chow, n = 12), or HFD with added atorvastatin (HFD + A, n = 11), for another 12 weeks. In addition to aortic histopathology, [^{18}F]FDG uptake was studied *in vivo* using PET/computed tomography (CT), and *ex vivo* by gamma counting of excised aorta.

Results: Total cholesterol levels were lower in the Chow and HFD + A groups than in the HFD group (10 ± 3.2, 23 ± 4.9 and 34 ± 9.2 mmol/l, respectively), with the Chow group also showing a lower plaque burden and lower numbers of macrophages in the lesions. Compared to the HFD group, [^{18}F]FDG uptake in the aorta (normalized for blood) was lower in the Chow group in both *in vivo* (2.1 ± 0.21 *vs.* 1.7 ± 0.25, $p = 0.018$) and *ex vivo* (5.2 ± 2.3 *vs.* 2.8 ± 0.87, $p = 0.011$) analyses, whereas atorvastatin had no effect on uptake (2.1 ± 0.42 *in vivo* and 3.9 ± 1.8 *ex vivo*). [^{18}F]FDG uptake correlated with plasma total cholesterol levels.

Conclusions: Atorvastatin therapy did not show cholesterol-independent effects on inflammation in atherosclerotic lesions in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice, as determined by histology and [^{18}F]FDG PET, whereas a cholesterol-lowering diet intervention was effective.

Abbreviations: [^{18}F]FDG, 2-deoxy-2-[^{18}F]-fluoro-D-glucose; ARG, autoradiography; HFD + A, high-fat diet with added atorvastatin; *Ldlr*$^{-/-}$Apob$^{100/100}$, mouse model deficient in low-density lipoprotein receptor expressing only apolipoprotein B-100; LRP, low-density lipoprotein receptor-associated protein; MCP-1, monocyte chemoattractant protein-1; PSL/mm^2, photo-stimulated luminescence per square millimeter; SUV, standardized uptake value; TBR, target-to-background ratio; IL-6, interleukin-6.

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1. Introduction

Inflammation plays a central role in the development of atherosclerosis and its complications, with macrophages being the most prominent inflammatory cells in atherosclerotic plaques [1]. Statin therapy can inhibit the progression of atherosclerotic plaques [2] and reduce the cardiovascular risk [3]. The main mechanism of action of statins is to reduce the blood cholesterol concentration by inhibiting 3-hydroxy-3- methyl-glutaryl-coenzyme A reductase, an enzyme that catalyzes endogenous cholesterol synthesis in the liver. However, statins have biological actions that are independent of cholesterol lowering, referred to as pleiotropic effects [4]. The reduction of cardiovascular risk is greater than expected on the basis of cholesterol reduction alone [5], and has been evident even before cholesterol lowering [6,7]. This is partly attributed to plaque-stabilizing effects related to the inhibition of the inflammatory response [8–10]. However, the contributions of pleiotropic effects of statins on cardiovascular risk reduction in individuals with elevated systemic inflammatory markers is very difficult to study in clinical trials, because cholesterol lowering itself is strongly atheroprotective [11,12].

Positron emission tomography/computed tomography (PET/CT) is a translational imaging method utilized in clinical studies and animal research. PET/CT imaging using 2-deoxy-2-[^{18}F]-fluoro-D-glucose ([^{18}F]FDG) has been used as a tool to assess the effects of statins on atherosclerosis-associated vascular inflammation [13,14]. [^{18}F]FDG is taken up by cells utilizing glucose, including active macrophages, which makes it a sensitive *in vivo* marker of atherosclerotic plaque inflammation [15]. A 4 week high-dose treatment with atorvastatin was shown to reduce [^{18}F]FDG uptake in the carotid arteries and aorta of patients at high risk of vascular inflammation, although the change in arterial [^{18}F]FDG uptake was not related to a change in blood low-density lipoprotein (LDL) level [13].

The responsiveness of LDL receptor-deficient (*Ldlr*$^{-/-}$) mice to statin therapy is limited, as the cellular intake of cholesterol cannot be increased via the LDL receptor [16–18]. *Ldlr*$^{-/-}$ mice expressing only apolipoprotein B-100 (*Ldlr*$^{-/-}$*Apob*$^{100/100}$) are expected to be even less responsive to statin-induced cholesterol lowering than *Ldlr*$^{-/-}$ mice, since the lipoprotein clearance via Ldlr-related proteins (LRPs) is also impaired [19,20]. These mice have a cholesterol profile resembling human familial hypercholesterolemia, and they develop extensive atherosclerosis in the aorta [19,21,22]. Therefore, to further study the possible cholesterol-independent effects of statin therapy on vascular inflammation in atherosclerosis, we used histology and [^{18}F]FDG PET/CT imaging to compare the effects of diet and atorvastatin interventions on inflammation in atherosclerotic plaques in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice. In this study, after 12 weeks on a high-fat diet (HFD) to induce atherosclerosis, mice were allocated to either continue on the HFD, or switched to a chow diet (Chow) or a HFD combined with atorvastatin (HFD + A) for an additional 12 weeks. At the end of the intervention, [^{18}F]FDG PET/CT was performed and the aortas were excised for gamma counting, histology of plaque burden and macrophage content, and evaluation of [^{18}F]FDG uptake into lesions and lesion-free vessel walls by autoradiography (ARG). Total cholesterol, triglycerides, and biomarkers of inflammation and metabolism were measured from plasma.

2. Materials and methods

2.1. Animals and study design

The study protocol was approved by the National Animal Experiment Board in Finland and the Regional State Administrative Agency for Southern Finland. Studies were carried out according to the respective European Union directives on animal experimentation. The mice were bred and housed in standard conditions and a 12/12 h light/dark cycle in the Laboratory Animal Unit of the University of Turku, with access to food and water *ad libitum*.

Ldlr$^{-/-}$*Apob*$^{100/100}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA; n = 41, both genders) were first fed a chow diet (9.1% of calories from fat, CRM [E], 801730, Special Diet Services, Essex, UK) for 8 weeks, and then a HFD (42% of calories from fat, 0.2% total cholesterol, TD 88137, Teklad, Harlan Laboratories, Madison, WI, USA) for 12 weeks. At the age of 20 weeks, a saphenous vein blood sample was taken from each mouse. Thereafter, the mice were divided into the following interventional groups for an additional 12 weeks: 1) continuous HFD (HFD, n = 18), 2) dietary intervention with chow diet (Chow, n = 12), and 3) HFD combined with atorvastatin (HFD + A, n = 12). The HFD + A diet contained 0.1 mg of atorvastatin per gram of food (Teklad Custom Research Diet, Harlan Laboratories, Madison, WI, USA). Food consumption and the weights of the mice were monitored weekly. For individual mice, the study was terminated if the mouse's well-being was compromised, judged on the basis of pre-defined criteria (weight reduction >15% or development of xanthomas affecting >10% of the skin area).

2.2. [^{18}F]FDG PET/CT

After the interventional period, all mice in the Chow and HFD + A groups, and eight mice in the HFD group, underwent *in vivo* [^{18}F]FDG PET/CT imaging using an Inveon multimodality small animal PET/CT scanner (Siemens, Knoxville, TN, USA), as described in a previous study [23]. The mice were fasted for 4 h, anesthetized with isoflurane (2–2.5%), and kept on a heating pad during the imaging. Glucose levels were measured with a glucometer (Contour, Bayer AG, Leverkusen, Germany). Approximately 10 MBq of [^{18}F]FDG was injected via a tail vein catheter, and attenuation-corrected PET imaging was performed for a duration of 20 min, at 50 min post-injection. After the PET data were acquired, 100–150 μl of intravenous contrast agent (eXia 160XL, Binitio Biomedical, Ottawa, Canada) was injected, and high-resolution CT was performed to visualize the blood vessels. The image reconstruction parameters were similar to those previously described [24]. The PET/CT image analysis was performed using Carimas 2.9 software (Turku PET Centre, Turku, Finland). Uptake was evaluated by defining regions of interest (ROIs) in the aorta, blood, myocardium, and muscle, using the contrast-enhanced CT images. The aortic ROI was placed in the arch, avoiding myocardial spillover. Blood radioactivity concentration was analyzed from the *vena cava.* Radioactivity concentrations were measured as mean standardized uptake values (SUV): (Tissue radioactivity/tissue volume)/(total injected radioactivity/mouse weight). Uptake in the aorta was normalized by calculating the target-to-background ratio (TBR) by dividing the aortic arch SUV by the blood SUV.

2.3. Ex vivo measurements and ARG

After imaging, the mice were sacrificed at 100-min post-injection by cardiac puncture and cervical dislocation. For the mice not imaged *in vivo*, [^{18}F]FDG studies were performed *ex vivo* at the same time point. Selected tissues were excised, weighed, and measured for radioactivity with a gamma counter (Triathler 425-010, Hidex, Turku, Finland). The thoracic aorta was excised, cleaned, and rinsed with saline. Radioactivity concentrations in organs and blood were calculated as SUV: (tissue radioactivity/tissue weight)/(total injected radioactivity/mouse weight). The aortic TBR was defined as described above.