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Statin-Induced Changes in Mitochondrial Respiration in Blood Platelets in Rats and Human With Dyslipidemia

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Summary

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are widely used drugs for lowering blood lipid levels and preventing cardiovascular diseases. However, statins can have serious adverse effects, which may be related to development of mitochondrial dysfunctions. The aim of study was to demonstrate the *in vivo* effect of high and therapeutic doses of statins on mitochondrial respiration in blood platelets. Model approach was used in the study. Simvastatin was administered to rats at a high dose for 4 weeks. Humans were treated with therapeutic doses of rosuvastatin or atorvastatin for 6 weeks. Platelet mitochondrial respiration was measured using high-resolution respirometry. In rats, a significantly lower physiological respiratory rate was found in intact platelets of simvastatin-treated rats compared to controls. In humans, no significant changes in mitochondrial respiration were detected in intact platelets; however, decreased complex I-linked respiration was observed after statin treatment in permeabilized platelets. We propose that the small *in vivo* effect of statins on platelet energy metabolism can be attributed to drug effects on complex I of the electron transport system. Both intact and permeabilized platelets can be used as a readily available biological model to study changes in cellular energy metabolism in patients treated with statins.

Key words

Statin • Mitochondria • Platelet

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Introduction

Statins, HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase inhibitors, have been demonstrated to reduce all-cause mortality and the risk of major cardiovascular events among people without evidence of cardiovascular disease (Taylor *et al.* 2013). However, evidence has not yet been found regarding the benefit of statin therapy on all-cause mortality in the setting of high-risk primary prevention (Ray *et al.* 2010), elderly at high risk of cardiovascular disease (Lloyd *et al.* 2013), or women with coronary artery disease (Bukkapatnam *et al.* 2010). In addition to their beneficial cardiovascular effects, statins seem to have multiple non-cardiovascular effects, which have not been sufficiently explored (Demierre *et al.* 2005, Vevera *et al.* 2005, Marzilli 2010, Gazzerro *et al.* 2012, Yu *et al.* 2013,

Vevera *et al.* 2016). Adverse effects of statins include a significant increase in the risk of diabetes mellitus (Ruscica *et al.* 2014), transaminase elevation (Naci *et al.* 2013), and myotoxicity (Sathasivam 2012), including a modest increase in the risk of myositis; severe myopathy (rhabdomyolysis) is rare. Non-cardiovascular benefits of statins include reduced incidence of contrast nephropathy (Desai *et al.* 2014).

Several potential mechanisms have been proposed to explain both desirable and detrimental effects of statins on muscle tissue and the brain, including impairment of membranes, improved endothelial function, reduction in free radical production, and reduction of inflammation (Bifulco *et al.* 2008, António *et al.* 2014). Higher doses of some statins are associated with transaminase and creatine kinase elevations (Naci *et al.* 2013), which can affect cellular energetics.

Converging evidence supports a mitochondrial foundation for muscle, liver, and brain adverse effects associated with statins (Kaufmann *et al.* 2006, Nadanaciva *et al.* 2007, Golomb and Evans 2008, Galtier *et al.* 2012, Sirvent *et al.* 2012, Abdoli *et al.* 2013). Patients treated with statins showed impairment of mitochondrial respiration that mainly involves complex I of the respiratory chain. Decreased coenzyme Q_{10} content and decreased maximal capacity of mitochondrial oxidative phosphorylation have been reported in statin-treated patients (Deichmann *et al.* 2010, Larsen *et al.* 2013, Vaughan *et al.* 2013). It seems that coenzyme Q_{10} improves the bioenergetics function of mitochondria treated with statins (Vaughan *et al.* 2013, Mohammadi-Bardbori *et al.* 2015). On the other hand, statins also exert antioxidative effects, lower intramitochondrial ionized calcium, lower oxidative stress, prevent mitochondrial membrane permeability transition pore opening, and prevent the release of cytochrome *c* from the mitochondria (Maes *et al.* 2012, Parihar *et al.* 2012, Zhao *et al.* 2015).

A high cholesterol level may increase the risk of developing Alzheimer's disease (Shepardson *et al.* 2011). Cholesterol lowering and mitochondrial effects of statins led to the assumption that statins could be useful in treatment or prevention of dementia. Novel drugs have been developed for the treatment of Alzheimer's disease that act on multiple targets, including cholinesterase activity, monoamine oxidase activity, Aβ aggregation, γ-secretase activity, serotonin transporter activity, production of reactive oxygen species, calcium channels, mitochondrial permeability transition pores, and interactions of amyloid-β with mitochondrial enzymes (Xie *et al.* 2006, Lim *et al.* 2011, Bolea *et al.* 2013). Statins also have been discussed as an alternative treatment approach in Alzheimer's disease (Kalra and Khan 2015). However, there is some controversy regarding the effect of statins on dementia: some studies have found beneficial effects, while other studies have not (Feldman *et al.* 2010, Padala *et al.* 2012, Valenti *et al.* 2014, Chatterjee *et al.* 2015). Recently, it was concluded that statins given late in life to people at risk of vascular disease and increased risk of vascular dementia or dementia due to Alzheimer's disease did not prevent cognitive decline or dementia (McGuinness *et al.* 2016). On the other hand, in randomized clinical trials, statin therapy was not associated with cognitive impairment (Ott *et al.* 2015).

Platelets are the cellular mediator of thrombosis, but they are also immune cells that initiate and accelerate many vascular inflammatory conditions (Morrell *et al.* 2014). Due to biochemical similarities between blood platelets and neurons, platelets have been established as a reliable model for the biochemical characterization of changes in brain neurons associated with mental disorders (Da Prada *et al.* 1988, Shad and Saeed 2007, Kaneez and Saeed 2009). Components of signaling pathways, with changes in platelets that are supposed to reflect changes in the brain, include serotonin receptors (Mendelson 2000), neurotransmitter transporters and storage vesicles (Mercado and Kilic 2010, Yubero-Lahoz *et al.* 2013), mitochondrial monoamine oxidase type B (Youdim 1988), brain derived neurotrophic factor (Pláteník *et al.* 2014), amyloid-β (Chen *et al.* 1995), complexes of the mitochondrial respiratory chain (Bosetti *et al.* 2002, Cardoso *et al.* 2004, Valla *et al.* 2006), and a number of blood-based biomarkers (Casoli *et al.* 2013, Donovan *et al.* 2013).

High-resolution respirometry (Gnaiger *et al.* 2000, Pesta and Gnaiger 2012) represents a sensitive technique to determine small mitochondrial dysfunctions. Studies examining mitochondrial function are typically measured in permeabilized skeletal muscle fibers. However, platelet mitochondria have been established as well suited for *ex vivo* analysis of cellular respiration (Sjövall *et al*. 2010, 2013, Hroudová *et al.* 2013) and the activity of respiratory chain complexes (Böhm *et al.* 2007). Platelets are an easily obtainable source of viable mitochondria and disturbances in the function of platelet mitochondria have been demonstrated in aging (Merlo Pich *et al.* 1996, Lenaz *et al.* 2000, Xu *et al.* 2007) and in

variety of diseases, including neurodegenerative disorders (Krige *et al.* 1992, Hauptmann *et al.* 2006, Shi *et al.* 2008, Hroudová *et al.* 2013, Fišar *et al.* 2016).

We suggest that intact and/or permeabilized platelets might serve as an appropriate and easy available biological model for studying energetic metabolism in statin-treated patients. The aim of our study was to demonstrate the effect of short-term (several weeks) statin application on mitochondrial respiration in human and rat platelets. Simvastatin-treated rats were compared to controls receiving vehicle jelly without simvastatin. In humans, mitochondrial respiration in blood platelets was compared before and after statin treatment in patients with disorders of lipoprotein metabolism; specifically, in subjects not contraindicated for statin use.

Materials and Methods

Chemicals and solutions

Mitochondrial respiration in intact platelets was measured in plasma diluted with inorganic Krebs and Henseleit isotonic medium (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, and 11.1 mM glucose; pH 7.4). Mitochondrial respiration medium (MiR05) used in assays with permeabilized platelets consisted of 110 mM sucrose, 60 mM K^+-lactobionate, 20 mM taurine, 3 mM $MgCl_2.6H_2O$, 10 mM KH_2PO_4, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid free, and 20 mM HEPES, adjusted to pH 7.1 with KOH (Gnaiger *et al.* 2000, Pesta and Gnaiger 2012).

Stock solutions used for platelet oxygen consumption rate measurements included 10 mg/ml digitonin, 2 M malate, 2 M pyruvate, 0.5 M ADP, 2 M glutamate, 1 M succinate, 4 mg/ml oligomycin, 1 mM or 10 mM carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone (FCCP), 1 mM rotenone, and 0.5 mg/ml antimycin A. All the chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

High-resolution respirometry

High-resolution respirometry measures electron transfer in the electron transport system (ETS) quantified by the oxygen consumption rate. An OROBOROS Oxygraph-2k (O2k; OROBOROS INSTRUMENTS, Austria) was used. Samples, with a volume of 2 ml, were measured in two glass chambers equipped with Clark polarographic oxygen electrodes. Common experimental conditions include a physiological temperature of 37 °C, stirring at 750 rpm, a closed-chamber mode of operation, calibration of the polarographic oxygen sensor before each measurement, and periodic measurement of instrumental background oxygen consumption (Gnaiger 2014).

The OROBOROS Oxygraph-2k represents a sensitive instrument used for analysis of mitochondrial respiration in research of cellular bioenergetics during physiological or pathophysiological processes, as well as for studying the effects of different drugs. Measurements were carried out on one set of 6 patients and 6 control and 6 experimental rats. Excellent measurement sensitivity and stability enabled us to obtain valid results even from these small sample sets.

Platelet preparation

Viable platelets are easily available components of peripheral blood, which may be useful for *in situ* studies of mitochondrial respiration (Sjövall *et al.* 2010, 2013, Hroudová *et al.* 2013). Thus, we used platelets as a biological model to analyze statin-induced changes in mitochondrial respiration under physiological conditions.

Six patients (4 males and 2 females, age of 43.7±14.3 years) with primary hypercholesterolemia (who were monitored at the Lipid Clinic of the 3rd Dept. of Medicine at Charles University in Prague, and were indicated for statin treatment) were enrolled in the study. Blood samples were collected at baseline (without any lipid lowering pharmacotherapy) and again after 6 weeks of treatment with rosuvastatin (5 patients) or atorvastatin (1 patient) at a dosage 0.2-0.5 mg/kg per day. Study participants characteristics at baseline and follow up are presented in Table 1. Two of the study participants were smokers, two had arterial hypertension, none had diabetes. Their transaminase levels and creatine phosphokinase activity were normal. Average body mass index was 24.8±2.6 kg/m^2 and did not change during the study.

Between 7:00 and 8:00 AM, peripheral blood samples were drawn from the antecubital vein of each fasting participant *via* BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ 07417 USA), with K_2EDTA as the anticoagulant. The study was carried out according to the principles expressed in the Declaration of Helsinki and the study protocol was approved by the Ethic Review Board of the First Faculty of Medicine and the General University Hospital in Prague, Czech Republic.

Table 1. Characteristics of study participants at baseline and after 6 weeks of statin treatment (N=6).

No	Age (years)	Gender	Chol 1 (mM)	Chol 2 (mM)	TG 1 (mM)	TG 2 (mM)	HDL 1 (mM)	HDL 2 (mM)	LDL 1 (mM)	LDL 2 (mM)	Glu 1 (mM)	Glu 2 (mM)	BMI 1 (kg/m^2)	Treatment (dose)
1	61	f	8.12	5.01	2.88	1.54	1.44	1.42	5.38	2.90	5.80	6.30	25.3	Rosuvastatin (10 mg)
2	35	m	6.35	5.01	1.48	1.19	1.24	1.39	4.44	3.08	4.60	4.80	24.8	Rosuvastatin (40 mg)
3	34	m	7.74	5.61	2.07	1.65	1.57	1.29	5.24	3.58	5.00	5.10	25.8	Rosuvastatin (20 g)
4	51	m	8.34	5.28	0.78	0.85	1.81	1.71	6.18	3.19	4.90	5.10	27.0	Rosuvastatin (40 mg)
5	22	f	10.20	5.90	2.67	1.43	1.96	1.70	7.05	3.56	4.60	4.40	19.3	Rosuvastatin (20 mg)
6	59	m	6.12	4.95	1.04	1.23	1.41	1.33	4.24	3.07	4.30	5.20	26.7	Atorvastatin (10 mg)
Mean	**43.7**		**7.81**	***5.29**	**1.82**	**1.32**	**1.57**	**1.47**	**5.42**	***3.23**	**4.87**	**5.15**	**24.8**	
SD	14.3		1.36	0.35	0.79	0.26	0.25	0.17	0.97	0.25	0.47	0.58	2.6	

Abbreviations: Index 1, baseline values; Index 2, post-treatment values; Chol, cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; Glu, fasting glucose concentration; BMI, body mass index
Significantly different mean values observed when values were compared before and after treatment (using the Wilcoxon signed-rank test) are marked: * $P<0.05$.

The experimental animals were adult naive male Long-Evans rats that were 3-month-old at the beginning of the experiment. Simvastatin (Simvastatin Mylan), dissolved in sweetened jelly, was administered at a dosage of 30 mg/kg per day for four weeks. The animals were randomly assigned to an experimental group of 6 rats receiving vehicle jelly with simvastatin and a control group of 6 rats receiving vehicle jelly alone. On day 28 of treatment, the animals were sacrificed by decapitation and blood samples were collected with K_2EDTA as the anticoagulant. All procedures were done in accordance with the Animal Protection Code of the Czech Republic and the European Council directives (2010/63/EC; 86/609/EEC). The study protocol was approved by the Animal Care Committee of the Institute of Physiology of Academy of Sciences of the Czech Republic.

Whole non-coagulable blood underwent centrifugation at 200 g for 20 min at 25 °C to separate a platelet rich plasma (PRP) aliquot from the red blood cells. The upper portion of the centrifuged samples, which contained the platelets dissolved in plasma, was drawn off, and the platelets were counted using a microscope and a counting chamber. The platelets in the plasma samples were used to measure mitochondrial respiration in intact platelets. Mitochondrial respiration in permeabilized platelets was measured in diluted PRP (Franco *et al.* 2012). Plasma samples containing platelets were centrifuged at 1500 g for 10 min at 25 °C. The plasma was then discarded and the pellet was resuspended in the same volume of MiR05.

Experimental protocols

Experimental protocols for intact or permeabilized platelets were adapted from Sjövall *et al.* (2010, 2013) as described previously (Hroudová *et al.* 2013).

Respiration of intact platelets with endogenous mitochondrial substrates was measured in PRP diluted (1:1) with Krebs and Henseleit isotonic medium. Endogenous basal rates of platelet respiration (resting state respiration, physiological respiration, *PR*) were measured without any additives. Respiration that was independent of adenosine diphosphate (ADP) phosphorylation (*LEAK*) was measured after the addition of 2 μg/ml oligomycin (an ATP synthase inhibitor). The maximal capacity of the electron transport system (*ETSC*) in the non-coupled state was achieved by titration with the protonophore carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP), in a range from 5 to 50 μM. Finally, mitochondrial respiration was inhibited using 2.5 μM of the complex I inhibitor, rotenone (*Rot*) and 2.5 μg/ml of antimycin A (a complex III inhibitor); residual oxygen consumption (*ROX*) was measured so that it could be subtracted from all other mitochondrial respiratory rates.

Respiration of permeabilized platelets was measured in washed (plasma free) platelets, whose plasma membranes were permeabilized using digitonin (50 μg/ml). Subsequently, substrates were added for complex I- and complex I+II-linked respiration. ADP stimulated respiration (*ADP*) was measured after the addition of 5 mM malate, 5 mM pyruvate, 1 mM ADP, and 5 mM glutamate. Respiratory capacity at saturating

concentrations of ADP, inorganic phosphate, oxygen, and defined complex I+II-linked substrates (*OXPHOS capacity*) was measured after the addition of 10 mM succinate. A non-phosphorylating resting state of intrinsic uncoupled or decoupled respiration (*LEAK*) was induced using 2 µg/ml oligomycin. Maximal convergent capacity of the ETS (*ETSC*) was obtained by titration with the uncoupling agent FCCP in each experiment (range 1 to 4 µM). Complex I-supported respiration was inhibited using 0.5 µM rotenone (*Rot*). Finally, 1.25 µg/ml antimycin A was added and residual oxygen consumption (*ROX*) was measured for subtraction from all other mitochondrial respiratory rates.

Data analysis

The respirometry data were collected and analyzed using DatLab 4.3 software (Oroboros Instruments, Innsbruck, Austria). Platelet oxygen consumption rates (platelet respiratory rate, O_2 flow per cell) were normalized for either platelet concentration (and expressed as pmol O_2 consumed per second relative to 10^6 platelets) or maximum oxygen flux in uncoupled respiration (and expressed as a control ratio in relative units). Net physiological respiration (*NetPR*) in intact platelets was calculated as the difference *PR – LEAK*. Statistical analyses were performed using Statistica (version 12, StatSoft, Inc., Tulsa, OK, USA). Data are expressed as arithmetic means. Standard deviations (SD) were calculated to characterize group variability. Hypothesis testing was performed using the Mann-Whitney U test and Wilcoxon signed-rank test.

Results

Effect of simvastatin on mitochondrial respiration in intact rat platelets

Mitochondrial respiration in intact blood platelets was measured, after 4 weeks of treatment, in a total of 6 control rats and 6 rats treated with simvastatin. The flux control ratio *LEAK/ETSC* in intact platelets was found to be 0.079±0.027 (mean ± SD, N=12) (Fig. 1B), which indicates the functional integrity of the inner mitochondrial membrane.

Mean mitochondrial oxygen consumption rates normalized for platelet concentration were measured before (*PR*) and after inhibition of ADP phosphorylation by oligomycin (*LEAK*), after uncoupling by FCCP (*ETSC*), and after inhibition of complex I-linked respiration by rotenone (*Rot*) (Fig. 1A). Control ratios were calculated as ratios of platelet oxygen consumption rates at different respiratory states to ETSC (Fig. 1B).

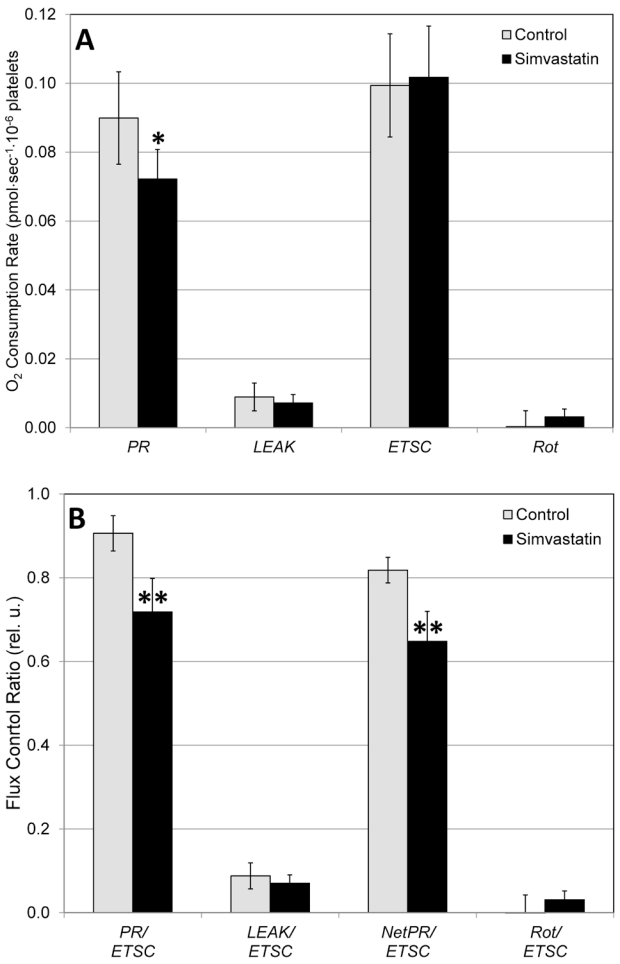

Fig. 1. Mitochondrial respiration in intact platelets of simvastatin-treated rats after 4 weeks of treatment. Platelet respiratory rates were normalized for (**A**) platelet concentration and (**B**) maximal capacity of the electron transport system (*ETSC*). Mean ± standard deviations are shown. Significantly different mean values found when compared with controls (using the Mann-Whitney U test) are marked: * P<0.05, ** P<0.01.
Abbreviations: *ETSC*, the maximal capacity of the electron transport system in the non-coupled state was achieved by titration with protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone); *LEAK*, respiration that was independent of adenosine diphosphate phosphorylation was measured after the addition of oligomycin; *NetPR*, net physiological respiration was calculated as the difference *PR-LEAK*; *PR*, endogenous basal rate of platelet respiration (physiological respiration); *Rot*, mitochondrial respiration after the addition of rotenone.

The endogenous basal rate of platelet respiration (*PR*) was significantly decreased in simvastatin-treated rats compared with controls (P=0.045) (Fig. 1A). The flux control ratio was significantly decreased for both *PR/ETSC* (P=0.0082) and *NetPR/ETSC* (P=0.005) in simvastatin-treated rats (Fig. 1B); a decrease in *PR* was

responsible for this effect.

Effect of statins on mitochondrial respiration in human platelets

Mitochondrial respiration in both intact and permeabilized platelets was measured in 6 patients before and after 6 weeks of treatment with a statin. The flux control ratio *LEAK/ETSC* was found to be 0.076±0.046 (mean ± SD, N=12) in intact platelets (Fig. 2B) and 0.145±0.024 (mean ± SD, N=12) in permeabilized platelets (Fig. 3B), which indicates the functional integrity of the inner mitochondrial membrane.

Mean mitochondrial oxygen consumption rates, normalized for platelet concentrations, are summarized in Figures 2A and 3A. Control ratios, calculated as oxygen consumption rates normalized to ETSC, are displayed in Figures 2B and 3B. No significant changes in mitochondrial respiration were detected in intact platelets (Fig. 2).

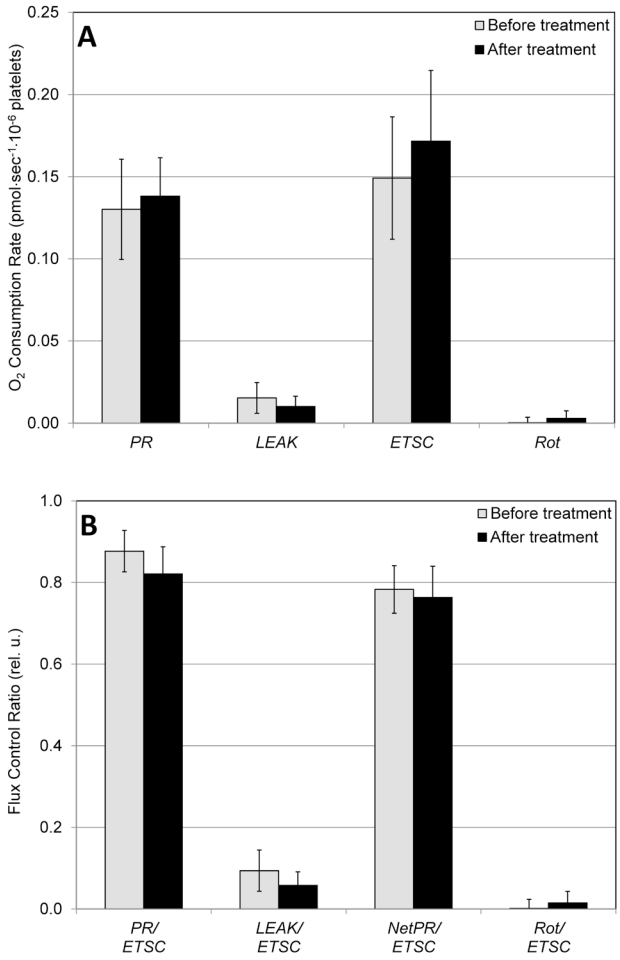

Fig. 2. Mitochondrial respiration in intact platelets of statin-treated humans before treatment and after 6 weeks of treatment. Platelet respiratory rates were normalized for (**A**) platelet concentration and (**B**) maximal capacity of the electron transport system (*ETSC*). Mean ± standard deviations are shown. No significant differences between before and after treatment mean values were found (using the Wilcoxon signed-rank test). Abbreviations: *ETSC*, the maximal capacity of the electron transport system in the non-coupled state was achieved by titration with protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone); *LEAK*, respiration that was independent of adenosine diphosphate phosphorylation was measured after the addition of oligomycin; *NetPR*, net physiological respiration was calculated as the difference *PR-LEAK*; *PR*, endogenous basal rate of platelet respiration (physiological respiration); *Rot*, mitochondrial respiration after the addition of rotenone.

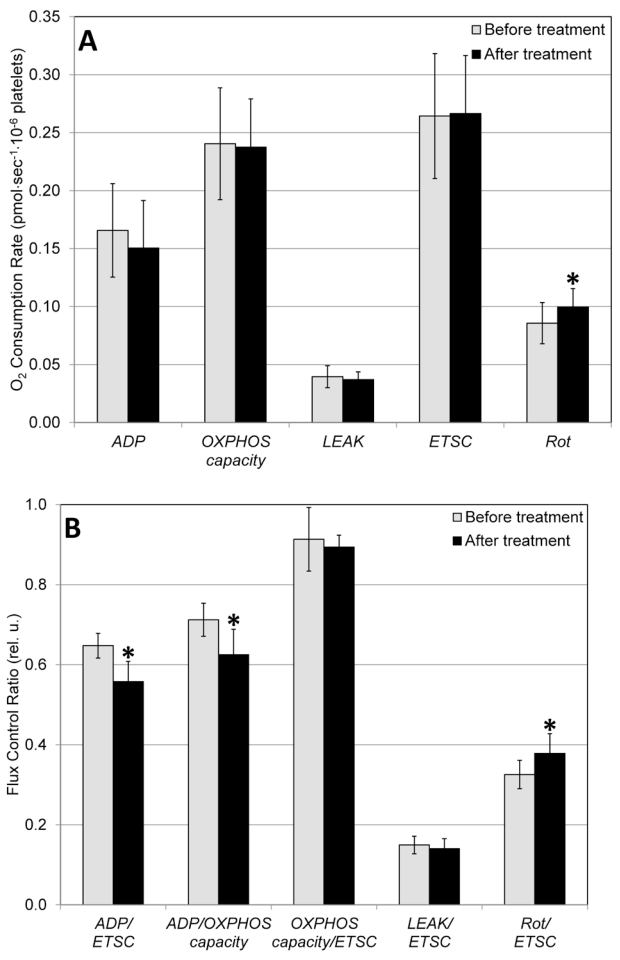

Fig. 3. Mitochondrial respiration in permeabilized platelets of statin-treated humans before treatment and after 6 weeks of treatment. Platelet respiratory rates were normalized for (**A**) platelet concentration and (**B**) maximal capacity of the electron transport system (*ETSC*) or maximal coupled oxidative capacity (*OXPHOS capacity*). Mean ± standard deviations are shown. Significantly different mean values found when comparing values before and after treatment (using the Wilcoxon signed-rank test) are marked: * $P<0.05$. Abbreviations: *ADP*, adenosine diphosphate stimulated respiration; *ETSC*, the maximal capacity of the electron transport system in the non-coupled state was achieved by titration using the protonophore FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone); *LEAK*, respiration that was independent of ADP phosphorylation was measured after the addition of oligomycin; *OXPHOS capacity*, respiratory capacity at saturating concentrations of ADP, inorganic phosphate, oxygen, malate, pyruvate, glutamate, and succinate; *Rot*, mitochondrial respiration after the addition of rotenone.

In permeabilized platelets, the *OXPHOS capacity/LEAK* ratio was found to be 6.36±0.89 (mean ± SD, N=12), which indicated good coupling of electron transport to ATP synthesis and was comparable to previous results (Sjöval *et al.* 2013). No change was observed during treatment with statins. The rotenone-induced decrease of the oxygen consumption rate was significantly lower after treatment with statins (Fig. 3A). A significant statin-induced decrease in ADP stimulated respiration (in the presence of substrates supporting respiration through complex I) was observed when the platelet respiration rate was normalized for maximal uncoupled capacity *ETSC* (P=0.027) as well as for maximal coupled oxidative capacity *OXPHOS capacity* (P=0.046); higher oxygen consumption rates after rotenone addition were also observed (Fig. 3B). These results indicate significantly decreased complex I-linked respiration after statin treatment.

Discussion

The respiratory rate of platelets may serve as an easily available biological marker for an *in situ* study of disease-related or drug-induced changes in mitochondrial function (Sjövall *et al.* 2010, 2013; Hroudová *et al.* 2013). Previous studies dealing with statin-induced *in vivo* changes in mitochondrial functions have been performed mostly on skeletal muscle mitochondria. The current pilot study is the first, as far as we know, to examine the effects of statins on the mitochondrial respiratory rate in intact blood platelets dissolved in plasma without any additives, i.e. when platelet respiratory rates are measured at the resting state respiration at or near physiological conditions.

Lowering of the physiological respiratory rate in intact platelets (Fig. 1A), as well as a decrease in the control ratio representing respiratory efficiency in simvastatin-treated rats (Fig. 1B), are in agreement with recently published *in vitro* inhibitory effects of simvastatin on phosphorylating respiration in permeabilized skeletal muscle fibers (La Guardia *et al.* 2013, Larsen *et al.* 2013). However, we did not find inhibition of uncoupled respiration associated with simvastatin (Fig. 1A). Physiological respiration, but not ETS capacity was observed to be affected by 4 weeks of simvastatin administration. This indicates that modulation upstream of mitochondrial metabolism and/or ATP synthase activity may participate in the *in vivo* effect of simvastatin on platelet respiratory rates. However, the effects of simvastatin on mitochondrial respiration in rat platelets are apparently linked to administration of high doses of simvastatin.

Pleiotropic effects of statins are frequently studied *in vitro* and in experiments on animal models, when the plasma concentrations of statins are much higher than those achieved during therapeutic use in humans (Björkhem-Bergman *et al.* 2011). To induce a similar effect in rats, we used simvastatin at a dosage of 30 mg/kg per day for 4 weeks. The effect of high doses of statins on mitochondrial respiration in intact platelets of rats (Fig. 1) was not present in human platelets at therapeutic statin doses (Fig. 2). We suggest that the observed differences between mitochondrial respiration in rat and human intact platelets (suspended in plasma) can be attributed to differences in plasma concentration of statins.

There is strong evidence that statins, as a class, are generally safe and adverse events associated with statin therapy are uncommon (Naci *et al.* 2013). However, the highest potency statins (rosuvastatin and atorvastatin) have shown higher muscle adverse event rates (Hoffman *et al.* 2012). We studied the effect of treatment with these newer statins on mitochondrial respiration in relatively young hyperlipidemic subjects who were not contraindicated for statin use (i.e. they were not selected for increased risk for problems on statins). The lipophilic or hydrophilic nature of statins seems to play an important role in the occurrence of side effects, with lovastatin and simvastatin being the most lipophilic, followed by atorvastatin, fluvastatin, rosuvastatin, and pravastatin. We did not observe any difference in mitochondrial respiration in platelets of patients treated with rosuvastatin and atorvastatin. Thus, mean values of respiratory parameters were calculated for the group of patients treated with rosuvastatin or atorvastatin as a whole. Disturbing effects on platelet mitochondrial respiration seem to be very low at therapeutic doses of atorvastatin and rosuvastatin, with no significant statin-induced changes in mitochondrial respiration being observed in intact human platelets (Fig. 2). Additionally, relatively little change in complex I-linked respiration was seen in permeabilized platelets (Fig. 3). This is consistent with the observation of a low incidence of clinically significant side effects associated with statin therapy. Our results do not support the claims of statin-related mitochondrial toxicity that have been predicted on the basis of *in vitro* experiments; such claims fail to appreciate the potential neuroprotective efficacy of statins

(Parihar *et al.* 2012, Malfitano *et al.* 2014, Li *et al.* 2014), which could be useful in the treatment of various neurological and neuropsychiatric disorders.

Limitations of this study include the following facts: the sample size was small, patients were not selected for a risk of having statin-induced problems, and the duration of treatment was short, relative to the time needed to develop potential statin problems. Our measurements after a few weeks of statin administration, in hyperlipidemic subjects without a significant risk of statin-induced problems, was unable to detect any risk of adverse effects that could be associated with long-term administration of statins. However, this study was designed as a pilot study to determine whether the respiration rate of mitochondria in intact or permeabilized platelets is impaired by therapeutic (in humans) or high doses (in rats) of statins. The results must be interpreted with caution, but we suggest that the data are sufficient to indicate that mitochondrial respiration in intact platelets is not significantly changed after short-term treatment at therapeutic statin doses; however, high doses of simvastatin, in rats, can induce a significant decrease in mitochondrial resting state respiration even after short-term applications. The results may be useful for the design of further studies of *in vivo* effects of statins on platelet mitochondrial respiration in people with established mitochondrial pathology, and in people with risk factors for mitochondria related problems. We hope our study will be the first step towards experiments designed to detect (using measurements of mitochondrial respiration in platelets) the risk of development of certain statin-induced adverse effects.

There is no expectation that everyone placed on statins will suffer adverse effects due to mitochondrial toxicity. However, it can be expected that mitochondrial function will be affected while achieving higher levels of statins in the blood, though this may not always be clinically manifested. In this pilot study, we wanted to determine whether such changes of mitochondrial function occur after short-term administration of statins, regardless of whether significant adverse effects from long-term administration of statins are produced or not. It can be expected that mitochondrial function will be affected while achieving higher concentrations of statins in the blood, even though it may not clinically manifest.

Mitochondrial respiration is a very complex system with many feedbacks. Our results suggest that statin-induced changes in the activity of respiratory chain complexes (described in earlier *in vitro* experiments) are either very small or are compensated for in intact platelets. We assume that the measurement of mitochondrial respiration in permeabilized platelets (with an excess supply of substrates and inhibitors) may reveal some drug-induced changes, which are hidden in intact platelets. Our measurement of respiration in permeabilized human platelets revealed that short-term administration of statins may slightly disrupt complex I-linked respiration; however, it does not cause a significant disruption in the physiological respiration in intact platelets.

Conclusions

The results of our study demonstrate decreased respiratory rates in intact platelets with unchanged respiratory capacity after short-term treatment with high doses of simvastatin in rats. Assuming that platelet respiration mirrors mitochondrial respiration in other cells, treatment with high doses of statins may disturb cellular bioenergetics in the organs most sensitive to ATP insufficiency, which might be related to its adverse effects. At therapeutic doses in humans, we did not observe a significant effect of statins on mitochondrial respiration in intact platelets. The reduction in complex I-linked respiration observed in permeabilized platelets shows small (*in vivo* compensated) disruptions in respiratory chain function even at therapeutic doses of statins. We suggest that platelets might be used as an easily available biological model to study the effects of statins on mitochondrial functions with a minimal burden on patients.

Conflict of Interest

There is no conflict of interest.

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