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Lovastatin induces platelet apoptosis

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ABSTRACT

Statins are widely used in the prevention of atherosclerosis and treatment of coronary artery disease because of pleiotropic effects on thrombosis. Thrombocytopenia and hemorrhage occurred in some statin-treated patients, but the reason remains unclear. In the current study, we show that lovastatin dose-dependently induces depolarization of mitochondrial inner transmembrane potential, leading to up-regulation of Bak, down-regulation of Bcl-X_L, and activation of caspase-3/8/9. Lovastatin treatment did not increase the surface expression of P-selectin or PAC-1 binding but led to strongly reduced collagen- and thrombin-induced platelet aggregation. The integrin $\alpha_{IIb}\beta_3$ antagonist, RGDS, inhibited lovastatin-induced apoptosis in both human platelets and Chinese hamster ovary (CHO) cells stably expressing integrin $\alpha_{IIb}\beta_3$. The number of circulating platelets in mice was significantly reduced after intraperitoneal injections with lovastatin. Taken together, these data indicate that lovastatin induced caspase-dependent platelet apoptosis. Lovastatin does not incur platelet activation, whereas impairs platelet function and reduces circulating platelets *in vivo*, suggesting the possible pathogenesis of thrombocytopenia and hemorrhage in patients treated with statins.

1. Introduction

The statin family of drugs, widely used in the control of hypercholesterolemia, is a first-line treatment of coronary artery disease and atherosclerosis (Moraes et al., 2013). Statins decrease cholesterol production by inhibiting hydroxymethyl glutaryl coenzyme A (HMG-CoA) reductase, which is responsible for the conversion of HMG-CoA to mevalonate, a rate-limiting step in synthesis of cholesterol. Independent from its cholesterol-dependent effects, the benefits of statins are pleiotropic. For instance, statins decrease vascular inflammation *via* downregulation of serum levels of C-reactive protein (CRP) and NFkB (Lahera et al., 2007). Moreover, statins have been reported to induce tumor cell apoptosis and have been trialed in cancer treatments (Johnson et al., 2002; Niknejad et al., 2014). However, like other medications, statins have many potential side effects. The most serious adverse effect associated with statin therapy is myopathy, which may develop fatal or nonfatal rhabdomyolysis (Sacher et al., 2005). Thrombocytopenia appears to be another side effect of statins, since the association of thrombocytopenic purpura with statin treatment has been reported (Ames, 2008; Gonzalez-Ponte et al., 1998; Groneberg et al., 2001; Possamai et al., 1992; Yamada et al., 1998), however, the pathophysiology of this relationship remains unclear.

Platelet apoptosis can be induced by either physiological or chemical compounds and occurs widely *in vitro* or *in vivo*, a mechanism which may control the number of circulating platelets or contribute in the development of platelet-related diseases (Leytin, 2012). We reported recently that an anticancer drug, arsenic trioxide (ATO), leads to platelet apoptosis *in vitro* and reduces circulating platelet count *in vivo* (Wu et al., 2014). In the current study, we show that lovastatin induces caspase-dependent platelet apoptosis. Lovastatin therapy did not cause platelet activation but led to platelet dysfunction and reduced circulating platelets *in vivo*.

2. Materials and methods

2.1. Ethics statement

For studies involving human subjects, approval was obtained from the Ethical Committee of Soochow University. Written informed consent was provided and studies were performed in accordance with the Declaration of Helsinki. Permission for animal

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experiments was granted by the Ethics Committee of Laboratory Animals of Soochow University. The proper housing, feeding and care, and all interventions relating to animal welfare were carried out in compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China).

2.2. Reagents and antibodies

Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, Missouri, USA). Lovastatin was purchased from Shanghai Oriental Pharmaceutical Science and Technology Co. Ltd. (Shanghai, China). Fluorescein isothiocyanate (FITC)-conjugated annexin V was purchased from Jiamay Biotech (Beijing, China). JC-1 dye and anti-caspase 3 antibody were purchased from Beyotime Institute of Biotechnology (Haimen, China). FITC-labeled PAC-1 antibody, which recognizes active integrin $\alpha_{IIb}\beta_3$, was purchased from BD Biosciences (San Jose, CA, USA). FITC-conjugated anti-human CD62P antibody, anti-caspase-8 and -9 antibodies were from Biolegend Inc. (San Diego, CA, USA). Annexin V-FITC & PI detection kit was purchased from Jiamay Biotech (Beijing, China). Anti-Bak, Bcl-X_L, glyceraldehyde 3-phosphate dehydrogenase (GADPH), actin antibodies, horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Santa Cruz (Santa Cruz, CA, USA). Arg-Gly-Asp-Ser (RGDS) peptides were synthesized by Ubio (Hangzhou, China).

2.3. Preparation of washed platelets

Human venous blood was obtained from healthy volunteers, and platelets were isolated and washed as previously described (Wu et al., 2014). Briefly, blood samples were anti-coagulated with ACD (2.5% trisodium citrate, 2.0% D-glucose, 1.5% citric acid). Platelets were washed with CGS buffer (0.123 M NaCl, 0.033 M D-glucose, 0.013 M trisodium citrate, pH 6.5) and resuspended in modified tyrodes's buffer (2.5 mM HEPES, 150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 5.5 mM D-glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) Washed platelets were placed at room temperature (RT) for 1 h to recover to the resting state.

2.4. Mitochondria membrane depolarization ($\Delta\Psi$m) assay

Washed platelets were incubated with different concentrations of lovastatin (6.25, 12.5, 25 μM), or vehicle control (DMSO/A23187, negative/positive control) at 37 °C for 5 h. For the integrin $\alpha_{IIb}\beta_3$ blocking experiment, washed platelets were pretreated with 2 mM RGDS peptides or PBS at 37 °C for 5 minutes (min) before 25 μM lovastatin or DMSO treatment at 37 °C for 5 h. The lipophilic cationic probe JC-1 was added and incubated in the dark for 15 min. The fluorescence was measured using a CytomicsTM FC500 Flow Cytometer (Backman Coulter, USA). The ratio of red fluorescence to green was used to measure the change of $\Delta\Psi m$.

2.5. Phosphatidylserine (PS) exposure assay

Washed platelets were treated with different concentrations of lovastatin (6.25, 12.5, 25 μM) or DMSO at 37 °C for 5 h. Annexin V binding buffer and FITC-conjugated annexin V were then added and incubated in the dark for 15 min. The fluorescence was then measured by flow cytometry, as above.

2.6. Platelet activation assay

Washed platelets were treated with different concentrations of lovastatin (6.25, 12.5, 25 μM) or DMSO at 37 °C for 5 h, and then added with PAC-1 or anti-human CD62P antibody conjugated with FITC. Flow cytometry analysis was performed to detect platelet activation, as above.

2.7. Cell apoptosis assay

Chinese hamster ovary (CHO) cells stably expressing wild-type integrin $\alpha_{IIb}\beta_3$ (CHO23) were established as previously described (Bodnar et al., 2002). Control CHO cells and CHO23 cells were maintained in DMEM media with 10% fetal bovine serum (FBS). Cells (1×10^5/mL) were seeded into 6-well plate, cultured for 24 h in complete media (10% FBS). Cells were then washed twice with PBS and treated with 25 μM lovastatin or DMSO in new serum-free media for another 24 h. After treatment, cell apoptosis was analyzed using an Annexin V-FITC & PI detection kit by flow cytometry according to the manufacture's procedure. Briefly, the cells were washed with PBS, added with 1× binding buffer, incubated with Annexin V-FITC for 15 min and PI for another 5 min, and the apoptosis level was measured by flow cytometry.

2.8. Platelet aggregation assay

Washed platelets were incubated with different concentrations of lovastatin (6.25, 12.5, 25 μM) or vehicle control (DMSO) at 37 °C for 1 h, and platelet aggregation was induced by the addition of collagen (5 μg/mL) or thrombin (0.5 U/mL) in a turbidimetric platelet aggregometer (Chrono-log, PA, USA) at a stirring speed of 1000 rpm.

2.9. Western blot assay

After washed platelets were treated with lovastatin or DMSO, the platelet suspension was frozen and thawed twice. Sodium dodecyl sulfate (SDS) sample buffer was added to the mixture, boiled at 100 °C for 10 min, and subjected to 10% or 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.10. Animal model

C57BL/6j mice (8–9 weeks) were purchased from Shanghai Institute for Biological Sciences (Shanghai, China). Mice were housed at 22–24 °C with a day–night light cycle of 12 h, randomly divided into lovastatin and control groups, and then intraperitoneally injected with lovastatin at a dose of 5 mg/kg or PBS. Before and 4 h after injection, blood samples were collected and platelet counted by a Sysmex XP-100 Blood Cell Analyzer (Sysmex Corporation, Kobe, Japan).

2.11. Statistical analysis

Data were shown as mean ± SD (standard deviation) and analyzed by the SPSS 19.0 statistical software. The statistical significance was determined by one-way ANOVA analysis of variance with the LSD test for multiple groups and by paired *t*-test for two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lovastatin dose-dependently induces apoptosis in platelets

It has been reported that lovastatin induces nucleate cell apoptosis through mitochondria pathways (Johnson et al., 2002; Liang et al., 2006; Niknejad et al., 2014; Sacher et al., 2005). However, it is still unclear whether lovastatin induces anucleate platelet apoptosis. To investigate this, platelets were incubated with different concentrations of lovastatin and platelet $\Delta\Psi m$ depolarization was tested by flow cytometry (Fig. 1). As the experiments demonstrate,