Monitoring the Cellular Effects of HMG-CoA Reductase Inhibitors in vitro and ex vivo

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Materials and Methods

Reagents

RPMI 1640 medium, DMEM, penicillin/streptomycin were from PAA Laboratories, Pasching, Austria. Phosphate-buffered saline (PBS) and fetal calf serum (FCS) were purchased from Biochrom, Berlin, Germany. Mevalonic acid lactone was converted to sodium mevalonate as described by Essig et al. The specific inhibitor of geranylgeranyltransferase, L841491 (L841), and simvastatin were kindly provided by MSD, Sharp and Dohme, Munich, Germany. Simvastatin was dissolved in ethanol and activated as described by Jakobisiak et al. Atorvastatin was kindly provided by Pfizer, Karlsruhe, Germany. A mouse monoclonal antibody directed against human RhoA was purchased from Santa Cruz, Heidelberg, Germany. Mouse anti-human Cdc42 and Rac1 antibodies were from BD Transduction Laboratories, Heidelberg, Germany. Secondary horseradish peroxidase-conjugated anti-mouse IgG was obtained from Amersham, Freiburg, Germany.

Fibroblast Cell Culture

Immortalized human renal fibroblasts were kindly provided by G. Muller, Goettingen, Germany. The cells were characterized in detail and shown to maintain the characteristics of primary fibroblasts. Culturing of the cells was done as previously described.

Phosphatase Treatment

After stimulation with simvastatin, fibroblasts were collected in 50 mM Tris/HCl, pH 7.9, 10 mmol/L MgCl₂, 100 mmol/L NaCl and 1 mmol/L dithiotreitol. Cellular homogenates were obtained by three freeze-thaw cycles in liquid nitrogen followed by three sonification cycles, 15 sec each. Cellular proteins (160 µg) were incubated with 30 U alkaline phosphatase (calf intestinal phosphatase, Sigma, Munich, Germany), for 1 h at 37 °C. The proteins were ethanol-precipitated before analysis by isoelectric focusing.

Separation of Isoprenylated and Non-Isoprenylated RhoA

Isoprenylated RhoA was determined essentially as described by Chappell et al. After treatment with or without simvastatin, whole cell lysates were prepared in lysis buffer (1 mmol/L Na₃PO₄, 50 mmol/L Heps, 5 mmol/L MgCl₂, 150 mmol/L NaCl, 0.05% SDS, 1%
Triton X-100). Cells were sonicated 2 x 10 s at 50% output (Branson sonifier), and debris was removed by centrifugation. Lysate containing about 450 µg protein was mixed with an equal volume of 4% Triton X-114 and warmed to 37 °C for 3 min. Separation of a Triton-rich lower phase containing the isoprenylated form of RhoA and an upper buffer phase containing the non-modified form was carried out at room temperature. Before analysis by SDS-PAGE, the protein of the lower phase was precipitated by ethanol, and the pellet was resuspended in 20 mmol/L Tris/HCl pH 8.0 and 1 mmol/L EDTA. For isoelectric focusing (IEF) the total upper and lower phase were precipitated.

**Isoelectric Focusing**
One-dimensional IEF was performed according to the manufacturer’s instructions (Invitrogen, Karlsruhe, Germany). Briefly, cell pellets were resuspended in Novex™ 3-10 IEF sample buffer (Invitrogen) and sonicated twice for 10 s. After measuring the total protein concentration with Bradford reagent (BioRad, Munich, Germany), 20 µg of protein was loaded per lane on one-dimensional Novex™ IEF gel pH 3-10. Electrophoresis was run using the buffer system specified by the manufacturer.

**Two-Dimensional Gel Electrophoresis (2D Electrophoresis)**
Cell pellets were resuspended in lysis buffer containing 9 mol/L urea, 4% CHAPS, 1% dithiothreitol (DTT), and 2% Biolyte 3-10 (Amersham) followed by sonication. Protein concentration was determined in each sample using Bio Rad protein assay. Immobiline DryStrip gels pH 3-10 NL (Amersham) were rehydrated for 12 h at 20 °C using rehydration solution containing 8 mol/L urea, 1% CHAPS and 0.4% DTT. One hundred µg of total protein was loaded with rehydration solution. First-dimension isoelectric focusing of proteins was run at 20 °C on a IGPhor isoelectric focusing unit with the voltage program specified by the manufacturer. Following the first-dimension separation, proteins were separated on 12% SDS-PAGE gels.

**Western Blot Analysis**
For Western blot analysis, the proteins were transferred to polyvinylidene difluoride membranes. After blocking with 5% horse serum in Tris-buffered saline containing 0.1% Tween 20, RhoA was detected using anti-RhoA antibody (1:200) overnight at 4 °C, followed by the secondary anti-mouse IgG (1:10 000) for 1 h. Anti-Cdc42 and anti-Rac1 antibodies were used at dilutions of 1:200 and 1:1500, respectively. Immunoreactive proteins were visualized using enhanced chemiluminescence system (ECL-Plus, Amersham). For quantification purposes, densitometric measurement was performed using the image analysis software from Bioprofil (Froebel, Germany). To analyze the minor spots,
luminescence measurements were performed using a luminescent image analyzer (LAS-1000, Fujifilm) and Aida 2D image analysis software (Raytest, Berlin, Germany).

**Preparation of Peripheral Blood Mononuclear Cells (PBMCs)**

Venous blood from adult volunteers was drawn on acid-citrate-dextrose buffer. Three volunteers took no medication, and two subjects were taking low prophylactic doses of statins (simvastatin 10 mg/d or atorvastatin 20 mg/d). One healthy subject was asked to take 40 mg of atorvastatin per day for 1 week. Blood from this volunteer was drawn before the start of medication, on day 2 and on day 7 after the start of medication. PBMCs were isolated immediately after collecting blood.

In a subsequent clinical study, 10 healthy volunteers (mean age 31 ± 7.1 y) were given 40 mg/d atorvastatin for 5 consecutive days. Blood was drawn before the start of the study and after 5 days of treatment. PBMCs were isolated within 1 h of blood collection. Total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglyceride levels in the sera were measured before and after the study. The protocol was approved by the local ethics committee, and written informed consent was obtained from all the volunteers.

PBMCs were separated by density gradient centrifugation using Vacutainer cell preparation tubes (CPT, Becton Dickinson). After washing the cells 3 times in PBS, the pellets were frozen at -80°C until the analysis. For *in vitro* experiments, PBMCs were washed twice in PBS and once in RPMI 1640 medium, and resuspended in RPMI 1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FCS. The cells were seeded on 60 mm diameter culture dishes at the density of 6 x 10⁶ cells per dish. Viability of cells was greater than 95% as estimated by Trypan blue exclusion. The cells were cultured for 24 h with presence or absence of 10 µmol/L atorvastatin and 100 µmol/L mevalonate at 37 °C in a humidified 5% CO₂ incubator. After harvesting, the cells were washed with PBS, and the pellets were frozen at -80°C until analysis.

**References**


