

Fluvastatin increases tyrosinase synthesis induced by UVB irradiation of B16F10 melanoma cells

Ryszard Galus^{1,2}, Emir Sajjad¹, Justyna Niderla¹, Katarzyna Borowska²;
Krzysztof Włodarski¹, Paweł Włodarski¹, Jarosław Józwiak¹

¹Chair and Department of Histology and Embryology, Center for Biostructure, Medical University of Warsaw, Warsaw, Poland

²Department of Dermatology, Military Institute of Health, Warsaw, Poland

Abstract: Statins are widely used to lower plasma concentrations of lipids, *e.g.* cholesterol. One of the main effects of statin treatment is inhibition of hydroxymethyl glutaryl-coenzyme A reductase. The role of fluvastatin, a frequently used statin, was examined in potential modulation of tyrosinase (key enzyme of melanogenesis) synthesis. Levels of tyrosinase mRNA induced by UVB irradiation of B16F10 melanoma cell line were measured by real time PCR. Fluvastatin increases tyrosinase mRNA production induced by UVB irradiation in B16F10 melanoma cell line. Fluvastatin treatment may potentially influence melanin synthesis and protection against UV irradiation.

Key words: melanogenesis, melanoma, statins, tyrosinase

Introduction

Statins inhibit cholesterol synthesis by reducing 3-hydroxy-3-glutaryl-coenzyme A (HMG-CoA) reductase activity. It has also been shown that statins stimulate formation of substantial amounts of new bone, *e.g.* by increasing expression of bone morphogenetic protein-2 (BMP-2), which leads to osteoblast differentiation and bone formation [1,2]. Administration of statins may also affect heterotopic ossification [3]. BMP-2 treatment of neural crest cells increases melanogenesis by promoting the synthesis of melanin [4]. Recently, it has been demonstrated that BMP signaling plays an important role in melanocytes residing at hair follicles providing the cross-talk with the melanocortin receptor-1 pathway [5]. A number of studies investigated the effects of statins on melanoma cells. Specifically, lovastatin induces apoptosis in A375 melanoma cells and also enhances response to chemotherapy drugs in the B16 mouse model of melanoma [6-9].

In numerous experiments investigating pleiotropic effects of statins, reports regarding their effect on

melanogenesis are lacking. Thus, the aim of the present work was to examine the effects of fluvastatin, a frequently used statin that effectively lowers serum cholesterol level, on melanin synthesis in melanoma B16F10 cell line.

Materials and methods

Cells. B16F10 melanoma cells were kindly donated by Dr Tomasz Stokłosa from Department of Immunology, Center for Biostructure, Medical University in Warsaw, Warsaw, Poland. B16F10 mouse melanoma cells were cultured in 75 cm² flask and grown in DMEM+GlutaMAX medium (Gibco, NY, USA) with 10% fetal bovine serum (Gibco, NY, USA) and 1 % antibiotic/antimycotic solution (Gibco, NY, USA). Incubation was carried out at 37 °C under atmosphere of air-CO₂ (95:5).

Antibodies. Antibodies against: tyrosinase, β -actin and secondary antibodies (HRP-goat anti-rabbit or HRP-bovine anti mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assay. All experiments were performed using cells of the same passage. After incubating B16F10 cells overnight in 25 cm² flasks (5x10⁴ cells/flask), cells were passaged to Petri dishes (Costar, Corning, NY, USA). When cells reached 40% of confluence, the medium was changed to DMEM supplemented with either 10% FCS alone or 10% FCS with fluvastatin at concentrations: 0.1 μ M; 1 μ M; 10 μ M. Cell viability was determined in triplicates, using crystal violet assay [10,11]. After incubating B16F10 cells with test substances for up to 72 hours, culture medium was removed and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at 37°C and rinsed four times. Crystal

Correspondence: R. Galus, Chair and Department of Histology and Embryology, Center for Biostructure Research, Medical University of Warsaw, Poland, 02-004 Warsaw, Chalubinskiego Str. 5, Poland; tel.: (+48) 501834369, fax: (+4822) 6295282, e-mail: rgalus@ib.amwaw.edu.pl

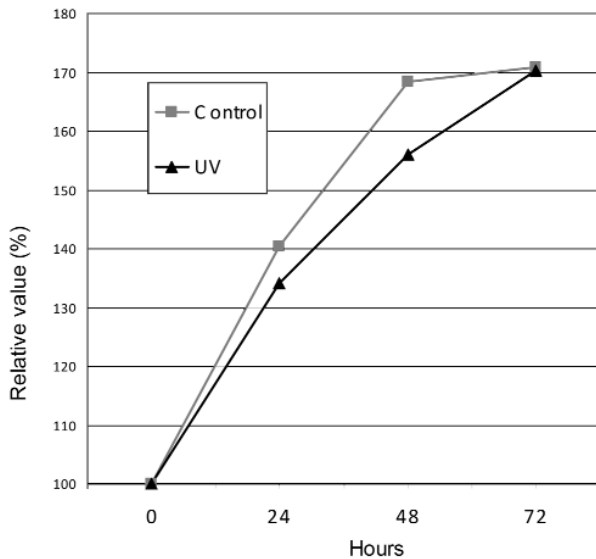


Fig. 1. Effects of UVB irradiation in B16F10 melanoma cell line at 100 mJ/cm² daily on cell viability 24, 48 and 72 hours after irradiation procedure vs. non-irradiated control cells.

violet-stained adherent cells were then extracted with 95% ethanol, and absorbance was determined at 590 nm using ELISA reader (Tecan, Salzburg, Austria). Cell viability was also tested with UVB irradiated/non-irradiated cells.

Irradiation procedure. The source of ultraviolet was a Cosmedico stimulator (Cosmedico Medizintechnik, Schweningen, Germany), which emits energy in the UVB range, with the peak at 311 nm. Cells were irradiated in PBS to avoid formation of medium-derived toxic photo-products [12]. After irradiation, PBS was replaced with fresh warm growth medium with/without 1 μ M fluvastatin. Cells were irradiated once daily (100 mJ/cm²) at 24, 48 and 72 hours of experiment and cell viability was performed after 24 hours, following each irradiation. To examine modulation of tyrosinase mRNA, cells were irradiated once at 12 hours of experiment. In order to investigate protein content, cells were irradiated twice (at 24 and 48 hours of experiment) and the measurements were done after 24 hours following last irradiation. Non-irradiated control cells were maintained in PBS during the time of irradiation procedure.

Total RNA isolation. RNA was isolated with NucleoSpin®RNA II kit (Macherey-Nagel, Neumann-Neander D-52355 Duren, Germany), according to producer's protocol. Quality of isolated RNA was tested by electrophoresis in 1% agarose denaturing gel, containing 6% formaldehyde and buffered with MOPS (Sigma, Genosys, St. Louis MO, USA). After electrophoresis, the gel was scanned with the digital imaging system GDS9000 using GRAB-IT. 2.0 software (UVP, Cambridge, UK). Densitometric analysis of bands was performed with GelWorks software (UVP, Cambridge, UK).

Reverse transcription. RT was carried out in 20 μ l of a reaction mix containing 1 \times transcription buffer, 5.5 mM MgCl₂, 0.5 mM of deoxynucleosides (dNTP), 2.5 μ M oligo dT primer, 3 U of RNase inhibitor and 6 U of reverse transcriptase (Reverse Transcription Kit, Promega). 1 μ g of total RNA was heated at 70°C for 10 min, cooled to 2°C and added to the reaction mix. RT was performed in Eppendorf Master Cycler Gradient. Samples were incubated in 42°C for 2 h and then heated to 94°C; cDNA was stored at -20°C.

RealTime PCR. Real Time PCR was performed in ABI Prism 7500 (Applied Biosystems, Warrington, UK) in 96-well optical

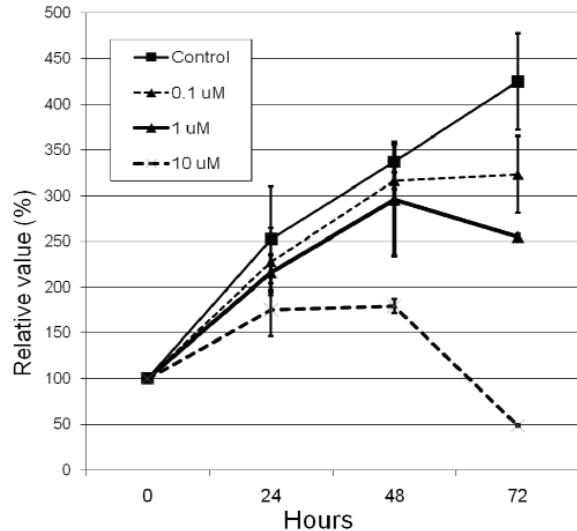


Fig. 2. Dose- and time-dependent effects of various concentrations of fluvastatin on cell viability in B16F10 melanoma cells. Data are presented as the averaged values \pm SE for three independent experiments.

plates. Each sample was tripled and supplied with endogenous control (β -actin). For tyrosinase expression, the sequence of the forward primer was 5'-GGC CAG CTT TCA GGC AGA GGT -3' and the sequence of the reverse primer was 5'-TGG TGC TTC ATG GGC AAA ATC -3' (Sigma, Genosys, St. Louis MO, USA). Reactions were run in 25 μ l volume with Power Sybr® Green PCR Master Mix (Applied Biosystems, Warrington, UK), appropriate primer set and 50 ng of cDNA template. Universal thermal conditions, that is 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C, were used. Data analysis was done with sequence detection software ver. 1.2 (Applied Biosystems, Warrington, UK).

Results and discussion

UVB irradiation of B16F10 melanoma cell line at 100 mJ/cm² daily did not show any important effect on cell viability 24, 48 and 72 hours after irradiation procedure, when compared to control non-irradiated cells (Fig. 1).

To determine which fluvastatin concentration shows minimal/no cytotoxic effect, melanoma cells were treated with fluvastatin at various concentrations. Fluvastatin at 0.1 μ M and 1 μ M induced relatively low cytotoxic effect during 48 hours, but after 72 hours cell viability was markedly reduced, thus indicating that optimal (maximal, yet not toxic) fluvastatin concentration in medium of the melanoma cell line in further experiments would be 1 μ M and incubation time should not exceed 48 hours (Fig. 2).

UVB irradiation increased the level of tyrosinase mRNA in B16F10 cells. Surprisingly, in cells treated with 1 μ M fluvastatin, the level of tyrosinase mRNA was higher when compared to the level resulting from UVB irradiation. Finally, simultaneous irradiation and fluvastatin treatment of B16F10 cell line triggered additive effect of tyrosinase mRNA increase, in comparison to stimulation of tyrosinase by UVB (Fig. 3).

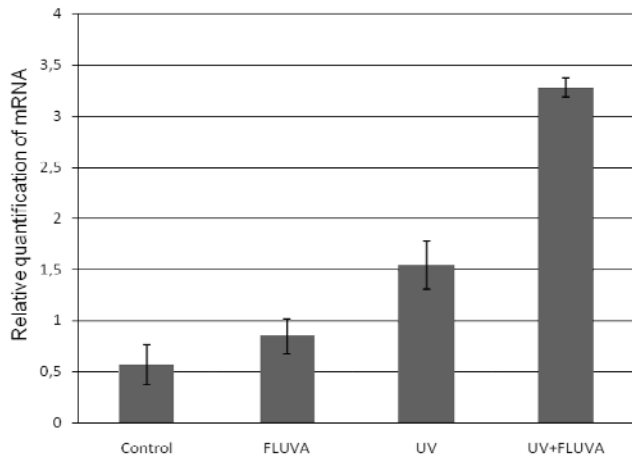


Fig. 3. Effects of UVB irradiation, 1 μ M fluvastatin (FLUVA) and combination of UVB irradiation/1 μ M fluvastatin (UVB + FLUVA) in B16F10 cell line on the level of tyrosinase mRNA. Relative quantification of mRNA levels from triplicates was averaged \pm SE.

In the current study we show that UVB-induced tyrosinase synthesis of cultured B16F10 melanoma cells is increased by fluvastatin. Levels of tyrosinase mRNA evaluated with use of real time PCR after UVB irradiation of B16F10 melanoma cell line and fluvastatin incubation were increased.

Statins increase expression of bone morphogenetic protein-2 (BMP-2) gene in cells [1] which specifically targets tyrosinase gene expression in primary quail neural crest cultures resulting in increased melanin synthesis [4]. On the other hand, unlike BMP-2 effect in quail melanocytes, BMP-4 stimulates proliferation of melanocytes, down-regulates tyrosinase mRNA and protein levels, decreases melanin content, decreases tyrosinase mRNA stability [13], and purified BMP-4 reduces the number of melanocytes in cultures of avian neural crest cells [14].

Several intracellular signaling pathways have been reported to involve transcriptional activation of tyrosinase gene. Activation of microphthalmia-associated transcription factor (MITF), that regulates tyrosinase gene expression, is known to be a critical event during melanogenesis [15]. Extracellular signal-regulated kinase (ERK) phosphorylates MITF and promotes its degradation, thereby resulting in inhibition of tyrosinase expression and melanogenesis [16]. Further investigations are required to evaluate these proposed signaling pathways.

Since fluvastatin is usually administered orally at daily doses of 20 to 80 mg in patients with hypercholesterolemia, and the plasma concentration typically reaches around 1.0 μ mol/L [17,18] our results seem particularly interesting from the clinical point of view. Melanin protects the skin against UV radiation [19]. According to the above results, fluvastatin treatment may potentially increase melanin synthesis and skin protection against UV irradiation.

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