Biochimica et Biophysica Acta 1843 (2014) 894–901

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Lovastatin inhibits human B lymphoma cell proliferation by reducing intracellular ROS and TRPC6 expression

Xiang Song [a,b,1], Bing-Chen Liu [a,b,1], Xiao-Yu Lu [b,1], Li-Li Yang [b,c], Yu-Jia Zhai [a], Amity F. Eaton [b,d], Tiffany L. Thai [b,d], Douglas C. Eaton [b,d], He-Ping Ma [b,d,*], Bao-Zhong Shen [c,e,**]

[a] Department of Cardiology, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China
[b] Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322, United States
[c] Department of Radiology, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China
[d] Center for Cell and Molecular Signaling, Emory University School of Medicine, Atlanta, GA 30322, United States
[e] Key Laboratory of Molecular Imaging of College of Heilongjiang, Harbin, Heilongjiang, China

ARTICLE INFO

Article history:
Received 7 October 2013
Received in revised form 17 January 2014
Accepted 2 February 2014
Available online 8 February 2014

Keywords:
Anti-cancer drugs
Cell cycle
Membrane cholesterol
Oxidative stress
Intracellular calcium
Confocal microscopy

ABSTRACT

Clinical evidence suggests that statins reduce cancer incidence and mortality. However, there is lack of *in vitro* data to show the mechanism by which statins can reduce the malignancies of cancer cells. We used a human B lymphoma Daudi cells as a model and found that lovastatin inhibited, whereas exogenous cholesterol (Cho) stimulated, proliferation cell cycle progression in control Daudi cells, but not in the cells when transient receptor potential canonical 6 (TRPC6) channel was knocked down. Lovastatin decreased, whereas Cho increased, the levels of intracellular reactive oxygen species (ROS) respectively by decreasing or increasing the expression of p47-*phox* and gp91-*phox* (NOX2). Reducing intracellular ROS with either a mimetic superoxide dismutase (TEMPOL) or an NADPH oxidase inhibitor (apocynin) inhibited cell proliferation, particularly in Cho-treated cells. The effects of TEMPOL or apocynin were mimicked by inhibition of TRPC6 with SKF-96365. Lovastatin decreased TRPC6 expression and activity via a Cho-dependent mechanism, whereas Cho increased TRPC6 expression and activity via an ROS-dependent mechanism. Consistent with the fact that TRPC6 is a Ca^{2+}-permeable channel, lovastatin decreased, but Cho increased, intracellular Ca^{2+} also via ROS. These data suggest that lovastatin inhibits malignant B cell proliferation by reducing membrane Cho, intracellular ROS, TRPC6 expression and activity, and intracellular Ca^{2+}.

1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme-A (HMG-coA) reductase inhibitors, commonly known as statins, are widely used for reducing hypercholesterolemia, a risk factor of cardiovascular diseases. Randomized controlled trials for preventing cardiovascular disease have surprisingly indicated that statins can reduce the incidence of colorectal cancer and melanoma [29]. Large population-based studies have also shown that statins reduce the incidence of overall cancers [15]. Therefore, it has been suggested that statins can prevent cancer [11]. Although epidemiological analyses suggest that statin use is not associated with 10 common cancers [10], more recent studies show that statin use can reduce the risk of several types of cancers [3,9,14,30]. Despite the debate, long-term use of statins, at least, reduces the incidence of selected cancers; such as: melanoma, endometrial cancer, and non-Hodgkin's lymphomas (NHL) [21]. Statin use also decreases the risk of NHL in HIV-positive persons [7]. In addition to reducing cancer incidence, statin use also reduces the overall cancer mortality [28]. Experimental data suggest that the anti-cancer effects of statins involve several mechanisms. In a mouse lymphoma model, both atorvastatin and lovastatin were shown to decrease lymphoma mortality by inducing apoptosis of the lymphoma cells [1,2]. In murine tumor models, lovastatin enhanced the antitumoral and apoptotic activity of doxorubicin [34]; whereas, in murine melanoma cells, statins stimulate membrane FasL expression and induce lymphocyte apoptosis through the RhoA/ROCK pathway [37]. Lovastatin also inhibits metastasis of B-cell lymphoma [27] and enhances radiation-induced apoptosis of the lymphoma cells [35]. Besides inducing apoptosis, statins also inhibit proliferation of thyroid cancer cells and cholangiocarcinoma cells [22,50]. However, it remains unknown whether statins can also inhibit lymphoma cell proliferation; and, if so, what is the underlying mechanism.

Recent studies suggest that the transient receptor potential canonical channel 6 (TRPC6) is overexpressed in cervical and breast cancers [17,44] and plays an important role in malignant cell proliferation in a variety of

* Correspondence to: H.-P. Ma, Department of Physiology, Emory University School of Medicine, 615 Michael ST, Suite 601, Atlanta, GA 30322, United States. Tel.: +1 404 727 0617; fax: +1 404 727 0329.
** Correspondence to: B.-Z. Shen, Department of Radiology, the Fourth Affiliated Hospital of Harbin Medical University, 31 Yinhang Street, Nangang District, Harbin, 150001, China. Tel.: +86 451 82576508; fax: +86 451 82576509.
E-mail addresses: heping.ma@emory.edu (H.-P. Ma), shenbzh@vip.sina.com (B.-Z. Shen).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.bbamcr.2014.02.002
0167-4889/

cancers [6,13,39]. Interestingly, it has been shown that Cho elevates TRPC6 activity by promoting the physical interaction between TRPC6 and podocin (a Cho-binding protein) [19] and that intracellular Cho is elevated in lymphoma cells [31]. Since TRPC6 is a Ca^{2+}-permeable channel [18], it is very likely that the elevated intracellular Cho promotes lymphoma cell proliferation by increasing intracellular Ca^{2+} via TRPC6. Although podocin mediates the stimulatory effect of Cho on TRPC6 activity [19], other pathways may be also involved. As a component of lipid rafts, Cho maintains NADPH oxidase activity in breast carcinoma cells [32]. Intracellular oxidative stress occurs in several types of cancer cells [12] including lymphoma cells [41]. Since TRPC6 is stimulated by ROS [23,26], it is very likely that Cho stimulates TRPC6 also by elevating intracellular ROS. These studies together suggest that statins may inhibit lymphoma cell proliferation by reducing TRPC6 channel activity through its inhibition of Cho synthesis. The present study suggests that lovastatin inhibits, whereas cholesterol stimulates, human lymphoma B cell proliferation by respectively decreasing or increasing intracellular ROS, TRPC6 expression/activity, and intracellular Ca^{2+}.

2. Materials and methods

2.1. Cell culture

Human B lymphoma Daudi cells were purchased from American Type Culture Collection. Daudi cells were suspended and maintained in a medium containing RPMI 1640, 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, which was prepared fresh each week. Every two days, the cell suspension was centrifuged and resuspended in flasks containing fresh medium. Cells were cultured continuously in a humidified incubator at 37 °C in 5% CO_2. To knock down TRPC6 expression, Daudi cells were transiently transfected with TRPC6 silencing short hairpin RNA (shRNA) carried by a lentiviral vector (Santa Cruz Biotechnology). Before the TRPC6 knockdown cells were used, the reduction of TRPC6 expression was confirmed by Western blot experiments. All the experiments in this study were performed at room temperature.

2.2. Cell proliferation assays

Cell proliferation was evaluated by performing CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) and by analyzing cell cycle. MTS assay was carried out according to the manufacturer's instruction. Briefly, Daudi B cells, either under control conditions or after experimental manipulations (as described in the Results), were transferred into 96 well plates. 10 μL of CellTiter 96® AQ_{ueous} One Solution was added into each well. After the cells were incubated at 37 °C for 3 h, the absorbance was detected at 490 nm with the Synergy 4 Microplate Reader (BioTek). All experiments were repeated three times. Cell cycle analysis was carried out using the fluorescent ubiquitin-based cell cycle indicator (Fucci®), as described previously [36]. After the cells were labeled with Fucci, confocal microscopy experiments were performed using either 488 nm or 543 nm laser to excite the indicator. The emissions at either 519 nm or 603 nm were measured and used for imaging analysis.

2.3. Confocal microscopy

Confocal microscopy experiments were performed as we previously reported [47]. Briefly, to detect cholesterol levels in the plasma membrane of Daudi cells, live cells were incubated with 5 μg/mL filipin (Sigma, Cat#: F9765) for 30 min. Since filipin can be easily oxidized, the stock solution of filipin was freshly prepared in methanol at 10 mg/mL. The relative cholesterol levels were semi-quantified according to the total fluorescence intensity. For cell cycle analysis, Daudi cells were transiently transfected with Premo™ FUCCI Cell Cycle Sensor (Molecular Probes, Cat#: P36238) according to the protocol provided by the manufacturer. Premo™ FUCCI contains Premo™ geminin-GFP to label cells in G2/M phases shown in green and Premo™ Cdt-RFP to label G1/S phases shown in red. To detect the levels of intracellular ROS, the cells were incubated with 25 μM 2′,7′-dichlorodihydrofluorescein diacetate (Molecular probe) a membrane-permeable, ROS-sensitive, fluorescent probe, for 15 min. Prior to the confocal microscopy experiments, the cells were washed twice with a NaCl bath solution and transferred on a glass slide with a drop of the NaCl bath solution to keep the cells alive. In each set of experiments, images were taken using the same parameter settings.

2.4. Western blot and biotinylation

Either control or treated Daudi cells were cultured as described above. Cell surface levels of gp91-*phox* were evaluated with biotinylation experiments. Cell lysates (100 μg) were loaded and electrophoresed on 10% SDS-PAGE gels for 60 to 90 min. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes for 1 h at 90 V. After 1 hour blocking with 5% BSA-PBST buffer, PVDF membranes were incubated with primary antibodies (1:1000 dilution) of rabbit polyclonal antibody to TRPC6 (Sigma, Lot # 8831P1), goat polyclonal antibody to p47-phox (anti-NCF1, Abcam, Cat# ab795), or rabbit polyclonal antibody to gp91-phox (EMD Millipore, Cat# 07-024) overnight at 4 °C, and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG secondary antibody (1:5000 dilution, GE healthcare) for 1 h after 4 vigorous washes. Finally, blots were visualized with chemiluminescence using the ECL Plus Western Blotting Detection System (GE healthcare).

2.5. Patch-clamp techniques

The whole-cell recordings were performed as we described previously [47]. Briefly, before electrophysiological analysis, the Daudi cell suspension was centrifuged and resuspended with the NaCl bath solution (see Chemicals and Solutions). After repeating this procedure twice, the cell suspension was added into the patch chamber mounted on the stage of a Nikon inverted microscope. Polished patch pipettes of borosilicate glass typically with about 5 MΩ were used for patch-clamp recording. Patch pipettes were filled with a NaCl pipette solution (see Chemicals and Solutions). Only patches with high resistance seals (above 5 GΩ) were used in the experiment to form the whole-cell configuration. Whole-cell currents were recorded using an Axopatch-200B amplifier and pClamp 10 software (Molecular Devices) and low-pass filtered at 2 kHz. A voltage-ramp protocol from −100 to 100 mV was used to quickly get the current–voltage relationship; the protocol was given at an interval of 1 min. All the experiments were performed at 22–23 °C.

2.6. Measurement of $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ were monitored using fura-2, a UV-excitable Ca^{2+} indicator, as we reported previously [45]. Daudi cells were incubated with 5 μM fura-2 acetoxymethyl ester for 30 min in the incubator, and washed twice with the NaCl bath solution. Using a fluorescence microscope equipped with dual-excitation and single-emission monochromators, the fluorescent intensity of fura-2 was measured at excitation wavelengths of 340 and 380 nm with 2.5-nm bandwidth and at an emission wavelength of 510-nm with 4-nm bandwidth. The emission signals were excited at both 340 and 380 nm and the ratio of these signals (340/380) was recorded and calibrated with EGTA (2 mM) and ionomycin (5 μM). Axon Imaging Workbench version 6.0 software (Axon instruments) was used for acquisition of intensity images and conversion to ratios.