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

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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 a NADPH oxidase inhibitor (apocynin) inhibited cell
proliferation, particularly in Cho-treated cells. The effects of TEMPO 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\textsuperscript{2+}-permeable channel, lovastatin decreased, but Cho increased, intracellular Ca\textsuperscript{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\textsuperscript{2+}.

**Keywords**

Anti-cancer drugs; Cell cycle; Membrane cholesterol; Oxidative stress; Intracellular calcium; Confocal microscopy; Patch-clamp techniques

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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 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\textsuperscript{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 manufacture. 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 NaCl bath solution and transferred on a glass slide with a drop of 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 hour at 90 volts. 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 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 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 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 [Ca²⁺]i

Changes in [Ca²⁺]i were monitored using fura-2, a UV-excitable Ca²⁺ 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 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 emission wavelength of 510-nm with 4-nm bandwidth. The emission signals 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.

2.7. Chemicals and solutions

Reagents were obtained from the following sources: RPMI 1640, fetal bovine serum, glutamine, penicillin/streptomycin, and anti-rabbit IgG from Invitrogen; ionomycin from Calbiochem. The NaCl bath solution contained (in mM): 145 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES, adjusted pH to 7.4 with NaOH. The NaCl pipette solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 ATP-Na₂, 10 HEPES and 50 nM (1 μM) free Ca²⁺ (after titration with 2 mM EGTA), adjusted to pH 7.2 with NaOH.

2.8. Statistical analysis

Results are shown as means ± SD. Student’s t-test was used for comparison between two groups. One-way analysis of variance was applied for comparison among multiple groups. Two-way analysis of variance was applied for comparison among multiple groups with two subgroups. Differences with a P value of < 0.05 were considered statistically significant.

3. RESULTS

3.1. Lovastatin inhibits human B lymphoma cell proliferation via a Cho- and TRPC6-dependent mechanism

To determine whether Cho affects human Burkitt’s lymphoma Daudi B cell proliferation, Daudi cells were treated for 72 h with 5 μM lovastatin, 30 μg/ml Cho, or 5 μM lovastatin plus 30 μg/ml Cho. MTS assays were performed to evaluate cell proliferation (Fig. 1A). The data showed that treatment of Daudi cells with lovastatin significantly reduced cell proliferation. In contrast, treatment of the cells with exogenous Cho strongly stimulated cell proliferation. To determine whether lovastatin inhibits cell proliferation by reducing Cho, the cells were co-treated with both lovastatin and Cho. The data showed that the co-treatment did not affect cell proliferation. These effects were abolished when the expression of TRPC6 was knocked down (Fig 1A and 1B), indicating that TRPC6 mediates the effects of Cho on Daudi cell proliferation. Although lovastatin is a well-known Cho synthesis inhibitor, it remains unclear whether lovastatin can reduce Cho levels in Daudi cells, particularly the Cho levels in the plasma membrane. Since the levels of Cho in the plasma membrane can be easily labeled with a fluorescent Cho probe (filipin), confocal microscopy experiments were performed in the cells stained with filipin. The data showed that lovastatin significantly decreased the levels of Cho in the Daudi cell membrane (Fig. 1C and 1D). To determine whether these treatments modulate cell cycle, the cells were incubated with a cell cycle probe, Fucci. As shown in Fig. 2A and 2B, lovastatin caused cell cycle arrest at G1 phase as shown by cells in red; the percentage of cells in the G₁ phase was increased from 56% ± 5% (control level) to 90% ± 6% (after lovastatin) as shown by green bars (n = 45; p <
Conversely, Cho caused cells to be accumulated at S-G2/M phases as shown by cells in green; the percentage of cells in S-G2/M phases was increased from 44% ± 5% to 93% (control level) ± 5% (after Cho), as shown by green bars (n = 45; P < 0.001). However, co-treatment of the cells with both lovastatin and Cho did not affect cell cycle progression. Daudi cell growth also arrested at G1 phase when TRPC6 expression was knocked down. These data suggest that lovastatin inhibits proliferation by reducing Cho synthesis and inhibiting cell cycle progression in malignant Daudi cells.

3.2. Lovastatin decreases, whereas cholesterol increases, intracellular ROS and TRPC6 expression

Since it has been shown that Cho stimulates NADPH oxidase [32] and that oxidative stress is closely associated with proliferation of cancer cells [12] including lymphoma cells [41], lovastatin-induced reduction of membrane Cho may improve oxidative stress in Daudi cells. Therefore, intracellular ROS were examined with an ROS probe (DCF). The data showed that lovastatin significantly reduced, but Cho increased, intracellular ROS and that co-treatment of the cells with both lovastatin and Cho did not affect intracellular ROS (Fig. 3A and 3B). Cho-induced elevation of ROS was abolished both by TEMPOL (a mimic superoxide dismutase) and by apocynin (an NADPH oxidase inhibitor), indicating that superoxide is involved. Taken together, these data suggest that lovastatin reduces superoxide levels by decreasing Cho. To determine whether lovastatin reduces ROS in Daudi cells by decreasing the expression of p47phox, a regulatory subunit of NADPH oxidase, we performed Western blot experiments to examine p47-phox and gp91-phox protein levels. In parallel with the effects on ROS, lovastatin significantly reduced, whereas Cho significantly increased, p47-phox and gp91-phox expression in Daudi cells. In contrast, co-treatment of the cells with lovastatin and Cho did not affect p47-phox and gp91-phox expression (Fig. 3C-3G). Recent studies suggest that TRPC6 plays an important role in cancer cell proliferation [6,13,39]. To determine whether Cho can stimulate TRPC6 expression by elevating superoxide, one of ROS, we also examined TRPC6 protein levels. The data showed that Cho significantly increased, whereas lovastatin significantly reduced, TRPC6 expression. In contrast, co-treatment of the cells with lovastatin and Cho did not affect TRPC6 expression (Fig. 4A and 4B). In parallel with the results of Cho-induced cell proliferation, reducing intracellular ROS with either TEMPOL or apocynin abolished Cho-induced elevation of TRPC6 protein levels.

3.3. Lovastatin inhibits cell proliferation by reducing intracellular ROS and inhibiting TRPC6

To determine whether intracellular ROS and TRPC6 mediate the effects of lovastatin on cell proliferation, Daudi cells were treated with lovastatin, Cho, or lovastatin plus Cho for 72 h in the absence or presence of TEMPOL, apocynin, or SKF-96365. MTS assay were performed to evaluate cell proliferation (Fig. 5A-5C). The data showed that both TEMPOL and apocynin significantly reduced cell proliferation, particularly in the cells treated with Cho, but did not further reduce the proliferation in the cells treated with lovastatin. In parallel, SKF-96365 significantly reduced cell proliferation, also particularly in the cells treated with Cho, but did not further reduce the proliferation in the cells treated with...
Lovastatin. These data suggest that intracellular ROS and TRPC6 mediate lovastatin-induced reduction of proliferation in malignant Daudi B cell cells.

3.4. Lovastatin decreases, whereas Cho increases, intracellular Ca\(^{2+}\) respectively by inhibiting or stimulating TRPC6

Previous studies have shown that TRPC6 is directly activated by the membrane-permeable diacylglycerol analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG) [18]. To determine whether manipulation of Cho levels regulates TRPC6 activity, we evaluated TRPC6 activity by whole-cell patch-clamp recordings of OAG-sensitive currents and found that lovastatin significantly reduced, but Cho significantly increased, OAG-sensitive currents (Fig. 6A). Application of SKF-96365 (a TRPC6 blocker) reversibly reduced OAG-sensitive currents. The experiments were performed using the same patch as a control (Fig. 6C). After the basal currents (trace a) were recorded, OAG was applied to the cell. Then the currents at 5 min after OAG addition were recorded and shown increased (trace b). In the presence of OAG, SKF-96365 was applied to the cell; the currents at 5 min after SKF-96365 were recorded and shown inhibited (trace c). After SKF-96365 was washed out, the currents were recovered (trace d), as shown in Fig. 6B. To confirm the involvement of TRPC6, the cells were transiently transfected with TRPC6 shRNA. In parallel with the effects on Daudi cell proliferation, knockdown of TRPC6 significantly reduced OAG-sensitive currents, particularly in the cells treated with Cho, but only moderately reduce OAG-sensitive currents in the cells treated with lovastatin (Fig. 6C). Since TRPC6 is a Ca\(^{2+}\)-permeable channel, the levels of intracellular Ca\(^{2+}\) were measured using the Fura-2 method (Fig. 7).

Consistent with the results from patch-clamp experiments, the data showed that lovastatin significantly reduced, but Cho significantly increased intracellular Ca\(^{2+}\), and that in the presence of lovastatin, exogenous Cho not only abolished the lovastatin-induced reduction of intracellular Ca\(^{2+}\), but even increased intracellular Ca\(^{2+}\). However, the increase induced by Cho in the presence of lovastatin was still significantly smaller than the increase induced by Cho alone. Treatment of the cells for 48 h with SKF-96365, TEMPOL, or apocynin significantly reduced intracellular Ca\(^{2+}\), particularly in the cells treated with Cho, but did not reduce intracellular Ca\(^{2+}\) in the cells treated with lovastatin (Fig. 7). These data suggest that Cho regulates intracellular Ca\(^{2+}\) via a TRPC6- and ROS-dependent mechanism.

4. DISCUSSION

NHL ranks fifth in cancer incidence and mortality in the United States during the period from year 2004 to year 2008 [40]. According to the American Cancer Society, the incidence rates for NHL have nearly doubled in recent decades. Recent studies suggest that statins can reduce both incidence and mortality of several cancers, including lymphomas [21]. The present study shows that lovastatin inhibits the proliferation of human Burkitt’s lymphoma Daudi B cells by reducing the levels of Cho in the plasma membrane, intracellular ROS, TRPC6 expression and activity, and intracellular Ca\(^{2+}\). Since the assembly and activity of NADPH oxidases are dependent on the cholesterol-rich membrane microdomains [32,43], we show that lovastatin reduces intracellular ROS by decreasing the expression of p47-phox and gp91-phox. Since we have shown that ROS stimulate TRPC6 in podocytes [26], we know that lovastatin reduces intracellular Ca\(^{2+}\), at least in part, by decreasing TRPC6.
activity in ROS-dependent manner. It has been shown that intracellular Ca\(^{2+}\) provides important regulatory signals during the cell cycle [5] and that H\(_2\)O\(_2\) stimulates TRPC6 [16]. Therefore, intracellular oxidative stress due to elevated levels of p47-phox and gp91-phox may account for malignant proliferation via TRPC6-mediated elevation of intracellular Ca\(^{2+}\). However, it remains largely unknown how Cho regulates the expression of p47-phox and gp91-phox.

It appears that TRPC6 plays a dual role in regulating cell fate. In podocytes, TRPC6-dependent increases of intracellular Ca\(^{2+}\) mediate apoptosis induced by albumin overload [8], sustained activation of atypical N-methyl-d-aspartate (NMDA) receptors [24], and high glucose [26]. Gain-of-function mutations of TRPC6 account for focal segmental glomerulosclerosis [49], which is caused by podocyte apoptosis [4,46]. Since intracellular Ca\(^{2+}\) regulates both cell proliferation and cell death [38], we argue that TRPC6-mediated increases in intracellular Ca\(^{2+}\) may regulate both cell proliferation and cell death, depending on its activity. Moderate activation of TRPC6 should result in a moderate elevation of intracellular Ca\(^{2+}\), which should promote cell proliferation. In contrast, excess activation of TRPC6 should result in a massive increase in intracellular Ca\(^{2+}\), which causes apoptosis. The present study shows that Cho elevates intracellular Ca\(^{2+}\) only up to 600 nM. It appears that intracellular Ca\(^{2+}\) at this concentration stimulates Daudi cell proliferation rather than induces apoptosis. However, it remains completely unknown what prevents cancer cells from intracellular Ca\(^{2+}\) overload and allows them to escape from apoptosis.

The concentration of lovastatin we used (5 μM) is much higher than the plasma concentrations in human subjects administrated with common dose of lovastatin [33]. However, we found that lovastatin at 5 μM did not induce apoptosis in podocytes [25]. Consistently, in the present study, we also noticed that lovastatin at 5 μM did not cause apoptosis or any other types of cell death in Daudi cells. Recent studies suggest that simvastatin can be safely used for patients with myeloma and lymphoma at a relatively high dose of 15 mg/kg/day [42]. This dose of simvastatin used for assisting cancer chemotherapy is almost 100 times higher than that commonly used for treating hypercholesterolemia. Assuming the average blood volume is 5000 ml, this high dose would result in a calculated plasma concentration of simvastatin at about 7 μM. Therefore, the pharmacological concentrations of lovastatin we used in the present in vitro study provide useful information to guide the use of statins in cancer chemotherapy. Since recent studies indicate that MTS assay may underestimate the effects of anti-cancer drug on cell proliferation [48], the effects of lovastatin on Daudi cell proliferation may be more promising than what we detected by the MTS assay. In conclusion, the present study provides strong evidence at the molecular level for supporting the clinical trials focused on the use of statins in cancer therapy.

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Highlights

- Lovastatin inhibits, whereas cholesterol stimulates, lymphoma proliferation via TRPC6
- Lovastatin inhibits, whereas cholesterol stimulates, both NOX2 and TRPC6 expression
- Superoxide and TRPC6 control lymphoma cell proliferation
- Lovastatin decreases, whereas cholesterol increases, intracellular Ca\(^{2+}\) via TRPC6
Fig. 1. Lovastatin inhibits, but Cho promotes, human lymphoma Daudi cell proliferation via TRPC6

(A) MTS assay of cell proliferation. In all the experiments through the study, otherwise indicated, Daudi cells were either under control conditions or transiently transfected with TRPC6 shRNA and then treated for 72 h with 5 μM lovastatin, 30 μg/ml Cho, or 5 μM lovastatin plus 30 μg/ml Cho, respectively. (B) Western blot of TRPC6 from control Daudi cells or the cells transiently transfected with either TRPC6 shRNA or control shRNA. Data represent three experiments showing similar results. (C) Summary plots of normalized Cho levels in the plasma membrane either after each treatment as listed in (A) or after treatment with 20 mM methyl-β-cyclodextrin (Mβ-CD, a Cho-depleting compound, as a positive control) for 30 min. Cho levels were evaluated with a fluorescent Cho-binding molecule, filipin, as described in the methods. Data are from four separate experiments, showing similar results. In each experiment, we randomly circled an area with the similar size and the similar density of cells for measuring the total fluorescent intensity. In each group, five such areas were used to get an average of the fluorescent intensity. (D) Representative confocal microscopy images of Daudi cells stained with filipin. Since the fluorescence was hardly seen after treatment with Mβ-CD, a DIC image was inserted into the lower left corner in the last image to show the same microscopic field containing Daudi cells with normal morphology.
Fig. 2. Lovastatin inhibits, but exogenous Cho stimulates, Daudi cell cycle progression

(A) Representative confocal microscopy images of cell cycle analysis with Fucci. Cells in G\textsubscript{1} phase show in red whereas cells in S or G\textsubscript{2}/M phase show in green. (B) Summary plots of G\textsubscript{1} phase (red bars) versus S-G\textsubscript{2}/M phase (green bars) after each treatment. Cells were treated for 72 h with 5 μM lovastatin, 30 μg/ml Cho, 5 μM lovastatin plus 30 μg/ml Cho, TRPC6 shRNA, or 0.5 ng/ml transforming growth factor β (TGF-β, as a positive control because TGF-β is known to induce G1 arrest [20])
Fig. 3. Lovastatin reduces, but Cho elevates, intracellular ROS via gp91-phox NADPH oxidase
(A) Representative confocal microscopy images of Daudi cells treated as in Figure 1, with
30 μg/ml Cho plus 250 μM TEMPO (a mimic superoxide dismutase), or with 10 mM
apocynin. Intracellular ROS levels were indicated with a fluorescent ROS probe, DCF. (B)
Summary plots of normalized fluorescence intensity of DCF showing relative ROS levels in
Daudi cells after each treatment. (C and D) Representative Western blot of p47-
phox and gp91-
phox. (E) Normalized p47-phox protein levels in Daudi cells from three separate
experiments. (F and G) Normalized cell surface (F) and total (G) gp91-phox protein levels in
Daudi cells from three separate experiments.
Fig. 4. Lovastatin reduces, whereas Cho elevates expression of TRPC6 via a NADPH oxidase-dependent production of superoxide

(A) Representative Western blot of TRPC6. (B) Normalized TRPC6 protein levels from four separate experiments. Daudi cells were treated as in Figure 1, but also treated with 30 μg/ml cholesterol either plus 10 μM apocynin or plus 250 μM TEMPOL.
Fig. 5. Lovastatin inhibits, but Cho promotes, Daudi cell proliferation via an ROS- and TRPC channel-dependent mechanism

(A, B, and C) MTS assay of cell proliferation after Daudi cells were treated as in Fig. 1, but in the absence or presence of 250 μM TEMPOL (A), 10 μM apocynin (B), or 10 μM SKF-96365 (C).
Fig. 6. Lovastatin reduces, but Cho elevates, TRPC6 whole-cell current in Daudi cells

(A) Summary plots of whole-cell currents at 100 mV under each condition as indicated. (B) Representative whole-cell currents from Daudi cells treated as in Fig. 1. A voltage-ramp protocol for −100 mV to 100 mV at a holding potential of −60 mV was given every minute. After recording basal current (a), the cell was exposed either to 100 μM OAG to activate TRPC6 channels (b) or to SKF-96365 to block TRPC6 channels (c). Blockade of TRPC6 was reversed after washout of SKF-96365. (C) Summary plots of OAG-sensitive currents from Daudi cells treated as described in Fig. 1, but either under control conditions or transiently transfected with TRPC6 shRNA. OAG-sensitive currents were achieved by subtracting the basal current from the current at 5 min after activated by 100 μM OAG. All the currents were measured at 100 mV.
Fig. 7. Lovastatin reduces, but Cho elevates, intracellular Ca^{2+} ([Ca^{2+}_i]) in Daudi cells via an ROS- and TRPC channel-dependent mechanism

Daudi cells were treated as in Fig. 1, but in the absence (open bars) or presence of 10 μM SKF-96365 (black bars), 250 μM TEMPO (grey bars), or 10 μM apocynin (dark grey bars).