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Hydrophobic statins induce autophagy and cell death in human rhabdomyosarcoma cells by depleting geranylgeranyl diphosphate

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ABSTRACT

Statins are the most common type of medicine used to treat hypercholesterolemia; however, they are associated with a low incidence of myotoxicity such as myopathy and rhabdomyolysis. The mechanisms for the adverse effects remain to be fully elucidated for safer chronic use and drug development. The results of our earlier work suggested that hydrophobic statins induce autophagy in cultured human rhabdomyosarcoma A204 cells. In this study, we first confirmed the statin-induced autophagy by assessing other criteria, including induced expression of the autophagy-related genes, enhanced protein degradation of autophagy marker protein p62 and electron microscopic observation of induced formation of autophagosome. We next demonstrated that the extent of inhibition of HMG-CoA reductase in the cell is parallel with the ability of a statin to induce autophagy. Thus, the primary activity of statins causes autophagy in A204 cells. Considering the mechanism for the induction, we showed that statins induce autophagy by depleting cellular levels of geranylgeranyl diphosphate (GGPP) mostly through an unknown pathway that does not involve two major small G proteins, Rheb and Ras. Finally, we demonstrated that the ability of statins to induce autophagy parallels their toxicity to A204 cells and that both can be suppressed by GGPP.

1. Introduction

Statins block de novo synthesis of cholesterol by inhibiting the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely used for the treatment of hypercholesterolemia. Since the discovery of mevastatin by Endo et al. in 1973, a variety of statins have been discovered as natural products or developed by drug design (Endo et al., 1977). Although statins are widely prescribed as supposedly safe for many patients, there is a risk of myotoxicity, such as muscle pain or even severe muscle disorder rhabdomyolysis (Arora et al., 2006; Omar and Wilson, 2002). The hydrophilic statins are thought to be actively transported into hepatocytes by expressing the organic anion transporter (OATP), whereas hydrophobic statins diffuse non-selectively into extra-hepatic tissues such as muscle and cause the adverse effects (Neuvonen et al., 2006). However, the mechanism underlying statin-induced myotoxicity has not been elucidated. Importantly, it has not been clarified whether inhibition of HMG-CoA reductase itself or other targets causes myotoxicity.

In our earlier work, we reported that hydrophobic statins induced autophagy in cultured human rhabdomyosarcoma A204 cells based on both the biochemical observation of the enhanced processing of the autophagy marker protein LC3 and the morphological observation of accumulation of the green fluorescent protein-labeled LC3 (GFP-LC3) on autophagosomes (Araki and Motojima, 2008). These changes were induced only by hydrophobic statins in A204 cells but not HEK293, HepG2 and HuH7 cells and were blocked by mevalonate but not by cholesterol.

Autophagy, found in all mammalian cells, is a system for the non-selective degradation of proteins and organelles to promote protein turnover for cell survival and protection against a variety of stresses (Mizushima, 2007). Both insufficient and excess autophagy can be harmful for the cell and the process is stringently regulated by several signaling pathways (Mizushima et al., 2008). The mammalian target of the rapamycin complex 1 (mTORC1) plays a major regulatory role in autophagy in collaboration with the autophagy-related proteins (ATG proteins). The small G protein Rheb, when modified by farnesylation, activates mTORC1, which inhibits the induction of autophagy by inhibiting the kinase activity of ULK (the mammalian Atg1 homolog) (Hosokawa et al., 2009; Jung et al., 2009). Under starvation conditions, Rheb is inactivated by the Rheb GTPase-activating protein TSC1–TSC2 and mTORC1 is inactivated, leading to the induction of autophagy (Huang and Manning, 2008). In addition, the Ras/PI3K/Akt pathway regulates autophagy negatively. In this pathway, inactivated Ras inhibits polyisoprenylation and induces autophagy by inactivating Akt (He and Klionsky, 2009). Other signaling pathways are known to control autophagy but the highly complicated regulatory processes remain to be fully elucidated.

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Our previous results, which suggested that modulation of the isoprenoid metabolic pathway by statins is involved in the hydrophobic statin-induced autophagy, raised several questions. The aim of this study was to provide the answers to these questions. Do statins induce not only the processing of LC3 but the total process of autophagy? What are the mechanisms for statin and cell-type specificities? Is the effect of the statin-induced autophagy on the cell protective or harmful?

2. Materials and methods

2.1. Materials

Anti-LC3 antibodies have been described (Araki and Motojima, 2008). The antibodies to Rheb, RhoA, Cdc42 and RhoG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-S6 ribosomal protein (Ser235/236) antibody was obtained from Cell Signaling (Beverly, MA). Anti-GFP antibody was obtained from Molecular Probe (Invitrogen, Carlsbad, CA). Rheb (sc-41859) and, RhoA (sc-29471) siRNA were purchased from Santa Cruz Biotechnology. Negative control siRNA was obtained from Invitrogen. GGPP, FPP, GGTI 2133 and FTI 277 were purchased from Sigma (St. Louis, MO). The GFP-p62 expression plasmid (Ichimura et al., 2008) was a gift from Dr M. Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Cerivastatin was purchased from Sequoia Research Products (Pangbourne, UK) and Simvastatin and Pravastatin were purchased from Wako (Tokyo, Japan).

2.2. Cell culture

Human rhabdomyosarcoma A204 cells were maintained at 37 °C under a 5% CO_2 atmosphere in McCoy's 5A medium containing 10% (v/v) fetal bovine serum (FBS). A204 cells expressing GFP-LC3 (autophagy marker) were cultured in McCoy's 5A medium containing 10% FBS and 150 μg ml^{-1} G418 as described (Araki and Motojima, 2008). The human hepatoma cell line HuH7, which was provided by the Riken Cell Bank (Tsukuba, Japan), was cultured at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium containing 10% FBS.

2.3. Western blotting

SDS-PAGE and western blotting were done as described (Araki and Motojima, 2008). Whole cell lysates prepared with SDS sample buffer (1% SDS, 4.5 M urea, 0.125 M Tris–HCl, pH 6.8) were separated by SDS-PAGE and transferred to a polyvinylidene diflouride (PVDF) membrane. After the transfer, the membrane was treated with TBS-Tween 20 (150 mM NaCl, 10 mM Tris–HCl, 0.05% (v/v) Tween 20, pH 7.6) containing 5% (w/v) skim milk. When the anti-phosphoribosomal S6 was used as primary antibody, the membrane was treated with TBS-Tween 20 containing 5% (w/v) bovine serum albumin. The blots were then probed with respective antibodies. Anti-rabbit IgG, anti-goat IgG and anti-mouse IgG labeled with horseradish peroxidase were used as the secondary antibody, and an enhanced Super Signal West Pico (Pierce, Richmond, IL) was used for detection by exposure to X-ray film.

2.4. Protein degradation assay

To analyze the enhanced protein degradation by autophagy, we used GFP-labeled p62, which is known to be degraded selectively by autophagy (Ichimura et al., 2008). Transfections were done as follows. A204 cells cultured in 12-well plates were transfected with the expression plasmid of GFP-p62 (0.3 μg/well) using Plus reagent and Lipofectamine reagent (Invitrogen, Carlsbad, CA). After transfection for 3 h, the cells were treated with or without 1 μM Cerivastatin for 24 h, and whole cell extracts were prepared with SDS sample buffer. The samples were examined by SDS-PAGE and western blotting using anti-GFP antibody as primary antibody.

2.5. Real-time PCR analysis

Real-time PCR analysis was done as described (Ashibe and Motojima, 2009). Briefly, total RNA of cells was prepared with GenElute Mammalian Total RNA Miniprep Kit (Sigma) and reverse transcriptions were done using 1 μg of RNA of each sample with the PrimeScript RT reagent kit (TaKaRa Bio, Kyoto Japan). Quantitative real-time PCR (qPCR) assays were done with SYBR premix Ex Taq (TaKaRa Bio, Kyoto Japan) and a LightCycler 1.5 instrument. The primers for qPCR are given in Table 1. The data were analyzed by the $\Delta\Delta C_t$ method.

2.6. Detection of autophagy by GFP fluorescence imaging and electron microscopy

Induced-autophagy was determined with A204 cells stably expressing GFP-LC3 (GL3 cell) (Araki and Motojima, 2008). The GL3 cells cultured on poly-L-lysine-coated coverslips were treated with statins. The statin-treated cells were fixed with 4% (w/v) paraformaldehyde for 20 min at room temperature, and fixed in MOWIOL (Sigma) containing 2.5% (w/v) 1,4-diazabicyclo [2.2.2] octane (Wako). The samples were examined under a confocal fluorescence microscope (Fluoview FV500, Olympus, Tokyo, Japan).

Ultrastructural analysis was performed by Tokai-EMA Inc. (Nagoya, Japan). For electron microscopy, A204 cells treated with or without Cerivastatin for 24 h were fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4, and post-fixed in 2% (w/v) OsO_4 in 0.1 M sodium phosphate buffer. The samples were stained with uranyl acetate and lead citrate, and examined with a JEM1200EX electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

2.7. Estimation of inhibition of HMG-CoA reductase by statins in the cell

Localization of GFP-ERas was determined by confocal fluorescence microscopy to estimate the extent of inhibition of HMG-CoA reductase in the cell by various concentrations of statins. ERas is a major small G-protein that localizes on the plasma membrane when protein is modified by polyisoprenylation but stays in the cytosol when the modification is incomplete. The polyisoprenylation of ERas depends on the cellular concentrations of polyisoprenoids, which should be reflected by the activity of HMG-CoA reductase in the cell. Thus, ERas on the plasma membrane shows no or little inhibition, whereas its cytosolic localization indicates severe inhibition of HMG-CoA reductase in the cell. A204 cells cultured on poly-L-lysine-coated coverslips were transfected with GFP-ERas expression plasmid (0.15 μg/well) (Addgene plasmids 13826 and 13827, Add Gene Inc., Cambridge, MA) (Takahashi et al., 2005) using Lipofectamine and Plus reagent. After transfection for 3 h, the cells were treated with various concentrations of Cerivastatin, Simvastatin or Pravastatin for 24 h and localization of GFP-ERas was determined by confocal fluorescence microscopy. The

Table 1
Primers for real-time PCR.

Gene name	Left	Right
LC3b	cgcaccttcgaacaaagag	ctcacccttgtatcgttctattatca
Gabarapl1	tgggccaactgtatgagga	ctacccccaagtccaggtg
Bnip3	tgctgctctctcatttgctg	gactccagttcttcatcaaaaggt
Fbxo32	gcagcagctgaacaacattc	cacaaaggcaggtcagtgaa
GAPDH	agccacatcgctcagacac	gcccaatacgaccaaatcc