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Ginsenoside Rh2 induces ligand-independent Fas activation via lipid raft disruption

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ABSTRACT

Lipid rafts are plasma membrane platforms mediating signal transduction pathways for cellular proliferation, differentiation and apoptosis. Here, we show that membrane fluidity was increased in HeLa cells following treatment with ginsenoside Rh2 (Rh2), as determined by cell staining with carboxy-laurdan (C-laurdan), a two-photon dye designed for measuring membrane hydrophobicity. In the presence of Rh2, caveolin-1 appeared in non-raft fractions after sucrose gradient ultracentrifugation. In addition, caveolin-1 and GM1, lipid raft landmarkers, were internalized within cells after exposure to Rh2, indicating that Rh2 might disrupt lipid rafts. Since cholesterol overloading, which fortifies lipid rafts, prevented an increase in Rh2-induced membrane fluidity, caveolin-1 internalization and apoptosis, lipid rafts appear to be essential for Rh2-induced apoptosis. Moreover, Rh2-induced Fas oligomerization was abolished following cholesterol overloading, and Rh2-induced apoptosis was inhibited following treatment with siRNA for Fas. This result suggests that Rh2 is a novel lipid raft disruptor leading to Fas oligomerization and apoptosis.

Introduction

There are specific plasma membrane platforms that organize receptors and their downstream molecules and initiate signaling pathways. These platforms are called lipid rafts because they are mainly composed of glycosphingolipids and cholesterol [1]. Owing to the long and saturated fatty acids of glycosphingolipids and cholesterol, lipid rafts are present in the liquid-ordered (L_o) phase. On the contrary, other plasma membrane compartments are present in the liquid-disordered (L_d) phase because they are composed of phospholipids with short and unsaturated fatty acids. Since double bond in unsaturated fatty acids causes steric hindrance and limits interaction between phospholipids and cholesterol [2]. Thus cholesterol interacts more favorably with saturated fatty acid and these interactions lead to the formation of lipid rafts.

The sequestration of cholesterol or the administration of cholesterol derivatives have been known to change the biophysicochemical properties of lipid rafts. For example, methyl-β-cyclodextrin (MβCD) physically removes cholesterol from cellular membrane, and statins also weaken the integrity of lipid rafts by inhibiting cholesterol biosynthesis and by reducing intracellular cholesterol levels. In addition, treatment with MβCD or statins disrupts lipid rafts and inhibits lipid raft-dependent cellular events such as cell signaling, tumorigenesis and apoptosis [3,4]. Natural cholesterol derivatives including steroid hormones and phytosterols also change the integrity of lipid rafts, depending on their structural moiety [5,6].

Ginsenosides, known as phytosterols of *Panaxa ginseng*, also contain a cholesterol backbone with hydroxyl groups and oligosaccharide moieties. In particular, ginsenoside Rh2 (Rh2) is known to alleviate multi-drug resistance in cancer cells and attenuate amyloid-induced cytotoxicity [7,8]. Multi-drug resistance proteins and amyloid beta are predominantly localized in lipid rafts. The functions of these proteins are inhibited when lipid rafts are disrupted, it is tempting to speculate that Rh2 might disrupt lipid rafts [9,10].

It has also been reported that Rh2 induces apoptosis and it was abolished by caspase-8 inhibition [11]. Since lipid rafts harvest death receptors and are essential for the formation of death-inducing signaling complex [12], Rh2 might induce apoptosis via caspase-8-activating death receptors present in lipid rafts. Here, we unambiguously demonstrated that Rh2 induced apoptosis via lipid

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raft disruption and Fas oligomerization in HeLa cells. In addition, we have shown that the fortification of lipid rafts by cholesterol overloading and Fas downregulation prevented Rh2-induced apoptosis, suggesting that lipid raft disruption by Rh2 is essential for ligand-independent Fas activation.

Materials and methods

Materials. Ginsenosides were purchased from BTGin (Chung-Nam, Korea). The anti-caveolin-1 and anti-Fas antibodies were obtained from Transduction Laboratory (Lexington, KY) and the anti-caspase-3 and anti-caspase-8 antibodies from Cell Signaling (Berverly, MA).

Cell culture. HeLa cells were purchased from the ATCC (Manassas, VA) and grown in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. Before drug treatment HeLa cells were incubated with serum-free DMEM for 4 h. To overload cholesterol in HeLa cells, HeLa cells were treated with cholesterol/MβCD complexes (20 μg/ml of cholesterol with 0.0624% of MβCD in a final working concentration). For Si-RNA transfection, HeLa cells were transfected with SiRNAs for 48 h using electroporation according to the manufacturer's instructions (NanoEntek, Korea).

Two-photon fluorescence microscopy and GP imaging. Serum starved HeLa cells were treated with chemicals, stained with 10 mM C-laurdan for 40 min at 37 °C and fixed with 3.7% formaldehyde for 15 min. Three dimensional GP images of HeLa cells were obtained using two-photon microscopy previously described [13]. The GP image is pseudo-colored as indicated by the scale, with GP from −1 to +1. GP distributions were obtained from the histograms of the GP values of the images and fitted to one or two Gaussian functions by the non-linear fitting algorithm (Origin 7.0).

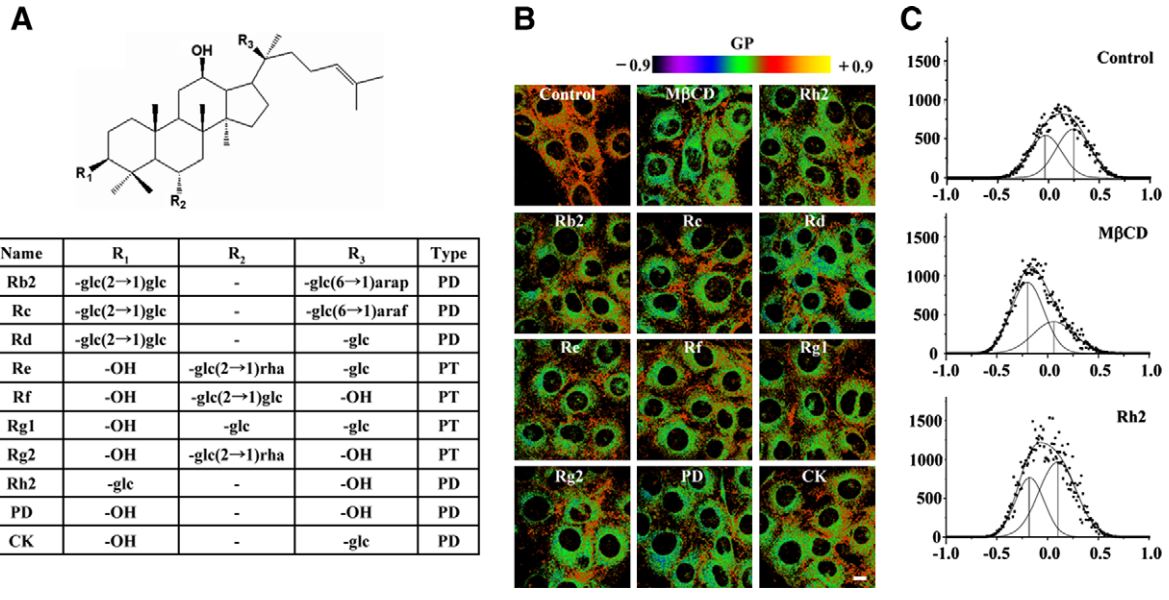
Lipid raft isolation. Lipid rafts were isolated as previously described [14]. Four 150-mm dishes of cells were mixed with 1 ml of lysis buffer (25 mM HEPES, pH 6.5, 150 mM NaCl, 1% TX-100, 1 mM EDTA, 1 mM PMSF and protease inhibitors), homogenized 20 times with a tight Dounce homogenizer and then incubated for 20 min at 4 °C. The lysate was mixed with 1 ml of 80% sucrose, transferred to SW41Ti centrifuge tube and overlaid with 7 ml of 30% sucrose solution and 3 ml of 5% sucrose solution. The discontinuous sucrose gradients were centrifuged for 18 h at 39,000 rpm, 4 °C. The gradient was then fractionated into 12 fractions from the bottom to the top.

Immunoblots. Proteins were separated on polyacrylamide gels. For immunoblotting, the proteins were transferred onto a PVDF membrane. The membranes were then blocked for 1 h at room temperature, and allowed to react with a sequence of primary and secondary antibodies. The antigen signals were visualized using ECL reagents.

Immunofluorescence. HeLa on a 12 mm cover slip were washed briefly with PBS. For immunofluorescence, cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 1% TX-100 in PBS, washed 3 times with PBS and then blocked with 5% BSA in PBS for 1 h. After blocking cells were incubated with primary antibodies and primary antibodies were detected using fluorescein-conjugated secondary antibodies. For CTB staining, HeLa cells were fixed with 3.7% formaldehyde for 10 min. After three washings with PBS, cells were incubated with FITC-conjugated CTB for 30 min at room temperature. Cells were observed with a confocal microscope (ZEISS LSM 510 META).

TUNEL assay. TUNEL assay was performed according to the manufacturer's instructions (Roche applied science, Mannheim). After the TUNEL reaction, cells were further labeled with DAPI. Cells were washed three times with PBS and mounted. Cells were observed using a confocal microscope (ZEISS LSM 510 META).

Caspase activity assay and MTT assay. The activities of Caspase-3 and -8 were measured with their specific substrates (Ac-DEVD-pNA and Ac-IETD-pNA, respectively). Whole cell lysates (100 μg) in HEPES buffer (100 mM HEPES, pH 7.5, 150 mM NaCl, 1% TX-100) were added to the reaction buffer (100 mM HEPES, pH 7.5, 10% Glycerol, 20 mM DTT, 2 mM EDTA and 0.1 M caspase substrates), and incubated at 37 °C for 8 h. Color changes of the cleaved substrate were measured at an absorbance wavelength of 405 nm. For MTT assay, cells were incubated with 5 mg/ml of



Name	R_1	R_2	R_3	Type
Rb2	-glc(2→1)glc	-	-glc(6→1)arap	PD
Rc	-glc(2→1)glc	-	-glc(6→1)araf	PD
Rd	-glc(2→1)glc	-	-glc	PD
Re	-OH	-glc(2→1)rha	-glc	PT
Rf	-OH	-glc(2→1)glc	-OH	PT
Rg1	-OH	-glc	-glc	PT
Rg2	-OH	-glc(2→1)rha	-OH	PT
Rh2	-glc	-	-OH	PD
PD	-OH	-	-OH	PD
CK	-OH	-	-glc	PD

Fig. 1. Ginsenosides increase the fluidity of plasma membrane. (A) The chemical structure and functional groups of ginsenosides (PD: Panaxadiol, PT: Panaxatriol, glc: β-D-glucosyl, arap: α-L-arabinosyl, araf: α-L-arabinofuranosyl, rha: α-L-rhamnosyl). (B) HeLa cells were treated with 10 mM MβCD or 50 μM ginsenosides for 1 h at 37 °C, stained with C-laurdan. Three dimensional GP images of HeLa cells were obtained using two-photon microscopy. Bar = 10 μm. (C) Representative distribution curves of GP images in control, MβCD-treated and Rh2-treated cells were fitted to one or two Gaussian functions.