SPANDIDOS PUBLICATIONS

Cholesterol-dependent induction of dendrite formation by ginsenoside Rh2 in cultured melanoma cells

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Abstract. Herbal remedies containing root extracts of Panax ginseng are commonly used for complementary or alternative therapies. Ginsenosides, the major components of root extracts, are responsible for ginseng's pharmacological and biological effects; however, their mechanisms of action are unclear. We examined whether membrane cholesterol was involved in the mechanism of action of ginsenoside Rh2 in cultured cells. In B16 melanoma cells, Rh2 (18.5 μM) induced dendrite formation within 2 h. Depletion of cholesterol by pretreatment with 10 mM methyl-ß-cyclodextrin suppressed this effect of Rh2. Rh2 did not change the cellular cholesterol content and the immunofluorescence staining pattern of the lipid-raft-associated molecules, ganglioside GM3, Caveolin-1, Flotillin-1, and Flotillin-2, for up to 3 or 6 h. However, within 2 min of addition, Rh2 changed the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) but not of 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH). DPH is more sensitive than TMA-DPH to changes in the physical properties of membrane lipid bilayers regulated by cholesterol. These results suggest that Rh2 affects the physical properties of cholesterol-regulated membrane lipid bilayers and could lead to changes in cellular functions.

Introduction

Panax ginseng has been used for over 2,000 years in the Far East to promote health. Today, herbal remedies containing ginseng root extracts are commonly used as complementary or alternative therapies. Ginseng has many pharmacological effects (1,2); however, clinical trial results regarding the efficacy of ginseng have been contradictory (3-6). Its long history and worldwide use in herbal remedies necessitate clarification of ginseng's efficacy and mechanism of action.

Ginsenosides are the major components of ginseng extracts and are responsible for ginseng's pharmacological and biological effects. The effects of purified ginsenosides have been investigated by many *in vitro* and *in vivo* experimental systems. In particular, the effects of ginsenoside Rh2 have been intensively investigated. We previously demonstrated that Rh2 inhibited cell proliferation and induced differentiation of B16 melanoma cells (7). Recently, many biological effects have been attributed to Rh2, such as inhibition of nitric oxide production (8), induction of apoptosis (9,10), and inhibition of invasiveness (11) in cultured cells. Animal studies have shown that Rh2 inhibits passive cutaneous anaphylaxis (12) and ischemic brain injury (13) and can increase plasma insulin levels (14) and insulin sensitivity (15). Despite the biological and pharmacological activities of Rh2, its mechanism of action is still unclear. To understand the mechanism of action of Rh2, it is worth noting that the latent period before Rh2 manifests its effects on cultured cells is very different for its various effects. For example, it takes more than two days for Rh2 to induce the expression of differentiation markers (7,16-18); however, it takes only 3 to 12 h for it to affect the activity of transcription factors (11,19,20). Rh2 affects cdks and cyclins (21), mRNA expression (18), and the phosphorylation level or activity of kinases (11,22-24) in less than 60 min. Taken together, these findings suggest that the primary actions of Rh2 occur within the first hour of addition, whereas later effects may be indirect and due to downstream signals. Accordingly, to understand the mechanism of action of Rh2 and its myriad effects, the primary action of Rh2 must first be clarified.

We have previously shown that within 2 min of addition, Rh2 changed the lipid fluidity of the cell membrane and subsequently changed cell surface properties related to differentiation (25). Furthermore, we have investigated the uptake of Rh2 by B16 melanoma cells and have raised the possibility that the mechanism of action of Rh2 is related to

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sterol function (26). Membrane cholesterol is a major component of lipid rafts that are crucial for various signal transductions. Previously, we reported an important role of lipid rafts for apoptosis and NK cell-mediated cytotoxicity (27,28). Altogether, it is possible that ginsenosides interact with the membrane lipids surrounding membrane proteins and somehow modify the functions of these proteins (1,29).

In the present study, we examined whether membrane cholesterols are involved in the mechanism of primary action of Rh2. Rh2 induced dendrite formation in B16 melanoma cells within 2 h after its addition and this induction was suppressed by the depletion of cholesterol. Furthermore, within 2 min of addition, Rh2 changed the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), which is sensitive to changes in the physical properties of membrane lipid bilayers regulated by cholesterol. These results suggest that membrane cholesterols are involved in the mechanism of primary action of Rh2.

Materials and methods

Cells and culture. The B16BL6 subline of B16 melanoma cells was kindly provided by Dr T. Sasaki (Department of Experimental Therapeutics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. For treatment with ginsenoside Rh2, cells were cultured in RPMI-1640 medium containing 2% FBS for one day and then cultured with Rh2 in the same medium. Rh2 was dissolved in ethanol at 40 mM and stored at -20˚C. This stock solution was diluted directly with the culture medium as needed. An equal concentration of ethanol was added to the control cultures.

Cholesterol depletion. Methyl-ß-cyclodextrin (MßCD) (Sigma-Aldrich, St. Louis, MO, USA), which extracts cholesterol from biological membranes, was used for cholesterol depletion of cultured cells (30). For cholesterol depletion, cells were washed with serum-free RPMI-1640 and incubated with MßCD in serum-free RPMI-1640 for the indicated time.

Measurement of dendrite induction by Rh2. B16 cells ($2x10^5$ cells/well) were inoculated into 6-well plates and cultured overnight. Rh2 (18.5 μM) was added to the culture and the same microscopic fields were photographed sequentially at the indicated time points. Cells with more than three dendrites were counted as dendrite-induced cells.

Quantification of Rh2. Quantification of Rh2 taken up by cells was performed as previously described (26). Briefly, total lipids were extracted from the cells and fractionated by high-performance thin-layer chromatography (HPTLC; Silica Gel 60) (E. Merck AG, Darmstadt, Germany) using chloroform/methanol (10:3, v/v) as the development solvent. The developed plates were sprayed with 10% H_2SO_4, heated at 90˚C for 10 min, and immediately covered with glass plates. Quantification was performed by scanning the chromatograms with a dual wavelength chromatoscanner (CS-900, Shimadzu Corporation, Kyoto, Japan) at 540 nm.

Measurement of cholesterol content. Cells cultured on 60-mm dishes were washed three times with PBS at room temperature. Ice-cold 1X cholesterol measurement reaction buffer (0.05 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, 0.1 M potassium phosphate, pH 7.4) was added to the dishes and cells were lysed for 20 min at 4˚C. Lysates were transferred to 1.5-ml Eppendorf tubes and sonicated briefly. Protein concentration was measured using the Bradford Ultra kit (Novexin Ltd., Cambridge, UK), and cholesterol was measured using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Inc., Eugene, OR, USA).

Antibodies. Anti-GM3 (clone GMR6) was purchased from Seikagaku Biobusiness Corporation (Tokyo, Japan). Anti-Caveolin-1 (clone 2297), anti-Caveolin-2 (clone 65), anti-Flotillin-1 (clone 18), and anti-Flotillin-2 (clone 29) were purchased from BD Biosciences (San Diego, CA, USA).

Immunoblotting. Samples were lysed with Laemmli buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membranes were then probed with a primary antibody, followed by a peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized using ECL Plus reagents (Amersham Biosciences, Little Chalfont, UK).

Staining of lipid-raft-associated molecules in cells. For immunofluorescence staining, cells grown on 35-mm culture dishes were washed with PBS and fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. Fixed cells were incubated with 0.5% bovine serum albumin (BSA) in PBS for 60 min at room temperature and then overnight at 4˚C with primary antibodies diluted 1:200 in 0.5% BSA in PBS. After three washes with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated or Alexa 488-conjugated secondary antibody diluted 1:200 in 0.5% BSA in PBS containing 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 60 min at room temperature. After washing with PBS, cells were mounted with Prolong Gold (Molecular Probes, Inc.). For staining of cholesterol, the cholesterol-binding antibiotic filipin (Sigma Chemical Co., St. Louis, MO, USA) was used. Filipin was dissolved at 25 mg/ml in DMSO as a stock solution. Cells grown on 35-mm culture dishes were washed with PBS and fixed with freshly prepared 4% paraformaldehyde for 30 min at room temperature. Cells were washed with PBS containing 50 mM glycine followed by incubation for 2 h at room temperature in PBS containing 50 μg/ml filipin. After brief washing with PBS, cells were mounted with Prolong Gold. Images were obtained using an Axiovert 200 inverted fluorescence microscope equipped with an AxioVision image processing and analysis system (Carl Zeiss, Jena, Germany). Filipin was imaged using filter set 49 (G365, FT395, BP445/50).

Analysis of gangliosides. Gangliosides were purified using the method of Suzuki (31). Briefly, $4x10^7$ cells were washed twice

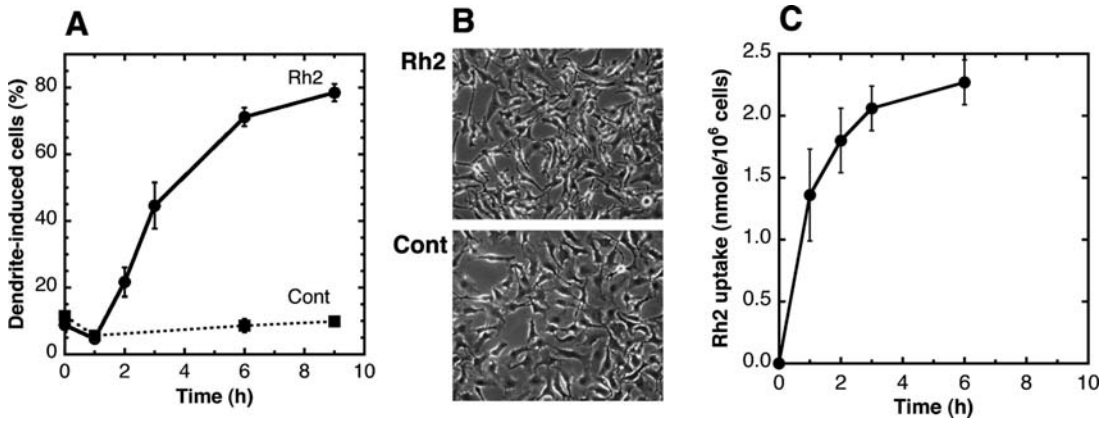

Figure 1. Induction of dendrite formation by Rh2 in B16 melanoma cells. (A) Cells were cultured with 18.5 μM Rh2 for the indicated time. Cells with more than three dendrites were counted as dendrite-induced cells. Values indicate the mean ± SD of three microscopic fields of view. Similar results were obtained in three independent experiments. (B) Morphology of cells cultured with (Rh2) or without (Cont) 18.5 μM Rh2 for 6 h. (C) Cells were cultured with 15 μM Rh2 for the indicated time and cellular Rh2 was measured as described in Materials and methods. Values indicate the mean ± SD of three independent experiments.

with PBS, extracted using 20 volumes of chloroform/methanol (2:1, v/v) for 30 min at room temperature, and then re-extracted twice with 10 volumes of chloroform/methanol (1:2, v/v) for 30 min at room temperature. The extracts were combined, dried under nitrogen gas flow, redissolved in 10 volumes of chloroform/methanol (2:1, v/v), and partitioned against 0.2 volumes of water. The organic phase was washed three times with the theoretical upper phase, chloroform/methanol/water (3:48:47, v/v). The combined upper phase was dialyzed against distilled water using a dialysis membrane (MW cut-off, 800 Da) at 4˚C overnight and lyophilized. The isolated gangliosides were separated by HPTLC (Silica Gel 60) using chloroform/methanol/0.025% $CaCl_2$ (60:35:8, v/v) and visualized using resorcinol reagent.

Measurement of fluorescence polarization. The compound DPH and its derivative, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) were purchased from Molecular Probes, Inc. Measurement of fluorescence polarization was performed as previously described (25). Briefly, cells ($1x10^6$/ml) were mixed with an equal volume of DPH ($1x10^{-6}$ M) or TMA-DPH ($1.5x10^{-6}$ M) dispersed in PBS. After incubation at 37˚C for 30 min with gentle shaking, cells were washed three times with PBS. Rh2 was added directly to the cell suspension in PBS, and fluorescence polarization was measured at 25˚C in the MV-1a apparatus (Elscint, Ltd. Haifa, Israel).

Statistical analysis. Differences between values were analyzed by the two-tailed Student's t-test using the built-in statistics function of Microsoft Excel (Version 11.2).

Results

Induction of dendrite formation by ginsenoside Rh2. We previously reported that Rh2 induced the differentiation of B16 melanoma cells and that treating B16 melanoma cells with Rh2 for 48 h induced melanin production (7,29). Dendrite formation is a hallmark of melanocytes, and factors such as keratinocyte-conditioned medium and α-melanocyte stimulating hormone induce dendrite formation within 1 h of addition to cultured S91 melanoma and B16 melanoma cells (32,33). To identify an Rh2-induced differentiation marker that is induced more rapidly than melanin, we examined the effect of Rh2 on dendrite formation. B16 cells were cultured with Rh2 (18.5 μM), and cells with induced dendrites (i.e. cells with more than three dendrites) were counted. Such dendrite-induced cells appeared within 2 h of Rh2 addition, and the number increased gradually; 9 h after Rh2 addition, 80% of the cultured cells were dendrite-induced cells (Fig. 1A and B). The time course of dendrite induction coincided with that of Rh2 uptake (Fig. 1C), except that dendrite induction was delayed by about 2 h relative to Rh2 uptake. Therefore, dendrite induction by Rh2 appeared to be a very early effect of Rh2.

Depletion of cholesterol suppresses dendrite induction by Rh2. To examine whether cholesterol is involved in the dendrite induction by Rh2, B16 cells were pretreated with MßCD and dendrite induction by Rh2 was examined. Pre-treatment with 10 to 40 mM MßCD for 15 min decreased the cholesterol content (Fig. 2A) and suppressed dendrite induction by Rh2 in a dose-dependent manner (Fig. 2B). Pretreatment with 10 mM MßCD for 2 h almost completely suppressed dendrite induction by Rh2 (Fig. 2C and D). More than 90% of the cholesterol in intact cells is localized on the cell membrane (34); therefore, these results strongly suggest that membrane cholesterol is involved in dendrite induction by Rh2. We also examined the effect of Rh2 on the cholesterol content of B16 cells. Rh2 did not change the cholesterol content for at least 2 h (data not shown).

Effect of Rh2 on lipid-raft-associated molecules. Lipid rafts are membrane microdomains enriched with cholesterol, glycosphingolipids, sphingolipids, and certain membrane proteins, and are involved in various signal transductions (27,28,35,36). MßCD depletes cholesterol from the cell membranes and has been used for disrupting lipid rafts in various cells (27,30), including B16 cells (37). Therefore, in order to examine whether Rh2 affects lipid rafts, we stained lipid-raft-associated

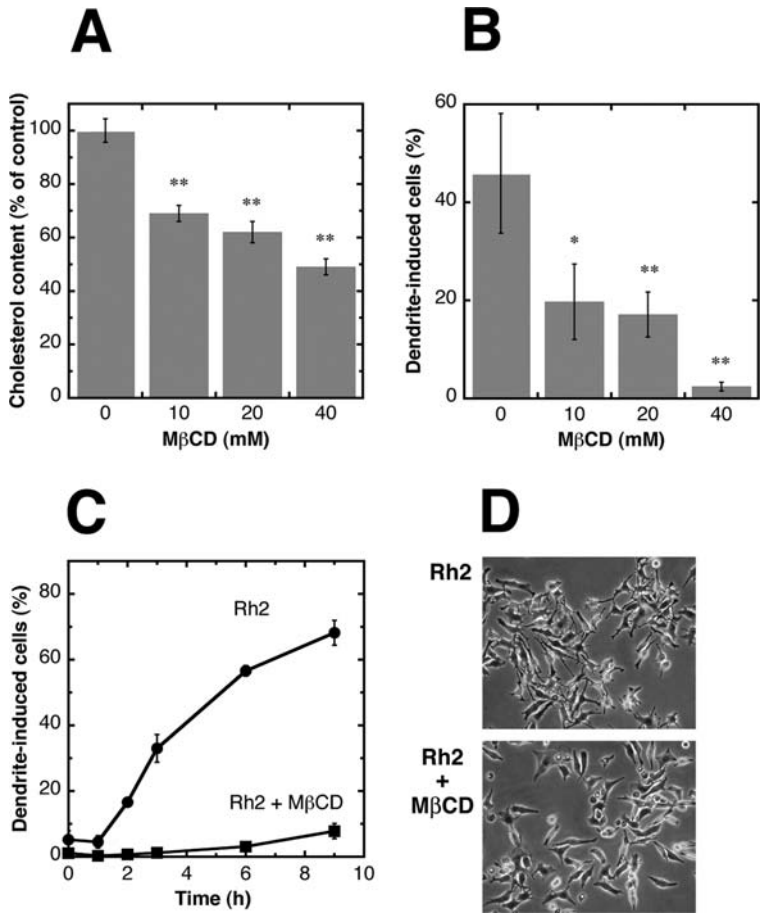

Figure 2. Disruption of lipid rafts by MßCD suppressed dendrite induction by Rh2 in B16 melanoma cells. (A) Cells were treated with the indicated concentration of MßCD in serum-free medium for 15 min and cellular cholesterol content was determined. (B) After treatment with the indicated concentration of MßCD in serum-free RPMI-1640 for 15 min, cells were cultured with 18.5 μM Rh2 in 2% FBS containing RPMI-1640 for 6 h and the number of dendrite-forming cells was counted. (C) After treatment of cells with or without 10 mM MßCD in serum-free RPMI-1640 for 2 h, cells were cultured with 18.5 μM Rh2 in 2% FBS containing RPMI-1640 for the indicated time and the number of dendrite-forming cells was counted. (D) Morphology of cells cultured with 18.5 μM Rh2 for 6 h with or without pretreatment with 10 mM MßCD for 2 h. In (A), (B), and (C) the values indicate the mean ± SD of 3-4 samples. Similar results were obtained in two independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with the control by the Student's t-test.

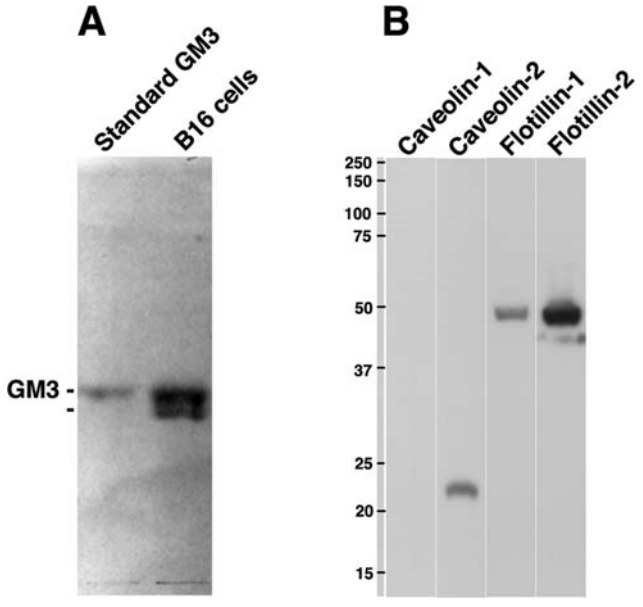

Figure 3. Expression of the lipid-raft-associated molecules in B16 melanoma cells. (A) Gangliosides were isolated from B16 cells and analyzed on TLC plates as described in Materials and methods. (B) Expressions of lipid-raft-associated proteins in B16 cells were examined by immunoblotting.

molecules, such as gangliosides, caveolins, and flotillins, in cells and examined whether Rh2 showed any effect on the staining pattern of these molecules. Before staining we examined the expression of these molecules in B16 cells. Gangliosides were isolated from B16 cells and were analyzed. The only ganglioside detected was GM3 (Fig. 3A). Protein expressions of Caveolin-1, Caveolin-2, Flotillin-1, and Flotillin-2 were examined by immunoblotting. We detected Caveolin-2, Flotillin-1, and Flotillin-2, but not Caveolin-1 (Fig. 3B). There was no difference in the staining pattern of the ganglioside GM3, Caveolin-2, Flotillin-1, and Flotillin-2 between control and Rh2-treated cells (Fig. 4A). We also stained cells with a cholesterol-binding antibiotic, filipin (Fig. 4B). We could not find a difference in the staining pattern between control and Rh2-treated cells.

Effect of Rh2 on the physical state of cholesterol-rich membrane lipid bilayers. To examine whether Rh2 affects the physical state of membrane lipid bilayers containing cholesterol, we measured membrane lipid fluidity using two fluorescent probes, DPH and TMA-DPH. Rh2 increased the fluorescence polarization of DPH within 2 min (Fig. 5A) but did not change the fluorescence polarization of TMA-DPH

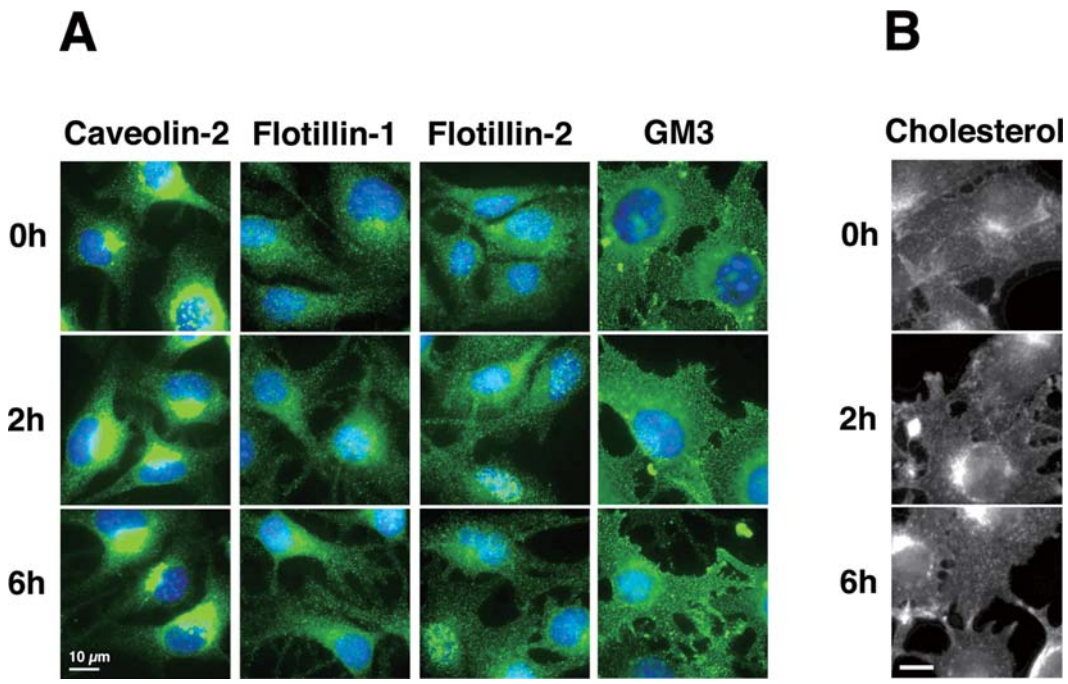

Figure 4. Effect of Rh2 on the staining pattern of lipid-raft-associated molecules in B16 melanoma cells. (A) Immunofluorescence staining of lipid-raft-associated molecules. Cells were cultured with 20 μM Rh2 in 2% FBS containing RPMI-1640 for the indicated time and stained with anti-Caveolin-2, anti-Flotillin-1, and anti-Flotillin-2, and anti-GM3 antibodies (green) and DAPI (blue). Bar, 10 μm. (B) Cholesterol was stained with filipin as described in Materials and methods. Bar, 10 μm.

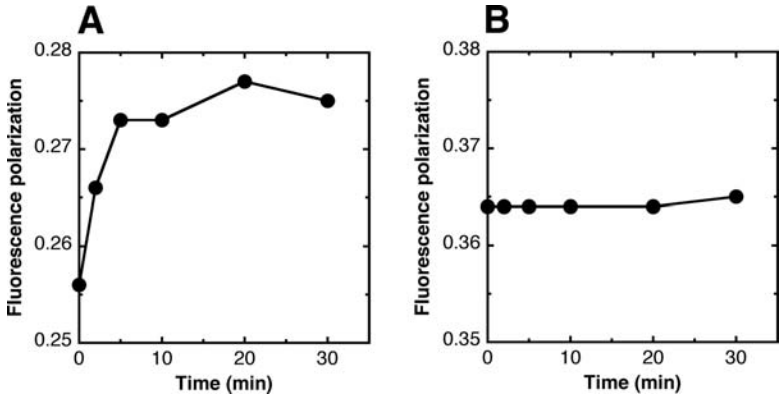

Figure 5. Effect of Rh2 on the fluorescence polarization of DPH (A) and TMA-DPH (B). Rh2 (20 μM) was added to suspensions of DPH- and TMA-DPH-labeled cells, and fluorescence polarization was measured at 25˚C using the MV-1a apparatus. Similar results were obtained from five experiments for DPH and three experiments for TMA-DPH, and representative results are shown.

over 30 min (Fig. 5B). This time kinetic study of the fluorescence polarization of DPH was in good agreement with our previous observation using cell membranes isolated from B16 cells (25). DPH monitors the fluidity in the lipid core region, while TMA-DPH monitors the fluidity of the phospholipid head group region (38). In erythrocytes, fluorescence polarization measured with DPH, but not TMA-DPH, is increased in the membrane core region of cholesterol-rich cells compared with that in control cells (39). Notably, DPH fluorescence polarization is more sensitive to membrane cholesterol content than TMA-DPH (40). Therefore, the observed increase in the fluorescence polarization of DPH but not TMA-DPH indicated that Rh2 affected the physical state of the cholesterol-rich membrane lipid bilayers.

Discussion

We examined the primary mechanism of action of ginsenoside Rh2 in cultured melanoma cells. We showed that Rh2 induced dendrite formation in B16 melanoma cells within 2 h and increased the fluorescence polarization of DPH within 2 min, but this was not the case for TMA-DPH. DPH is more sensitive than TMA-DPH to changes in the physical state of lipids in membrane bilayers owing to cholesterol alterations in the membrane (39,40). These results suggested that Rh2

modified the cellular function primarily by affecting the properties of the membrane lipid bilayer that are determined by cholesterol. It has been proposed that ginsenosides interact with membrane lipids around membrane proteins, such as receptors, ion channels, and signal transduction proteins, and modify the functions of these proteins, thereby exerting diverse cellular effects (1,29). It has been increasingly evident that the activities of many membrane proteins are affected by the composition of the surrounding lipid bilayer (41). Cholesterol is an essential component of the eukaryotic cell membrane (42) and modulates membrane organization as well as a broad spectrum of membrane functions (42-44). In particular, cholesterol plays an essential role in the formation of lipid rafts (36). Lipid rafts have a critical role in Ca^{2+} (45), insulin (46), and neurotransmitter signaling (47). Interestingly, Rh2 inhibits voltage-dependent Ca^{2+} channels (48), N-methyl-D-aspartate receptors (49), and catecholamine secretion (50) within 10 min. Furthermore, intravenous injection of Rh2 increases the plasma insulin level and decreases the plasma glucose level within 30 min in normal rats (14) and increases insulin sensitivity within 60 min in rats with insulin resistance induced by fructose-rich chow (15). These effects of Rh2 might be mediated by lipid rafts. It was recently suggested that Rh2 at a toxic high dose (50 μM) induced apoptosis by disrupting lipid rafts in HeLa cells (51). However, in our staining experiment for lipid-raft-associated molecules, such as cholesterol, ganglioside GM3, Caveolin-2, Flotillin-1, and Flotillin-2, we could not find changes in the staining pattern of these molecules by non-toxic doses of Rh2 (18.5 to 20 μM), indicating that Rh2 did not disrupt lipid rafts in our assay system. Although we could not define whether Rh2 affected the function of lipid rafts in the induction of dendrite formation in B16 cells, it is possible that Rh2 exerts its effect through lipid rafts.

We and others have reported that T cells from patients with systemic lupus erythematosus show altered lipid raft composition and function (52-54). Lipid rafts organize insulin signal transduction (55), and lipid raft perturbations have been suggested to participate in the pathogenesis of insulin resistance (56,57). These observations indicate that the nature of lipid rafts is different in the healthy state and the disease state. Ginseng has been used mainly as a tonic to enhance stress tolerance. Clinical trials have not verified the efficacy of ginseng in healthy individuals (4). These characteristics of ginseng might be derived from, in part, the differential effect of ginseng on lipid rafts in cells under normal versus pathogenic conditions.

Cholesterol has been shown to modulate the activity of G-protein-coupled receptors (44), which are involved in many cellular responses (58) and are the target of 30 to 50% of marketed drugs (59). This may be another reason for the multiple biological and pharmacological effects of Rh2 and also calls attention to the concomitant use of pharmaceuticals that target G-protein-coupled receptors and Rh2.

In conclusion, our results suggest that Rh2 exerted its effect through modifying the property of the membrane lipid bilayer regulated by cholesterol. Cholesterol modulates many signaling pathways, such as lipid rafts and G-protein-coupled receptors. These findings provide insights into the mechanisms of action of ginsenoside Rh2 and may explain why ginsenosides have so many biological and pharmacological effects. Further study is required to clarify whether ginsenosides modify the function of lipid rafts and/or G-protein-coupled receptors.

Acknowledgements

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