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b-series gangliosides crucially regulate leptin secretion in adipose tissues

Shuting Ji [a], Yuki Ohkawa [a], Kyohei Tokizane [b], Yuhsuke Ohmi [a], Ryoichi Banno [c], Keiko Furukawa [a, d], Hiroshi Kiyama [b], Koichi Furukawa [a, *]

[a] Department of Biochemistry II, Nagoya University Graduate School of Medicine, Nagoya 466-0065, Japan
[b] Department of Functional Anatomy and Neuroscience, Nagoya University Graduate School of Medicine, Nagoya 466-0065, Japan
[c] Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, Nagoya 466-0065, Japan
[d] Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, Kasugai 487-8501, Japan

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ABSTRACT

Gangliosides are widely involved in the regulation of cells and organs. However, little is known about their roles in leptin secretion from adipose tissues. Genetic deletion of b-series gangliosides resulted in the marked reduction of serum leptin. Expression analysis of leptin revealed that leptin accumulated in the adipose tissues of GD3 synthase-knockout (GD3S KO) mice. Analysis of primary cultured stromal vascular fractions (SVF) derived from GD3S KO mice revealed that leptin secretion was reduced, although leptin amounts in cells were increased compared with those of wild type. Interestingly, addition of b-series gangliosides to the culture medium of differentiated SVF resulted in the restoration of leptin secretion. Results of methyl-β-cyclodextrin treatment of differentiated 3T3-L1 cells as well as immunocytostaining of leptin and caveolin-1 suggested that b-series gangliosides regulate the leptin secretion from adipose tissues in lipid rafts.

1. Introduction

Although sialic acid-containing glycosphingolipids, gangliosides have been considered to be involved in the development and function of the nervous system [1], recent studies of mice with genetic modification of ganglioside profiles revealed that gangliosides play roles in the maintenance of integrity and regeneration of lesioned nervous tissues [2–4]. Gangliosides are widely expressed in almost all tissues and cells, and also play pivotal roles regardless of expression levels [5]. Generally speaking, genetic modification of ganglioside expression resulted in less serious phenotypes than expected. These results have been interpreted to be due to compensational effects of remaining glycolipids in the individual mutant mice [5]. In particular, GD3 synthase gene knockout (GD3S KO) mice lacking all b-series gangliosides showed no apparent abnormalities except disturbed neuroregeneration of lesioned hypoglossal nerves [6]. These results suggest that remaining a-series gangliosides could replace majority of functions of lost b-series gangliosides.

Recently, we found that serum levels of leptin were markedly suppressed in GD3S KO mice, while they did not show apparent changes in body weights. Leptin is a hormone secreted from adipose tissues, and plays an important role in the regulation of appetite and body weights [7,8]. Lack of leptin and/or leptin receptor causes obesity [9]. But there have been no reports on the roles of gangliosides in the regulation of leptin secretion.

In this study, mechanisms for the suppressed levels of leptin in GD3S KO mice were analyzed, elucidating novel regulatory functions of b-series gangliosides.

Abbreviations: GEM, glycolipid-enriched microdomain; GD3S, GD3 synthase; KO, knockout; WT, wild type; RT-PCR, reverse transcription-polymerase chain reaction; SVF, stromal vascular fractions; MβCD, methyl-β-cyclodextrin; CNS, central nervous system; DMEM, Dulbecco's modified Eagle's medium; WAT, white adipose tissue; BAT, brown adipose tissue; FBS, fetal bovine serum.

* Corresponding author. Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan. Fax: +81 52 744 2069.
E-mail address: koichi@med.nagoya-u.ac.jp (K. Furukawa).

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2. Materials and methods

2.1. Antibodies and reagents

Anti-β-actin antibody was purchased from Sigma (St. Louis, MO). Antibodies to caveolin-1, flotillin-1 or leptin (ob-A20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, dexamethasone, 3-isobutyl-1-methyl-xanthine, methyl-β-cyclodextrin (MβCD), type I collagenase (from Clostridium histolyticum) were purchased from Sigma. Indomethacin was from Wako (Osaka, Japan). Cell strainer (100 μm Nylon) was from BD Biosciences (San Jose, CA). Spin-X™ UF concentrator 5 K was from Life Sciences (Pittston, PA).

2.2. Mice

The mutant mice used in this study were generated and maintained in our laboratory [6]. Wild type (WT) and GD3S KO mice were mated, and resultant heterozygotes were mated each other, and genotypes of the offspring were screened as described [6]. All experimental protocols were approved by the animal experimental committee of the Graduate School of Medicine in Nagoya University along the guidelines of Japanese government, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1966). Alteration of ganglioside composition in GD3S KO mice was shown in Supplementary Fig. S1 as reported [6].

2.3. Primary culture of adipose-derived stromal vascular fractions (SVF)

Cells were isolated and cultured as described previously [10] with minor modifications as described in Supplementary materials and methods. Briefly, epididymal fat tissues were dissected and digested with collagenase I. After filtration, SVF cells were obtained by centrifugation. The SVF cells were cultured until reaching 75–90% confluency in Dulbecco's modified Eagle's medium (DMEM)/F-12 Ham supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin.

2.4. Differentiation of SVF and 3T3-L1

Induction of differentiation of SVF and 3T3-L1 to adipocytes was performed as described in Supplementary materials and methods. Briefly, serum-free medium consisted of DMEM/F-12 Ham, 1% penicillin and streptomycin was used. Induction medium consisted of serum-free medium, 5% FBS, 17 nM insulin, 0.1 μM dexamethasone, 250 μM 3-isobutyl-1-methyl-xanthine and 60 μM indomethacin. The differentiation was confirmed by a morphology resembling mature adipocytes and staining by Nile Red to examine the lipid droplets as shown in Fig. S2.

2.5. Preparation of lysates from cells and tissues

Cells were lysed in a cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml of leupeptin) (Cell Signaling) with Protease Inhibitor Mixture™ (Calbiochem, San Diego, CA) and 1 mM PMSF. Insoluble materials were removed by centrifugation at 15,000 rpm for 10 min at 4 °C. For tissues, epididymal white adipose tissues (WAT) and brown adipose tissues (BAT) were homogenized in a lysis buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 50 mM NaF, 1 mM $NaVO_4$, 1% Triton X-100, 200 mM PMSF, and 0.01–0.02 TIU/mL aprotinin) with Protease Inhibitor Mixture™ and 1 mM PMSF. After centrifugation of the lysates at 8000 × *g* for 60 min at 4 °C, the supernatants were centrifuged at 18,000 × *g* for 90 min at 4 °C, and clarified lysates were used for immunoblotting. Protein concentrations were measured using a protein assay Kit, DC™ (Bio-Rad, Hercules, CA).

2.6. Western immunoblotting

Lysates from cells and tissues and supernatants of cell culture were separated by SDS-PAGE using 10–15% gels. The separated proteins were transferred onto an Immunobilon-P™ membrane (Millipore, Billerica, MA). Blots were blocked with 5% skim milk in PBS containing 0.05% Tween 20. The membranes were first probed with primary antibodies. After washing, the blots were incubated with goat anti-rabbit IgGs (eBioscience Inc., San Diego, CA) or goat anti-mouse IgGs (Cell Signaling) (1:1000) conjugated with horseradish peroxidase (HRP). Bound conjugates were visualized with an Enhanced Chemiluminescence™ (ECL) detection system (PerkinElmer Life Sciences, Waltham, MA) and analyzed using LAS3000™ image analysis system (Fuji, Tokyo, Japan). After protein extraction from adipose tissues, fifty μg of lysates were subjected to 15% SDS-PAGE. For detection of leptin in culture medium, supernatants of cell culture were concentrated 200-fold using Spin-X™ UF concentrator 5 K. Eighty μg proteins were applied for detection of proteins using anti-leptin antibody (1:200).

2.7. Preparation of GEM/rafts fractions

Tissues were destroyed with a nitrogen cavitation apparatus, followed by removal of nucleus and cell debris by centrifugation at 1000 rpm for 10 min. The supernatant was centrifuged at 40,000 rpm at 4 °C for 1 h using a Beckman MLS50 rotor (Kent, MI). Then, insoluble materials were dissolved in 1 ml lysis buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml of leupeptin). The lysates were mixed with an equal volume of 80% sucrose in MNE buffer (25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/μl aprotinin), and a stepwise gradient was prepared by overlaying 2 ml of 30% sucrose in MNE followed by a final layer of 1 ml of 5% sucrose in MNE. After centrifugation for 16–18 h at 4 °C at 36,000 rpm using a Beckman MLS50 rotor (Kent, MI), fractions of 500 μl were separated from the top of the gradient, and were used for Western immunoblotting.

2.8. Immunohistochemistry

For paraffin sections of adipose tissues, immunohistochemistry was performed using the standard streptavidin-biotin-peroxidase complex (SAB) method. Deparaffinized sections (5 μm thick) were treated with 0.3% hydrogen peroxide (H_2O_2) in absolute methanol for 20 min to block endogenous peroxidase. After blocking with Protein Block Serum-Free™ (Dako, Carpinteria, CA), the sections were incubated with anti-leptin antibody (1:400). Biotinylated secondary antibody (Vector, Southfield, MI) was then applied for 45 min. The sections were incubated with peroxidase-conjugated streptavidin (Vector) for 30 min, and the reaction products were visualized using 3,3′-diaminobenzidine (Dako, Tokyo, Japan). Nuclear counterstaining was performed using hematoxylin. Negative control was prepared without primary antibodies.

2.9. Real time RT-PCR

Primers used for real time reverse transcription-polymerase chain reaction (RT-PCR) were designed according to Oligo4.0™ as shown in Table S1. Cells and tissues were homogenized in Trizol™ reagent (Ambion, Carlsbad, CA). Four μg of RNA was reverse-transcribed using M-MLV RT™ (Invitrogen, Carlsbad, CA). PCR was

performed on a CFX connect™ Real-Time System (Bio-Rad, Hercules, CA) by adding 2 ng sample of cDNA to the commercially available SsoAdvanced SYBR Green Supermix™ (Bio-Rad, Hercules, CA). The mRNA expression levels were normalized by GAPDH mRNA.

2.10. Incorporation of gangliosides into primary SVF

Gangliosides were dried in glass tubes and resuspended in ITS (insulin, transferrin, selenium) medium by vortexing and sonication as described previously [11].

2.11. MβCD treatment of 3T3-L1

After differentiation of 3T3-L1 cells were incubated with 10 mM or 15 mM MβCD in serum-free medium for 30 min or 1 h at 37 °C. After washing, cells were cultured in ITS medium for 12 h, and cell lysates and supernatants were collected.

2.12. Statistical analysis

Values obtained in the experiments were examined with Student's *t* test. When *P* values were <0.05, they were considered significant.

3. Results

3.1. Reduced serum leptin levels in GD3S KO mice

To investigate the metabolism in whole body of GD3S KO mice, we analyzed serum samples from mice at the age of 15 and 30 weeks. Very low levels of leptin were found in GD3S KO mice

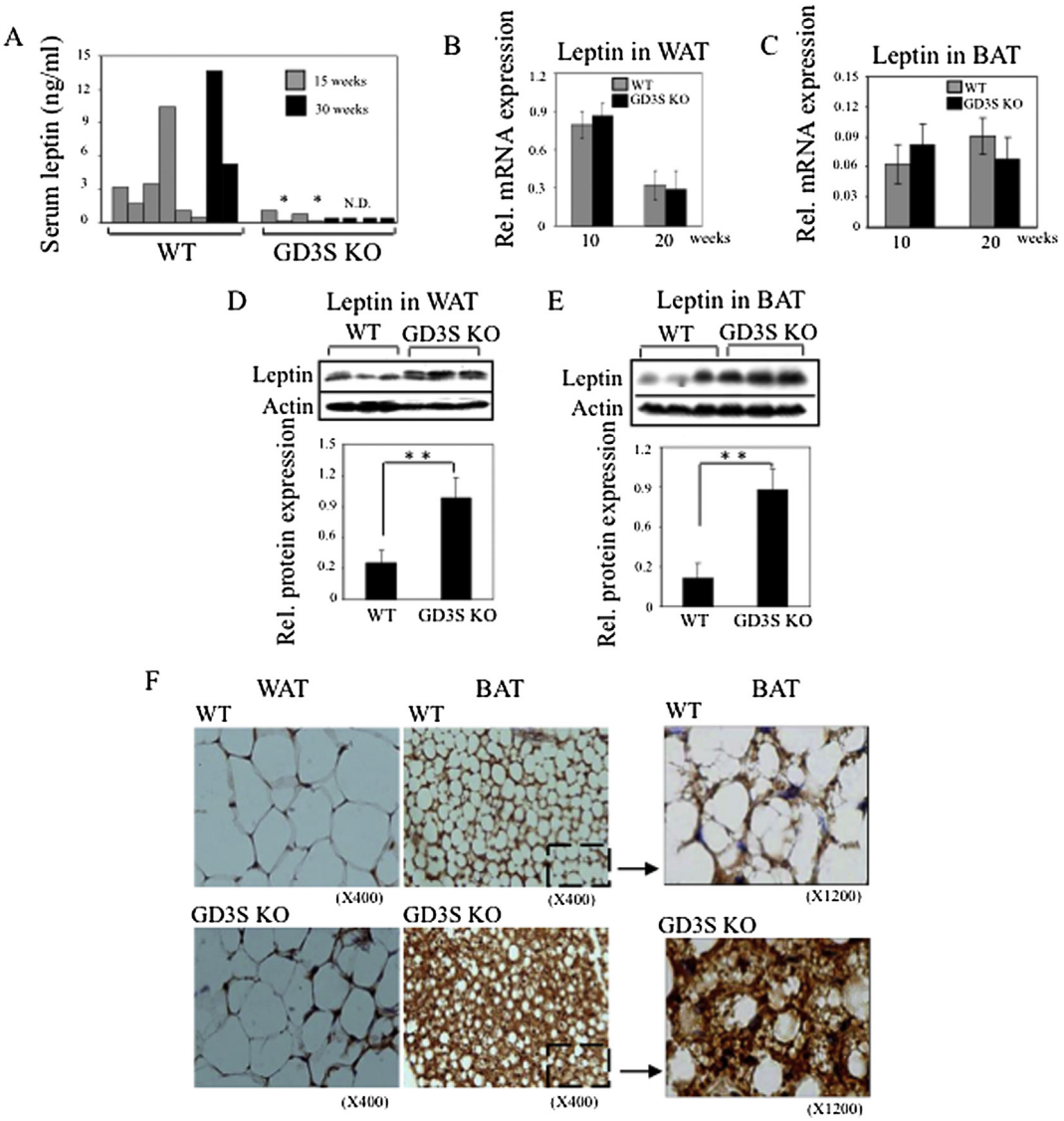

Fig. 1. Effects of lack of b-series gangliosides on leptin levels in sera, adipose tissues and cultured SVF cells. A, Serum levels of leptin were examined and results were presented in graphs. Fasting blood samples were collected at 12 h after removal of food. Numbers of 15 week and 30 week-old mice were 6 and 2 (*$p < 0.05$), respectively. N.D., not detectable. Leptin in sera was examined by FALCO biosystem (Kyoto, Japan). B–D, Accumulation of leptin in adipose tissues in GD3S KO mice due to impaired secretion. Gene expression levels of leptin in WAT (B) and BAT (C) were examined. WT and GD3S KO mice were at 10 and 20 weeks after birth. Protein levels of leptin in adipose tissues were examined. Results of Western blotting of leptin in WAT (D) and BAT (E) from WT and GD3S KO mice were shown. The intensity of bands was compared with that of actin. Samples were prepared from 15 week-old WT and GD3S KO mice. Results were presented as means ± S.D. (n = 3, **$p < 0.01$). F, Immunohistochemistry with an anti-leptin antibody of WAT and BAT from 15-week-old WT and GD3S KO mice (n = 3) was performed. HRP-labeled goat anti-rabbit secondary antibodies and ABC kit were used as described in Materials and methods. Magnifications are as indicated.

compared with WT mice (Fig. 1A). Then, we tried to elucidate the mechanism for low levels of leptin in blood of GD3S KO mice. Leptin, a product of *ob* gene, is produced by fat tissues to regulate appetite. Then, gene expression and protein levels of leptin in epididymal WAT and intercapular BAT were analyzed. Leptin protein levels were significantly higher in both WAT and BAT of GD3S KO mice (Fig. 1D and E), while mRNA levels were almost equivalent between WT and GD3S KO mice (Fig. 1B and C).

3.2. Leptin was accumulated in adipose tissues and in cultured adipose-derived SVF cells in GD3S KO mice

Since accumulation of leptin in adipose tissues was suspected, adipose-derived SVF cells were isolated from epididymal adipose tissues to examine the leptin accumulation *in vitro*. After differentiation, the SVF cells showed characters of adipocytes such as morphology change from spindle to round, and lipid accumulation was observed (Fig. S2). In cell lysates, high levels of leptin were detected in GD3S KO mice compared with WT mice (Fig. S3A). In the supernatants, leptin levels were decreased in GD3S KO mice (Fig. S3B). Consequently, accumulation of leptin was also observed in the cultured cells *in vitro*, while adiponectin showed no change in all these tests (Fig. S4). Immnohistochemistry using anti-leptin antibody also showed stronger staining of leptin in GD3S KO mice than in WT mice (Fig. 1F), suggesting impaired secretion of leptin in adipose tissues of GD3S KO mice.

3.3. b-series gangliosides restored leptin secretion in cultured adipose-derived SVF cells from GD3S KO mice

Ganglioside expression in adipocytes was analyzed using 3T3-L1 and SVF. For 3T3-L1 adipocytes, a-series gangliosides GM3, GM2,

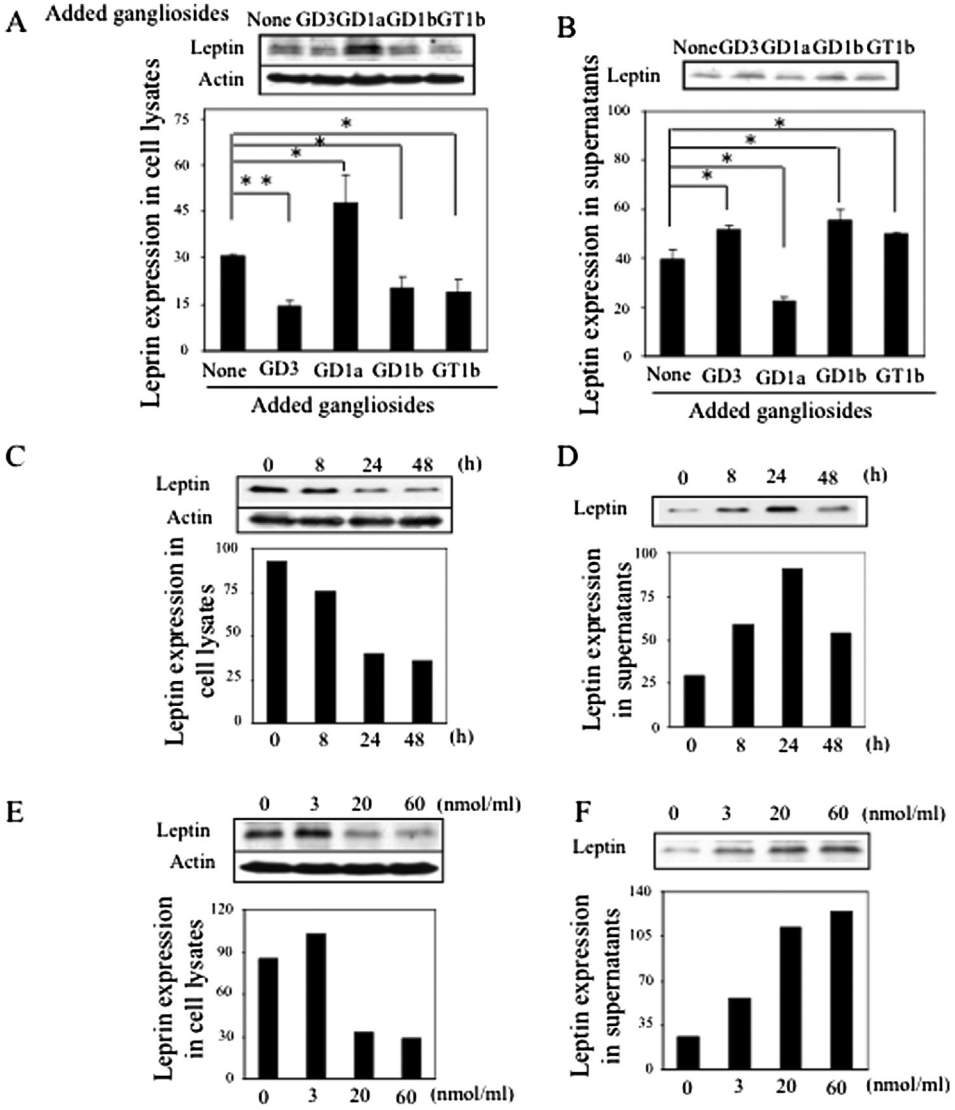

Fig. 2. Restoration of leptin accumulation by exogenous b-series gangliosides. A and B, Exogenous b-series gangliosides added to culture medium restored leptin secretion from cultured adipocytes. Gangliosides were added into culture medium of primary adipose-derived SVF from GD3S KO mice. When b-series gangliosides such as GD3, GD1b or GT1b were added to the cells (20 nmol/ml) and incubated for 18 h, the accumulation of leptin in cells was reduced (A). In turn, leptin secretion into supernatants was increased (B). Addition of GD1a resulted in further increase of accumulation in the cells and reduced secretion from cells. Levels of leptin protein in SVF cells from WT and GD3S KO mice were analyzed in Western immunoblotting. Bands were measured by Image J, and ratios to actin were presented as means ± S.D. ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$). C–F, Ganglioside GT1b increases leptin secretion in a dose and time dependent manner. After differentiation of adipose-derived SVF cells from GD3S KO mice, cells were cultured with GT1b-containing serum-free medium for 0, 8, 24 and 48 h (C, D). Serum-free medium with gradual dose of GT1b were also used to measure the leptin secretion (E, F). The cell lysates (E) and supernatants (F) were collected and leptin levels were measured by western blotting. The intensity of bands was measured by Image J.

GM1, GD1a, and b-series gangliosides GD1b, GT1b expression was observed (Fig. S5A). The SVF cells showed similar expression patterns to those of 3T3-L1 (Fig. S5B). Considering that the impaired secretion of leptin might be accounted for the lack of b-series gangliosides, gangliosides were added into culture medium of adipose-derived SVF cells from GD3S KO mice. When b-series gangliosides were added to the cells, the accumulation of leptin in cells was reduced (Fig. 2A), and the secretion into supernatants was increased (Fig. 2B). Addition of an a-series ganglioside, GD1a resulted in neither mitigation in leptin accumulation in the cells nor increase in the supernatants.

After addition of GT1b for 8–48 h, reduced accumulation of leptin was observed in cell lysates (Fig. 2C). In the supernatants, increased leptin seretion was observed from 8 to 24 h (Fig. 2D). Addition of gradual dose of GT1b (20 and 60 nmol/ml) resulted in the reduced leptin levels in the cells (Fig. 2E). In the supernatants, increased secretion of leptin was observed (Fig. 2F). Thus, GT1b enhanced leptin secretion in a dose and time dependent manner.

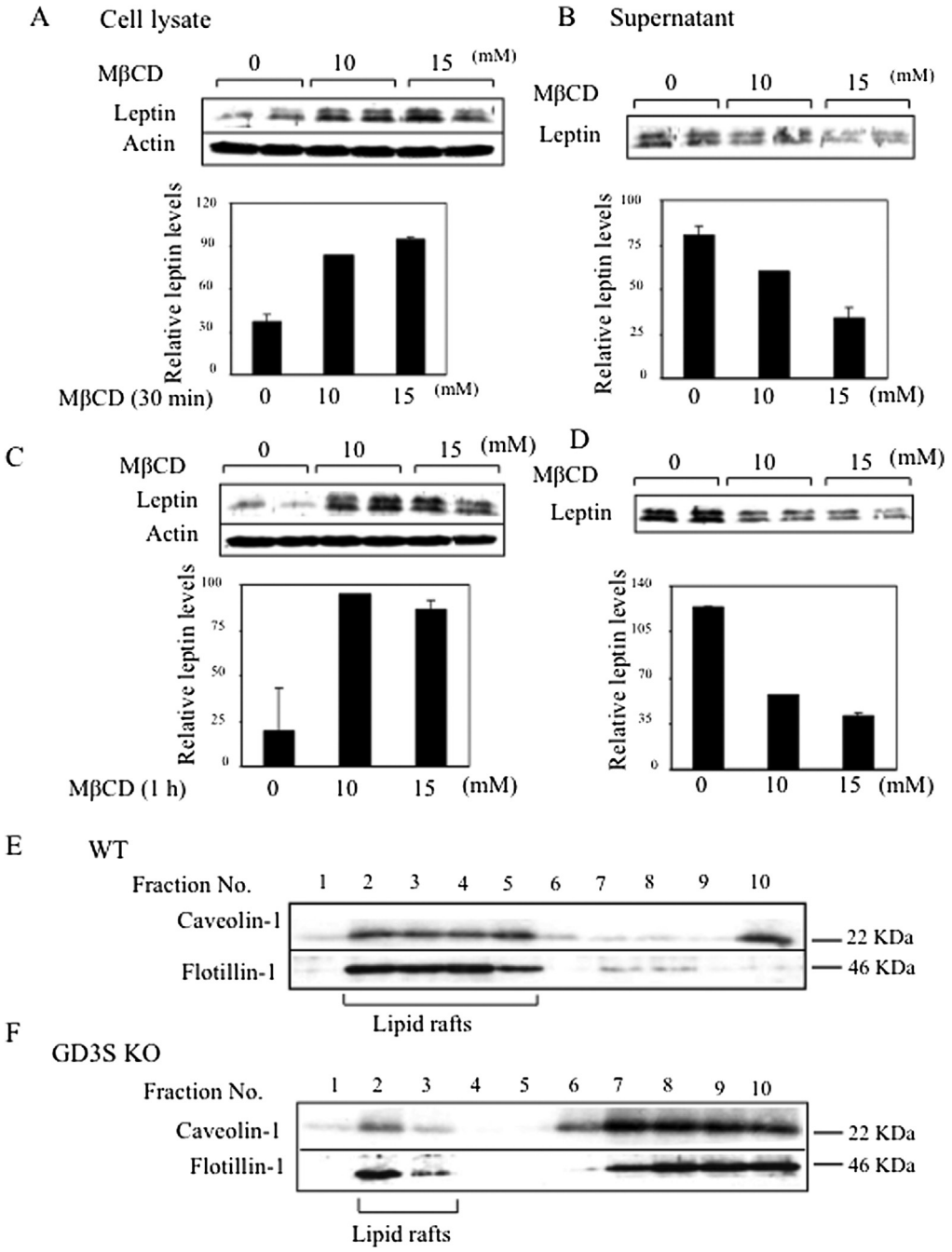

Fig. 3. Involvement of lipid rafts in the regulation of leptin secretion. A–D, Leptin secretion from 3T3-L1 cells was impaired by MβCD treatment. After differentiation, 3T3-L1 was cultured with 0, 10, 15 mM MβCD for 30 min (A, B) or 1 h (C, D), then the cell lysates and supernatants were served for immunoblotting. The intensities of bands were measured by Image J, and data were presented as ratios to actin. E and F, Altered distribution of raft markers in WAT of GD3S KO mice. Caveolin-1 and flotillin-1 in WAT from 23 week-old mice were analyzed by immunoblotting using fractions separated by sucrose density-gradient ultracentrifugation. Fractions were separated by 15% SDS-PAGE and analyzed by Western blotting using anti-caveolin-1 (×1000) and anti-flotillin-1 (×1000) antibodies.

3.4. Leptin secretion is regulated in lipid rafts, and lipid rafts are disordered in GD3S KO mice

The secretion pathway of leptin consists of ER, Golgi, secretion vesicles and final secretion through membrane, while definite pathway for vesicle transport to membrane of leptin has not been understood. Although cholesterol-enriched lipid rafts are present in adipocytes [12], no study has been reported on roles of lipid rafts in the secretion of leptin. To examine whether leptin secretion is regulated in lipid rafts, we applied MβCD for 30 min or 1 h on differentiated 3T3-L1 to deplete membrane cholesterol and disrupt lipid rafts, and leptin in cell lysates and supernatants were examined. Consequently, leptin secretion was impaired by MβCD in a dose and time dependent manner (Fig. 3A–D), suggesting that the secretion of leptin was regulated in lipid rafts.

Lipid rafts may be critical for leptin secretion, and its destruction might cause the accumulation of leptin. Utilizing natures of lipid rafts, i.e. insoluble properties in cold non-ionic detergent [13,14], distribution of lipid raft marker caveolin-1 and flotillin-1 was analyzed by preparing density-gradient fractions from WAT of 23-week-old mice. The results showed that distribution of caveolin-1 and flotillin-1 in GD3S KO mice markedly shifted from lipid rafts to non-rafts (Fig. 3E and F), suggesting the destruction of lipid raft in GD3S KO mice. Therefore, b-series gangliosides are considered to be essential for the maintenance of integrity of structure and function of lipid rafts.

3.5. Leptin and caveolin-1 were colocalized depending on gangliosides

In immunocytostaining of leptin and caveolin-1, merge of leptin and caveolin-1 was observed in adipocytes of WT mice (Fig. 4A, *upper*), while this colocalization disappeared in adipocytes of GD3S KO mice (Fig. 4A, *middle*). When b-series ganglioside GT1b was added to GD3S KO-derived cells, colocalization of leptin and caveolin-1 dramatically restored (Fig. 4A, *lower*). These results were quantified as shown in Fig. 4B. Taken together with results in Fig. 3, it is suggested that leptin secretion is regulated by b-series gangliosides in lipid rafts.

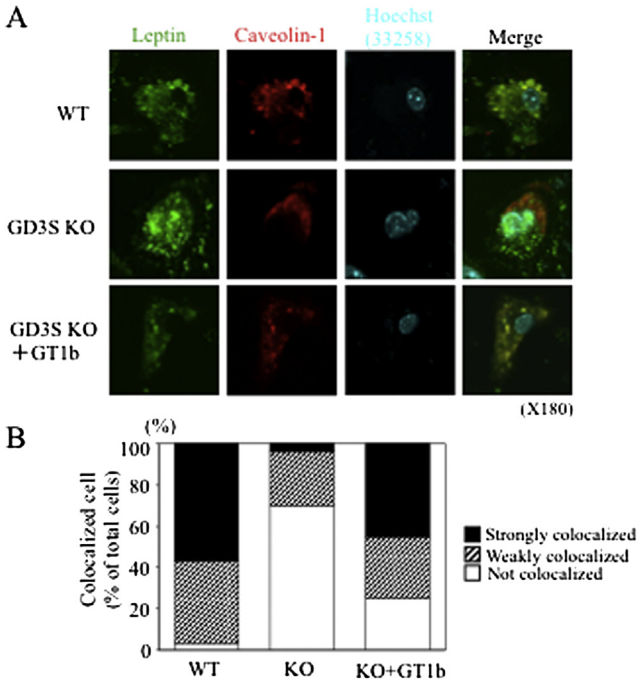

Fig. 4. Addition of ganglioside GT1b induced colocalization of leptin and caveolin-1 in adipocytes from GD3S KO mice. After differentiation of SVF cells from WT and GD3S KO mice, double staining of leptin and caveolin-1 was done. Cells were fixed with 4% PFA, and blocking was performed by Protein Block Serum-Free™ (Dako, Carpinteria, CA) for 60 min at room temperature. Then, samples were incubated with anti-caveolin-1 antibody (1:200) or anti-leptin-antibody (1:400) for 60 min. Goat anti-mouse Alexa568 and goat anti-rabbit Alexa488 were used as secondary antibodies. Nuclei were labeled with Hoechst33258. A, Double staining of caveolin-1 and leptin in WT adipocytes showed their colocalization (*upper*), while GD3S KO adipocytes showed separated staining (*middle*). When GD3S KO mice-derived adipocytes were treated with GT1b (20 nmol/ml), colocalization of leptin and caveolin-1 was restored (*lower*). These results were quantified and shown in B.

4. Discussion

As for expression of gangliosides in adipose tissues, few studies have been performed [15], and no information on glycolipids in cultured adipocytes and differentiated adipocytes were available. To our surprise, leptin secretion was disturbed by deficiency of b-series gangliosides, and addition of b-series gangliosides (not of a-series gangliosides) virtually resulted in the restoration of leptin secretion from cultured adipocytes in a time and dose-dependent manner. Although no precise mechanisms are known at this moment, gangliosides should be involved in the regulation of leptin secretion via lipid rafts. This is the first report on the role of gangliosides in the leptin secretion.

Immunocytochemistry and biochemistry studies revealed that soluble protein hormones are concentrated into secretory granules by specific secretory granule cargo proteins [16], and small GTPases and SNARE proteins have been considered to be involved in the exocytosis of peptide hormone vesicles [17]. Particularly, insulin-granule exocytosis via the SNARE system has been rigorously analyzed [18]. In fact, SNARE proteins are highly enriched in lipid rafts indicating their implication for the spatial control of exocytosis [19]. It was also demonstrated that adipocytes continuously synthesize and secrete leptin via a rough endoplasmic reticulum-Golgi secretory vesicles pathway [20]. Taken together, it seems likely that leptin secretion is exerted under regulation of gangliosides with secretory vesicles and exocytosis machinery such as SNARE proteins.

Our results of immunocytostaining for leptin and caveolin-1 suggested that b-series gangliosides are essential for the association of leptin with lipid rafts and resulting secretion. Involvement of GEM/rafts in the fusion of leptin-containing granules to plasma membrane will be clarified in the future. In this case, whether only leptin secretion is regulated by gangliosides may be an intriguing issue to be clarified.

There have been a number of studies indicating that gangliosides modulate functions of various membrane receptors such as adhesion receptor integrins [21], platelet-derived growth factor receptors [22], nerve growth factor receptors [23], and insulin receptor [24].

All these results suggest that complex gangliosides regulate leptin secretion from adipose tissues. Although GD3S KO mice showed low levels of serum leptin, they were not obese. Although they showed activated BAT features (data not shown), their body temperature and energy expenditure were almost equivalent with WT mice. Our results on responses of GD3S KO mice to HF/HS feeding and energy expenditure showed that GD3S KO mice showed almost similar results (Fig. S6), suggesting that they can adapt to some extent to the nutritional environments. How they regulate energy expenditure under low serum leptin levels is a quite intriguing issue to be investigated. Whether differential regulatory function of complex gangliosides in distinct organs lead apparently normal and balanced conditions, or some

compensational mechanisms lying under these phenomena affect remains to be clarified.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.143.

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