主論文の要旨

b-series gangliosides crucially regulate leptin secretion in adipose tissues

（b 系列ガングリオシドは脂肪組織のレプチン分泌に重要な調節作用を示す）

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Introduction

GD3 synthase gene knockout (GD3S KO) mice lacking all b-series gangliosides showed no apparent abnormalities except disturbed neuroregeneration of lesioned hypoglossal nerves, suggesting that remaining a-series gangliosides could replace the major 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. Lack of leptin and/or leptin receptor causes obesity. 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.

Materials and methods

1. Mice

The mutant mice used in this study were generated and maintained in our laboratory. Wild type (WT) and GD3S KO mice were mated, and resultant heterozygotes were mated each other.

2. Primary Culture of Stromal Vascular Fractions (SVF)

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.

3. Differentiation of SVF and 3T3-L1

Serum-free medium consisted of DMEM/F-12 Ham, 1% penicillin and streptomycin. Induction medium consisted of serum-free medium, 5% FBS, insulin, dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin. Cells were first cultured in serum-free medium for 24 h, then in the induction medium for 48 h, and in insulin-containing medium for 48 h. The cells began to accumulate lipids and to exhibit a morphology resembling mature adipocytes.

4. Preparation of GEM/rafts Fractions

Tissues were destroyed with a nitrogen cavitation apparatus, followed by removal of nucleus and cell debris by centrifugation. The supernatant was centrifugated using a Beckman MLS50 rotor. Then, insoluble materials were resuspended in 1 ml lysis buffer, mixed with an equal volume of 80% sucrose in MNE buffer, 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, fractions of 500 µl were separated from the top of the gradient,

and were used for Western blotting.

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) with Protease Inhibitor Mixture™ 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 8,000 × *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™.

6. Western blotting

Lysates from cells and tissues and supernatants of cell culture were separated by SDS-PAGE. The separated proteins were transferred onto an Immunobilon-P™ membrane, and blocked with 5% skim milk in PBS. The membranes were first probed with primary antibodies. After washing, the blots were incubated with goat anti-rabbit IgGs or goat anti-mouse IgGs conjugated with horseradish peroxidase (HRP). Bound conjugates were visualized with an Enhanced Chemiluminescence™ (ECL) detection system and analyzed using LAS3000™ image analysis system.

7. Incorporation of Gangliosides into Primary SVF

Gangliosides were dried in glass tubes and resuspended in ITS (insulin, transferrin, selenium) or serum free medium by vortexing and sonication.

8. MβCD Treatment of 3T3-L1

After differentiation of 3T3-L1 cells were incubated with MβCD (methyl-beta-cyclodextrin). After washing, cells were cultured in ITS medium for 12 h, and cell lysates and supernatants were collected.

9. Real Time RT-PCR

Primers used for RT-PCR were designed according to Oligo4.0™. Cells and tissues were homogenized in Trizol™ reagent. Four μg of RNA was reverse-transcribed using M-MLV RT™. PCR was performed on a CFX connect™ Real-Time System by adding 2 ng sample of cDNA to the commercially available SsoAdvanced SYBR Green Supermix™. The mRNA expression levels were normalized by GAPDH mRNA.

10. Immunohistochemistry and immunocytochemistry

Deparaffinized sections were treated with 0.3% hydrogen peroxide (H_2O_2) in absolute methanol to block endogenous peroxidase. After blocking with Protein Block

Serum-Free™, the sections were incubated with anti-leptin antibody. Biotinylated secondary antibody was then applied. The sections were incubated with peroxidase-conjugated streptavidin, and the reaction products were visualized using 3,3′-diamino- benzidine. Nuclear counterstaining was performed using hematoxylin. Cells were fixed with 4% PFA, and blocking was performed by Protein Block Serum-Free™. Then, samples were incubated with anti-caveolin-1 antibody or anti-leptin-antibody. Goat anti-mouse Alexa568 and goat anti-rabbit Alexa488 were used as secondary antibodies. Nuclei were labeled with Hoechst33258. Sections were observed by light microscopy or confocal fluorescence microscopy. Negative control was prepared without primary antibodies.

11. Statistical Analysis

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

Results

Genetic deletion of b-series gangliosides resulted in the marked reduction of serum leptin (Fig.1 A). Expression analysis of leptin revealed that leptin accumulated in the adipose tissues of GD3S KO mice (Fig.1 B-F). 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 (Fig.1 G, H). Interestingly, addition of b-series gangliosides to the culture medium of differentiated SVF resulted in the restoration of leptin secretion (Fig.2 A, B) in time and dose dependent manner (Fig. 2 C-F). Results of methyl-β-cyclodextrin treatment of differentiated 3T3-L1 (Fig. 3 A-D) as well as isolation of GEM/rafts of adipose tissues suggested leptin secretion was regulated by lipid raft and which was destructed in GD3S KO mice (Fig.3 E, F). Immunocytostaining of leptin and caveolin-1 revealed collocation of them when gangliosides existed, suggesting that b-series gangliosides regulate the leptin secretion from adipose tissues in lipid rafts (Fig.4 A, B).

Discussion

As for expression of gangliosides in adipose tissues, few studies have been performed, and no information on glycolipid functions 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 have revealed that soluble protein hormones are concentrated into secretory granules by specific secretory granule cargo proteins, and small GTPases and SNARE proteins have been considered to be involved in the exocytosis of peptide hormone vesicles. It was also demonstrated that adipocytes continuously synthesize and secrete leptin via a rough endoplasmic reticulum-Golgi secretory vesicles pathway. 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.

Conclusion

Results of adipose tissues analysis using GD3S KO mice suggest that b-series gangliosides crucially regulate leptin secretion from adipose tissues in lipid rafts.