Supplementary Information

Materials and methods

Animals

This study was approved by the Animal Care Committee of Nara Women's University. Six-week-old male rats (SLC: Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). Diabetes was induced in overnight-fasted rats by intraperitoneal injection of STZ at a dose of 40 mg/kg body weight dissolved in citrate buffer (0.1 M, pH 4.5). Control rats received an equivalent volume of the buffer alone. Rats were fed a commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum for 2 and 8 weeks (wk).

Analytical methods

Two and 8 wk after STZ administration, rats were anaesthetized with diethyl ether and killed by exsanguination of the inferior vena cava using the anticoagulant sodium heparin. After perfusion of ice-cooled saline through the portal vein, the liver and kidney were removed. Plasma glucose levels, plasma aspartate aminotransferase (AST: EC 2.6.1.1) and alanine aminotransferase (ALT: EC 2.6.1.2) activities, and blood urea nitrogen (BUN) levels were measured using commercial kits (Wako Pure Chemical Industries, Osaka, Japan). Vitamin C level was determined by a sensitive method involving chemical derivatization and HPLC.[1]

Measurement of ceramide species

Lipids from each tissue were extracted according to the method of Folch *et al*.[2] Quantitative measurements of ceramide species was made by LC-MS/MS (Finnigan MAT TSQ 7000) as described previously.[14]

Assay of SMase activity

The enzyme assay of SMase (EC 3.1.4.12) was performed as described previously.[3] The excised tissues were homogenized in 3 volumes of ice-cold phosphate-buffered saline (10 mM, pH 7.4). Protein concentrations were determined according to the method of Lowry *et al.*[4] The homogenate (10-20 mg protein/ml) was dissolved in 300 µl of 0.2% Triton X-100 containing 10 mM Tris, pH 7.4 (supplemented with 25 µM genistein) for 10 min on ice. Nitrobenzofurazan (NBD) C_6-SM (Molecular Probes Inc., Eugene, OR, USA) was added to the lysates to achieve a final concentration of 20 µM and the lysates were incubated at 4°C for 10 min. The lysates were added to a solution of 5 mM $MgCl_2$ in 10 mM Tris pH 7.4 for nSMase or 0.5 M acetate buffer pH 4.5 for the aSMase and the final volume was made 300 µl. Incubation was performed for 1 h at 37°C. For sSMase analysis, the 200-µl assay mixture consisted of 10 µl of a 1:20 dilution of the plasma in an assay buffer (0.1 mM Zn^{2+} sulphate, 1 nmol NBD C_6-SM, 0.1% NP-40/62, 0.1 M sodium acetate, pH 5.0), which was incubated for 1 h at 37°C. The reaction was stopped by adding 1 ml of methanol. Lipids were extracted according to the method of Bligh and Dyer. The samples (20 µl each) were directly analyzed by HPLC analysis, which was performed using a Nova Pak 4 µm C18 column (3.9 × 150 mm, Waters). Elution was performed at a flow rate of 1 ml/min with a mixture of water, acetonitrile, and phosphoric acid at a volume ratio of 35:65:0.2. The fluorescence of NBD in these compounds was determined using a fluorescence detector (Shimadzu, RF-10AXL, excitation at 466 nm and emission at 536 nm).

Statistical analysis

The data are expressed as means ± SEM. Differences between group means were considered significant at $P < 0.05$ using Fisher's protected least significant difference test.

References and Notes

1 E. Kishida, Y. Nishimoto and S. Kojo, *Anal. Chem.* 1992, **64**, 1505-1507.

2 J. Folch, I. Ascoli, M. Lees, J. A. Meath and N. LeBaron, *J. Biol. Chem.* 1966, **191**, 833-841.

3 S. A. Lightle, J. I. Oakley and M. N. Nikolova-Karakashian, *Mech. Ageing Dev.* 2000, **120**, 111-125.

4 O. H. Lowry, N. J. Rosebrough, A. L. Farr and J. Randall, *J. Biol. Chem.* 1951, **19**, 265-275.