

Correction

In an article by Katagiri et al (*Circ Res.* 2007;101:27–39), the authors incorrectly cited reference 4. The correct reference appears below. The corrected article is now available at <http://circres.ahajournals.org>.

4. International Diabetes Federation. The IDF consensus worldwide definition of the metabolic syndrome. April 14, 2005: http://www.idf.org/webdata/docs/Metac_syndrome_def.pdf.

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Increased hepatic expression of ganglioside-specific sialidase, *NEU3*, improves insulin sensitivity and glucose tolerance in mice

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Abstract

Membrane microdomains rich in gangliosides are recognized as being critical for proper compartmentalization of insulin signaling. Plasma membrane-associated sialidase, *NEU3*, is a key enzyme for ganglioside hydrolysis. We previously reported that mice overexpressing *NEU3* mainly in muscles developed severe insulin-resistant diabetes. To examine the possible contributions of *NEU3* to in vivo insulin sensitivity and glucose tolerance, *NEU3* was expressed by using adenoviral vectors in the livers of C57BL/6 mice on standard and high-fat diets, and insulin-resistant KKAy mice on standard diets. Hepatic *NEU3* overexpression paradoxically improved glucose tolerance and insulin sensitivity in the C57BL/6 mice fed standard diets, and glucose tolerance in the C57BL/6 mice fed high-fat diets and in KKAy mice. Hepatic *NEU3* overexpression increased hepatic glycogen deposition and triglyceride accumulation, and enhanced the hepatic peroxisome proliferator-activated receptor γ and fetuin expression in the C57BL/6 mice on standard and high-fat diets, and in KKAy mice. Thin-layer chromatographic analysis demonstrated increased levels of GM1 and markedly reduced GM3 in the livers of mice with hepatic *NEU3* overexpression (*NEU3* mice). Basal and insulin-stimulated tyrosine phosphorylations of insulin receptor substrate 1 were significantly increased, but tyrosine phosphorylations of the insulin receptor and insulin receptor substrate 2 in the *NEU3* liver were unchanged. Insulin-stimulated tyrosine phosphorylations of the insulin receptor were increased in adipose tissues of *NEU3* mice. These results suggest that hepatic *NEU3* overexpression improves insulin sensitivity and glucose tolerance through modification of ganglioside composition and peroxisome proliferator-activated receptor γ signaling. Our findings also provide further evidence that *NEU3* is an important regulator of insulin sensitivity and glucose tolerance.

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1. Introduction

Gangliosides are a family of sialic acid-containing glycosphingolipids present on cell surface membranes. Several lines of evidence suggest their functional roles in regulating a wide range of biological processes including cell growth, cell differentiation, and transmembrane signaling [1-4]. Gangliosides are involved in cell-signaling functions as ligands and as modulators of receptor activity

[3,5-7]. Sialylparagloboside and the structurally related GM3 ganglioside have been found to inhibit the intrinsic tyrosine kinase activity of soluble insulin receptors [8,9]. Furthermore, GM3 ganglioside depressed insulin-mediated signaling in cultured cells [9,10].

Mice with disrupted *GM3S* gene, which encodes GM3 synthase, such that they lack the capacity to synthesize GM3 ganglioside, had enhanced tyrosine phosphorylation of the skeletal muscle insulin receptor after ligand binding and showed improved responses on glucose and insulin tolerance tests [11]. The *GM3S* knockout (KO) mice were protected from high-fat diet-induced insulin resistance [11]. Insulin resistance with uncoupling insulin receptor activity

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against insulin receptor substrate 1 (IRS-1), which was induced by tumor necrosis factor α in 3T3-L1 adipocytes, was reportedly accompanied by increased GM3 expression caused by elevations of GM3 synthase activity and its messenger RNA (mRNA) [9,12]. When the adipocytes were incubated with exogenous GM3, tyrosine phosphorylation of insulin receptors and IRS-1, as well as glucose uptake in response to insulin stimulation, were suppressed. Obese Zucker fa/fa rats and ob/ob mice, models of insulin resistance, had significantly higher levels of GM3 synthase mRNA in adipose tissues than their lean counterparts [9].

Plasma membrane-associated sialidase, *NEU3*, is a key enzyme for ganglioside hydrolysis [13,14]. Membrane microdomains rich in gangliosides are now recognized as being critical for proper compartmentalization of insulin signaling [4,6,15]. We generated mice overexpressing *NEU3* and reported that these mice developed a diabetic phenotype associated with hyperinsulinemia, islet hyperplasia, and increased beta cell mass [16].

To investigate the role of *NEU3* in insulin and glucose metabolism, we investigated the effects of hepatic *NEU3* overexpression on in vivo glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard and high-fat diets, as well as on KKAY mice receiving a standard diet. Our results show that hepatic overexpression of *NEU3* improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diets, as well as glucose tolerance in C57BL/6 mice fed high-fat diets and in KKAY mice. Possible molecular mechanisms underlying improved glucose tolerance and insulin signaling are discussed.

2. Methods

2.1. Preparation of recombinant adenovirus

Adenoviral-mediated gene transfer was used to overexpress the human *NEU3* (*hNEU3*) gene in the livers of male mice. The active adenovirus (Ad*NEU3*) was constructed by using the AdEasy system (Stratagene, La Jolla, CA) and contained the human *NEU3* complementary DNA (cDNA) and a short hemagglutinin (HA) tag sequence under the control of cytomegalovirus (CMV) promoters. The adenovirus bearing the bacterial β -galactosidase gene (AdLacZ) was used as a control.

2.2. Animals

Male C57BL/6 mice were housed individually and given free access to a standard diet (65% carbohydrate, 4% fat, and 24% protein) or a high-fat diet (Quick Fat; 60.2% carbohydrate, 15.3% fat, and 24.5% protein; Clea Japan, Tokyo, Japan), starting at 5 weeks of age, for 5 weeks. KKAY mice also were housed individually and given free access to a standard diet. Four weeks after separation, the body weight-matched C57BL/6 mice and KKAY mice received a single dose of Ad*NEU3* or AdLacZ adenoviruses at a dose of 4×10^8 plaque-forming units by tail-vein injection, resulting in liver-specific infection. Animal study

protocols were in accordance with the institutional guidelines for animal experiments at Tohoku University.

2.3. Triglyceride and glycogen contents of the liver

Frozen livers were homogenized, and triglycerides were extracted with $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, vol/vol), dried, and resuspended in 2-propanol [17]. Triglyceride contents were measured using Lipidos liquid (TOYOBO, Osaka, Japan). Glycogen was isolated from 30 to 50 mg of frozen liver by dissolving the tissue in 30% potassium hydroxide saturated with sodium sulfate for 30 minutes at 100°C, followed by ethanol precipitation. Glycogen content was determined by the phenol-sulfuric acid spectrophotometric method at 490 nm [18] and expressed as micrograms of glycogen per milligram of liver.

2.4. Histologic analysis

Livers were removed from the mice on day 7 after adenovirus injection, fixed with 10% formalin, then embedded in paraffin. Tissue sections were stained with periodic acid-Schiff (PAS) and Oil Red O.

2.5. Tyrosine phosphorylation of the insulin receptor, IRS1, and IRS2

Insulin receptor antibody and anti-IRS2 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Affinity-purified antibody against IRS1 was prepared as described previously [19].

Mice that had been fasted for 16 hours received an injection of 100 μL of normal saline (0.9% NaCl) with or without insulin (standard diets, 2 U/kg body weight; high-fat diets, 10 U/kg body weight), via the tail vein [20]. Liver, epididymal fat tissues and hind-limb muscles were removed 300 seconds later and immediately homogenized. After centrifugation, the resultant supernatants were used for immunoprecipitation with anti-insulin receptor, anti-IRS1, or anti-IRS2 antibody. Immunoprecipitates were boiled in Laemmli buffer containing 10 mmol/L dithiothreitol and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then immunoblotted with antiphosphotyrosine antibody (4G10) as described previously [19]. The immunoblots were visualized with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.6. Blood analysis

Blood glucose was assayed with Glucose Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Serum insulin and leptin were determined with enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Yokohama, Japan). Serum adiponectin concentrations were measured with an ELISA kit (Ohtsuka Pharmaceutical, Tokyo, Japan). Serum total cholesterol, triglyceride, and free fatty acid (FFA) concentrations were determined with Cholescolor liquid, Lipidos liquid (TOYOBO), and NEFA C (Wako Pure Chemical, Osaka, Japan) kits, respectively.

2.7. Glucose and insulin tolerance tests

Glucose tolerance and insulin sensitivity were assessed with intraperitoneal glucose tolerance and insulin tolerance tests. Glucose tolerance tests were performed on fasted (16 hours) mice. Mice were given intraperitoneal glucose (2 g/kg body weight), and blood glucose was assayed immediately before and at 15, 30, 60, and 120 minutes after administration. Intraperitoneal insulin tolerance tests were performed on mice fed ad libitum. Mice received an injection of human regular insulin (0.75 U/kg body weight; Eli Lilly, Kobe, Japan) into the intraperitoneal space, and blood glucose was assayed immediately before and at 20, 40, 60, and 80 minutes after injection.

2.8. Quantitative reverse transcription-polymerase chain reaction-based peroxisome proliferator-activated receptor γ and fetuin gene expression

Total RNA was isolated from 0.1 g of mouse hepatic tissue with ISOGEN (Wako Pure Chemical), and cDNA synthesis was performed with a Cloned AMV First Strand Synthesis Kit (Invitrogen, Rockville, MD) using 5 μ g of total RNA. cDNA synthesized from total RNA was evaluated in a real-time polymerase chain reaction quantitative system (Light Cycler Quick System 350S, Roche Diagnostics, Mannheim, Germany).

2.9. Ganglioside-specific sialidase activity assays

Crude homogenates from livers were used for sialidase activity assays with mixed gangliosides (Sigma-Aldrich, St Louis, MO) as substrates in the presence of Triton X-100 as described previously [21,22]. One unit of sialidase was defined as the amount of enzyme catalyzing the release of 1 nmol of sialic acid per hour.

2.10. Glycolipid analysis by thin-layer chromatography

Glycolipids were extracted from mouse tissues as described elsewhere [23,24] and fractionated by thin-layer

chromatography (TLC) on high-performance TLC plates (J. T. Baker, Phillipsburg, NJ) in chloroform-methanol-0.5% CaCl₂ (60:40:9, vol/vol/vol). Each lane of the plate was loaded with the equivalent of 6 mg, wet weight, of liver tissues, and then was visualized with orcinol-H₂SO₄.

2.11. Statistical Analysis

The statistical significance of differences was assessed by the unpaired Student *t* test. A *P* value of <.05 was considered significant.

3. Results

3.1. Adenovirus-mediated NEU3 expression in the liver improved glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard diets

The NEU3 adenovirus vector was administered intravenously to C57BL/6 mice on the standard diet (AdNEU3 mice). Mice given the LacZ adenovirus were used as controls (AdLacZ mice). Compared with the controls, the AdNEU3 mice had significantly higher hepatic contents and enzymatic activities of NEU3 at 7 days after infection (Fig. 1A and B). HA-tagged NEU3 was not detected in skeletal muscles, adipose tissues, heart, and kidney by Western blotting with anti-HA antibody (data not shown). There were no changes in NEU3 enzymatic activities in skeletal muscle and adipose tissues (data not shown). Thin-layer chromatography demonstrated increases in GM1 and a decrease in GM3 in the livers of AdNEU3 mice compared with those of AdLacZ mice (Fig. 1C).

Food intakes of AdNEU3 mice were significantly lower than those of AdLacZ mice from the 4th through the 7th days after adenovirus injection (Fig. 2A). As a result, the body weights on the 7th and 14th days were significantly decreased in AdNEU3 mice, as compared with AdLacZ mice (Fig. 2B). Blood glucose levels in the ad libitum-fed state were markedly lower in AdNEU3 mice than in control

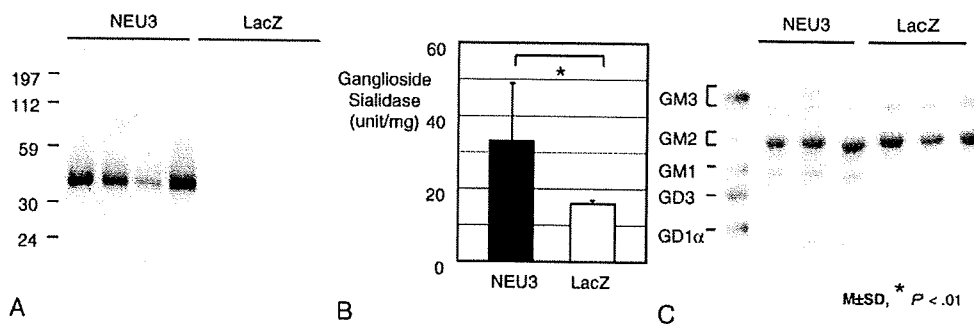


Fig. 1. Hepatic NEU3 overexpression and ganglioside composition changes in the livers: Western blotting of NEU3 (A), ganglioside-specific sialidase activity (B), and TLC analysis of gangliosides (C). C57BL/6 male mice fed standard diets were injected with 4×10^8 plaque-forming units of adenovirus-containing alpha-galactosidase (AdLacZ) or human NEU3 (AdNEU3) construct via the tail vein. Mice were killed on day 7 after adenoviral injection, and liver samples were collected. A, Western blotting of liver lysates. Liver lysates (45 μ g) from AdLacZ or AdNEU3 mice ($n = 4$) were electrophoresed and immunoblotted with anti-HA antibody (Roche Applied Science, Tokyo, Japan). B, Sialidase activity in liver homogenates was assayed with mixed gangliosides as substrates in the presence of Triton X-100. Similar representative results were obtained from 3 or more experiments, and the data are presented as means \pm SD of the 4 mice of each group. **P* < .05, assessed by unpaired *t* test. C, Thin-layer chromatography of glycolipids from the livers of AdLacZ and AdNEU3 mice ($n = 3$). Representative immunoblots and TLC analysis data are presented.

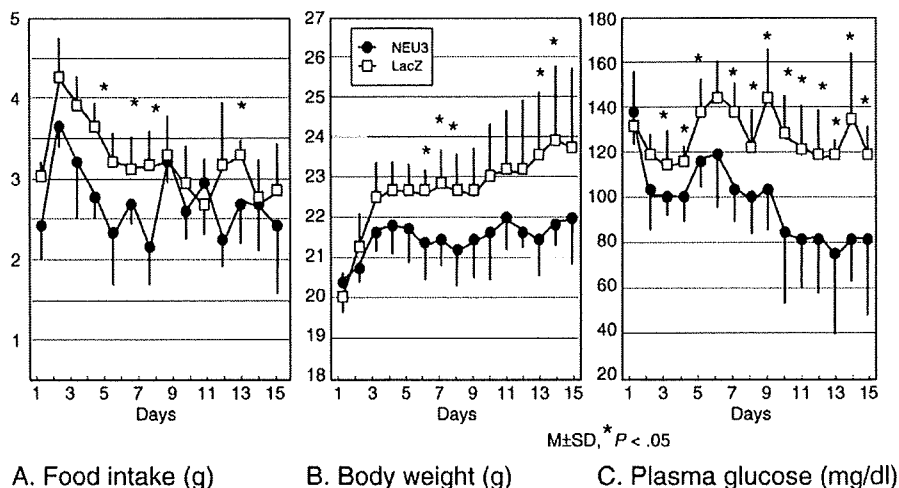


Fig. 2. Hepatic *NEU3* expression reduced food feeding (A), body weights (B), and blood glucose concentrations (C) in C57BL/6 mice fed standard diets. Food feeding (A), body weights (B), and blood glucose levels (C) in the ad libitum-fed state were measured every day after adenoviral administration in AdLacZ (squares) and AdNEU3 mice (circles) for 2 weeks ($n = 5$ per each group). Similar representative results were obtained from 3 or more experiments, and the data are presented as means \pm SD. **P* < .05, assessed by unpaired *t* test.

mice from the 3rd to the 15th day after adenovirus injection (Fig. 2C).

On day 4 after adenovirus injection, glucose tolerance tests, ie, intraperitoneal injection of 2 g glucose per kilogram body weight, were performed on C57BL/6 mice that had been fasted overnight. As shown in Fig. 3A, the fasting blood glucose levels did not differ significantly between the AdLacZ and AdNEU3 groups. However, after a glucose load, blood glucose levels were significantly reduced in AdNEU3 mice after 15, 30, 60, and 120 minutes (*P* < .05). Fasting insulin levels did not differ significantly between the AdLacZ and AdNEU3 groups, although plasma insulin levels

were significantly reduced in the AdNEU3 mice after 30 minutes (*P* < .05). These results suggest that hepatic *NEU3* expression improved glucose tolerance, possibly through increased insulin sensitivity, after 4 days of administration.

Insulin tolerance tests (0.75 U/kg body weight) were performed in mice fed ad libitum on day 5 after adenovirus injection. The glucose-lowering effect of insulin was significantly improved in the AdNEU3 mice at 20, 40, and 60 minutes after insulin injection, as compared with the AdLacZ mice, further confirming that hepatic *NEU3* over-expression significantly improves insulin sensitivity in C57BL/6 mice fed standard diets.

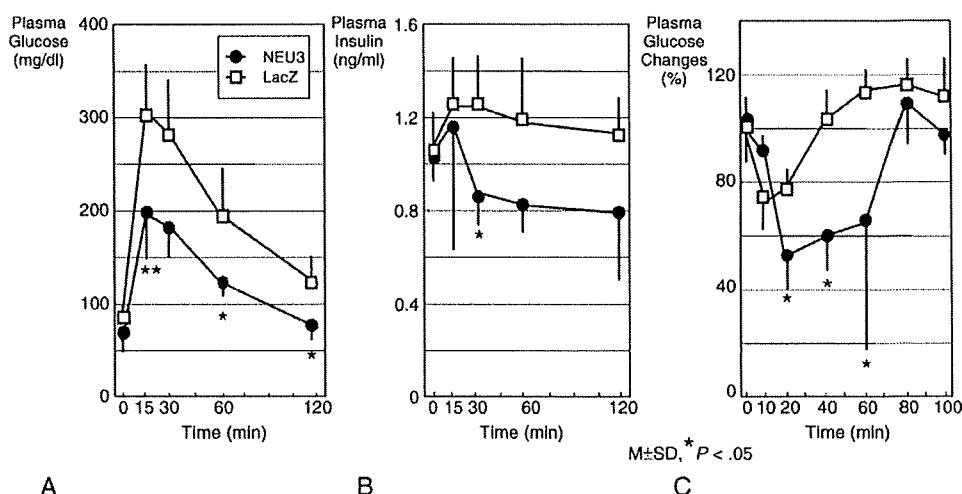
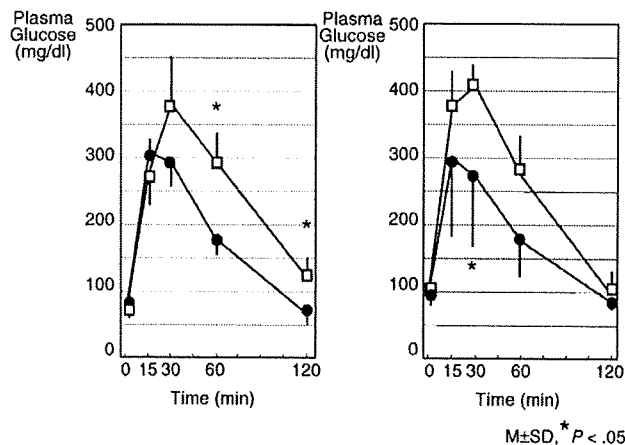


Fig. 3. Hepatic *NEU3* expression improved glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard diets. A and B, Glucose tolerance test. AdLacZ (squares, $n = 4$) or AdNEU3 mice (circles, $n = 4$) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose levels (A) and insulin levels (B) are determined. C, Insulin tolerance test. AdLacZ (squares, $n = 4$) and AdNEU3 mice (circles, $n = 4$) fed ad libitum were intraperitoneally challenged with 0.75 U/kg insulin, and blood glucose levels were determined. Data were expressed as percentages of blood glucose levels immediately before intraperitoneal insulin load. Similar representative results were obtained from 3 or more experiments, and the data are presented as means \pm SD. **P* < .05, assessed by unpaired *t* test.



A. High fat diets B. KKAY mice

Fig. 4. Hepatic *NEU3* overexpression improved glucose tolerance tests in C57BL/6 mice fed high-fat diets (A) and KKAY mice (B). A and B, glucose tolerance test. AdLacZ (squares, $n = 4$) or AdNEU3 mice (circles, $n = 4$) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose levels were determined. Significant difference from the AdLacZ value is shown. Data are mean \pm SD. * $P < .05$, assessed by unpaired t test.

3.2. *NEU3* overexpression improved glucose tolerance in C57BL/6 mice on high-fat diets and in KKAY mice

When AdNEU3 was administered to the C57BL/6 mice fed high-fat diets, there were no significant differ-

ences in food intake or body weight between AdNEU3 and AdLacZ mice (data not shown). However, blood glucose levels in the ad libitum-fed state were markedly decreased in AdNEU3 mice as compared with AdLacZ mice from the 3rd to 15th days after adenovirus injection (data not shown).

Glucose tolerance tests on mice fed high-fat diets demonstrated blood glucose levels to be significantly lower in AdNEU3 than in AdLacZ mice after 60 and 120 minutes (Fig. 4A). AdNEU3 treatment also improved glucose tolerance in KKAY mice (Fig. 4B). These findings indicate that hepatic *NEU3* expression exerted therapeutic effects on diabetic animal models with diet-induced obesity.

3.3. *NEU3* overexpression increased hepatic triglyceride and glycogen accumulation and induced hyperlipidemia

Hepatic overexpression of *NEU3* increased liver weight and hepatic triglyceride deposition in C57BL/6 mice fed standard diets (Fig. 5A and B). AdNEU3 mice had significantly higher hepatic glycogen deposition (Fig. 5C). *NEU3* overexpression also promoted hepatomegaly and triglyceride deposition in the livers of C57BL/6 mice on high-fat diets and of KKAY mice (data not shown). Hepatic *NEU3* overexpression increased plasma triglyceride and total cholesterol concentrations in C57BL/6 mice fed standard diets (Fig. 5D and E). However, there

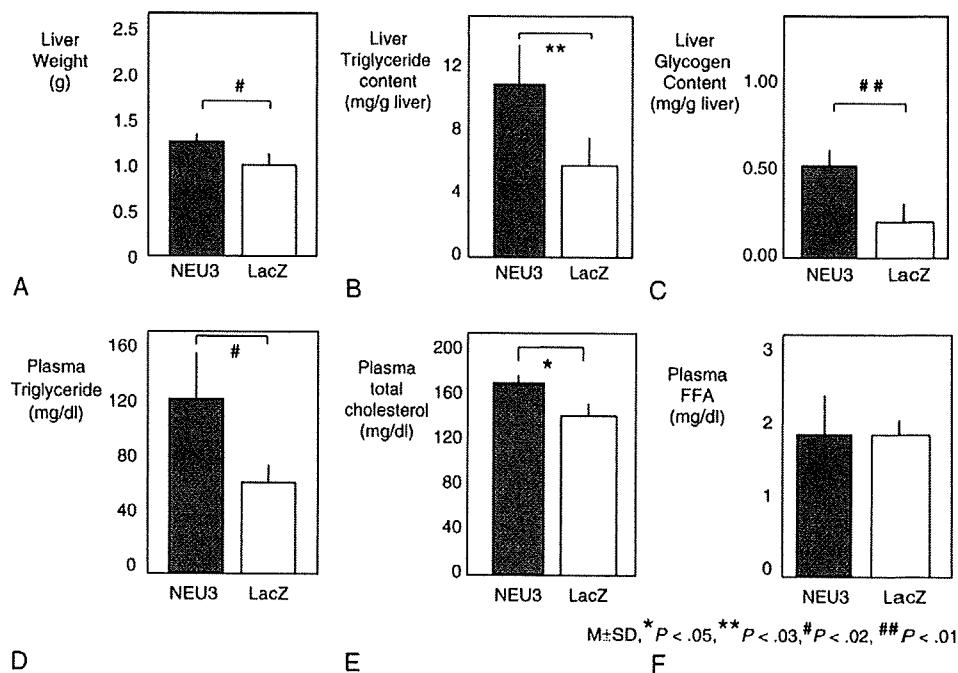


Fig. 5. Hepatic *NEU3* overexpression increased liver weights (A), hepatic contents of triglyceride (B) and glycogen (C), and plasma triglyceride (D) and total cholesterol concentrations (E), but not FFA (F) in C57BL/6 mice fed standard diets. AdLacZ (gray, $n = 8$) or AdNEU3 mice (white, $n = 8$) were killed after a 16-h fast on day 7 after adenoviral injection, and livers were removed. Liver weight (A), triglyceride content (B), and glycogen synthesis (C) were determined ($n = 8$ per group). Blood samples from AdLacZ (gray, $n = 8$) or AdNEU3 mice (white, $n = 8$) were collected after a 16-hour fast on day 7 after adenoviral injection. Plasma triglyceride (D), total cholesterol (E), and FFA concentrations (F) were determined. Similar representative results were obtained from 3 or more experiments, and the data are presented as means \pm SD. * $P < .05$, ** $P < .03$, # $P < .02$, ## $P < .01$, assessed by unpaired t test.

were no significant differences in FFA concentrations between AdNEU3 and AdLacZ mice fed standard diets (Fig. 5F). NEU3 overexpression also increased plasma triglyceride and total cholesterol concentrations, but not plasma FFA in C57BL/6 mice on high-fat diets, and also in KKAY mice (data not shown). There was no difference in serum levels of either adiponectin or leptin between AdNEU3 and AdLacZ mice (data not shown), suggesting that these adipocytokines are not involved in the improvement of glucose tolerance and insulin sensitivity seen in AdNEU3 mice.

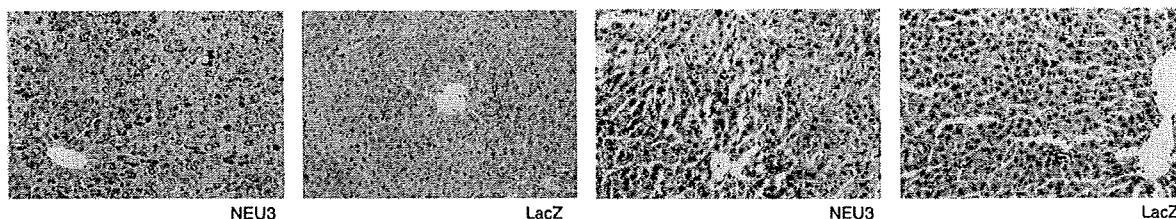
These results were confirmed by the histologic findings. PAS staining demonstrated markedly increased glycogen contents in the livers of AdNEU3 mice fed a standard diet compared with AdLacZ mice (Fig. 6A). AdNEU3 mice on standard diets had abundant lipid droplets in their livers compared with AdLacZ mice (Fig. 6B). In the cases of high-fat diet-fed mice (Fig. 6C and D) and KKAY mice (Fig. 6E and F), AdNEU3 mice had abundant glycogen granules and lipid droplets in their livers compared with AdLacZ mice.

3.4. NEU3 overexpression improved insulin-stimulated IRS1 tyrosine phosphorylation in the liver and insulin-stimulated insulin receptor tyrosine phosphorylation in adipose tissues

The results of intraperitoneal glucose tolerance and insulin tolerance tests on days 4 to 5 after adenoviral administration clearly showed that hepatic NEU3 expression markedly improved glucose tolerance and insulin sensitivity in mice. To explore the potential cellular mechanisms by which hepatic NEU3 overexpression influences insulin sensitivity and glucose tolerance, we obtained samples of hepatic, skeletal muscle, and adipose tissues after insulin injection on day 4 after adenoviral administration. There was no significant difference in food intake between AdNEU3- and AdLacZ-treated mice until day 4 after adenoviral administration (Fig. 2A).

We first determined whether NEU3 interferes with activation of upstream insulin signaling components by measuring tyrosine phosphorylation status of the insulin receptors, IRS1 and IRS2. There was no significant

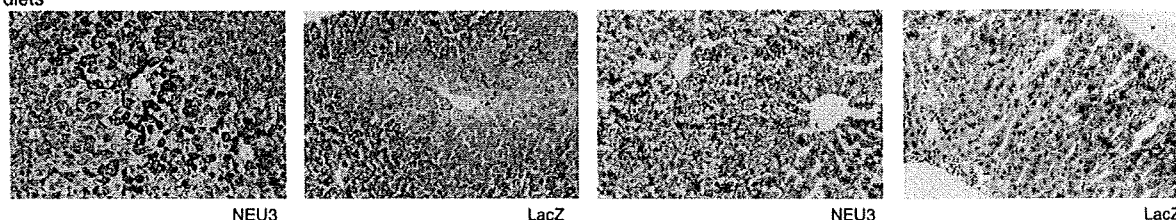
Standard diets



A. PAS staining

B. Oil Red O staining

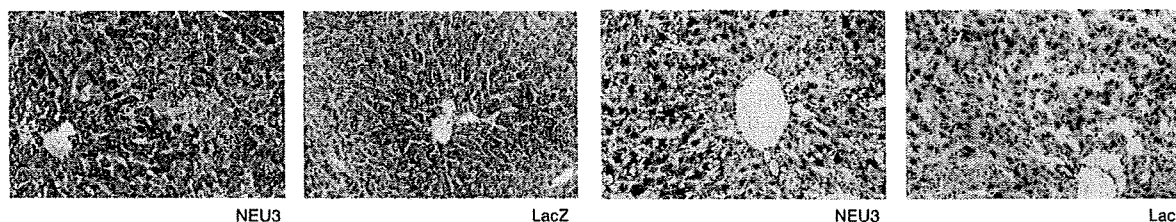
High fat diets



C. PAS staining

D. Oil Red O staining

KKAY mice



E. PAS staining

F. Oil Red O staining

Fig. 6. Hepatic glycogen and fat contents were increased in AdNEU3 mice. Mice were killed after a 16-hour fast on day 7 after adenoviral injection, and livers were removed and subjected to histologic examination. Histologic findings with PAS (A) and Oil Red O staining (B) of the liver from the standard diet C57BL/6 mice treated with AdNEU3 (left) or AdLacZ (right) are shown. PAS (C) and Oil Red O staining (D) of the livers from high-fat diet C57BL/6 mice with AdNEU3 (left) or AdLacZ (right), as well as PAS (E) and Oil Red O staining (F) of the livers from KKAY mice with AdNEU3 (left) or AdLacZ (right) are shown.

difference in insulin-stimulated insulin receptor tyrosine phosphorylation in the liver or skeletal muscles between AdNEU3 and AdLacZ mice (Fig. 7A and B). Basal and insulin-stimulated tyrosine phosphorylations of IRS1 were significantly higher in AdNEU3 than in AdLacZ mice (Fig. 7C). In contrast, IRS2 phosphorylation did not differ between AdNEU3 and AdLacZ mice (Fig. 7D). Basal and insulin-stimulated tyrosine phosphorylation of IRS1 was significantly increased (Fig. 7H), but there were no significant changes in insulin receptor tyrosine phosphorylation in the liver or skeletal muscles of AdNEU3 mice fed high-fat diets (Fig. 7E and F). Insulin-stimulated insulin receptor tyrosine phosphorylation was also increased in adipose tissues of AdNEU3 mice on high-fat diets (Fig. 7G).

Improved insulin sensitivity in the liver was consistent with enhanced basal and insulin-stimulated IRS1 phosphorylation, although insulin receptor phosphorylation was not affected. Improved insulin sensitivity in adipose tissues was also consistent with enhanced insulin receptor phosphorylation in response to insulin administration.

3.5. Hepatic *NEU3* expression increased hepatic peroxisome proliferator-activated receptor γ expression in C57BL/6 mice and KKAY mice

As shown in Fig. 8A, insulin-resistant diabetic *NEU3*-transgenic mice [16] had significantly reduced hepatic expression of peroxisome proliferator-activated receptor γ (PPAR γ), whereas hepatic overexpression of *NEU3* significantly enhanced hepatic PPAR γ expression in mice fed standard and high-fat diets, as well as in KKAY mice. Hepatic fetuin expression was significantly reduced in AdNEU3 mice fed standard diets and in KKAY mice as compared with AdLacZ mice fed standard diets, and KKAY mice (Fig. 8B). The expression level of these genes was consistent with improvement of insulin sensitivity.

4. Discussion

This study demonstrates that *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diets. Increased hepatic

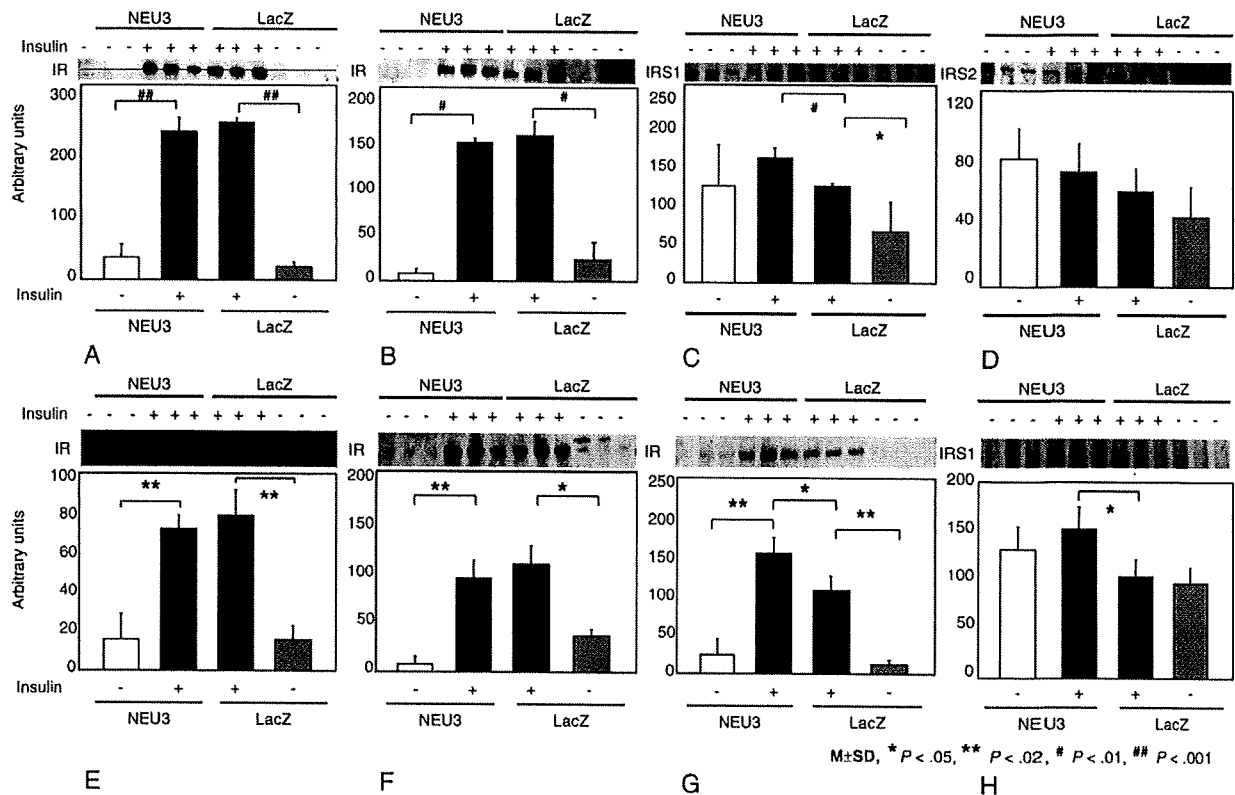


Fig. 7. Insulin-induced tyrosine phosphorylation of the insulin receptor (A, E), IRS1 (C, H), and IRS2 (D) in the livers, and tyrosine phosphorylation of the insulin receptor in skeletal muscles (B, F) and white adipose tissues (G) of C57BL/6 mice on standard (A-D) or high-fat diets (E-H). On day 4 after adenovirus injection, the mice that were fasted for 16 hours received an intravenous injection of 100 μ L of saline with or without insulin (standard diets, 2 U/kg body weight; high-fat diets, 10 U/kg body weight). Liver, epididymal fat tissues and hind-limb muscles were removed 5 minutes after the injections. Liver (A, C, D, E, H), adipose tissue (G), and muscle (B, F) lysates from C57BL/6 mice on standard (A-D) or high-fat diets (E-H) were immunoprecipitated with each antibody toward insulin receptor (A, B, E, F, G), IRS1 (C, H), and IRS2 (D) as indicated. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antiphosphotyrosine antibody (4G10, Upstate). Similar representative results were obtained from 3 or more experiments, and the data are presented as means \pm SD. *P < .05, **P < .02, #P < .01, ##P < .001, assessed by unpaired *t* test.

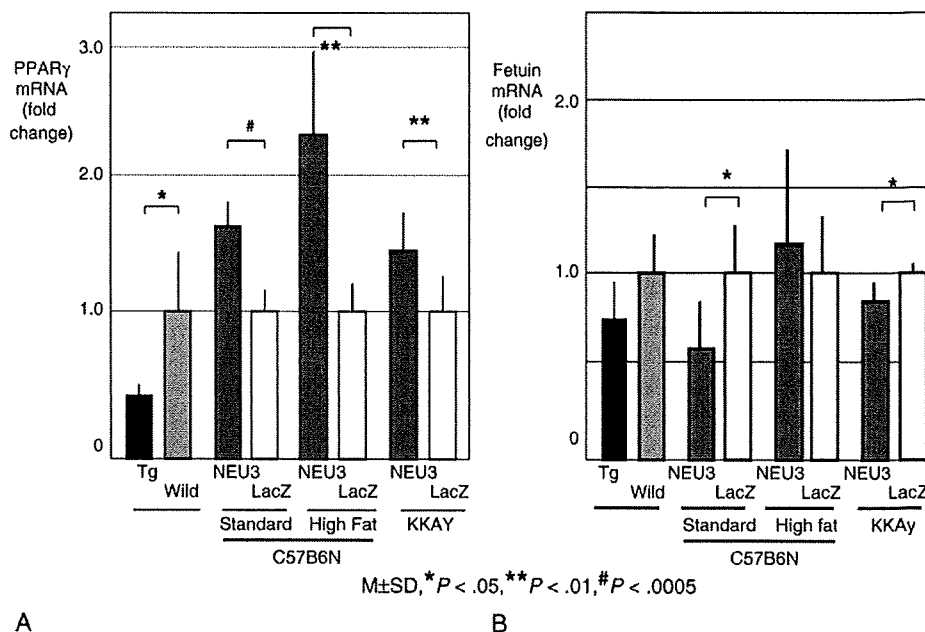


Fig. 8. The gene expression of PPAR γ (A) and fetuin (B) in the livers from *NEU3* transgenic and wild C57BL/6 mice and Ad*NEU3*- or Ad*LacZ*-treated C57BL/6 mice on standard or high-fat diets and Ad*NEU3*- or Ad*LacZ*-treated KKAY mice. The relative mRNA levels of PPAR γ and fetuin in the livers from *NEU3*-transgenic mice (Tg) and wild C57BL/6 mice [16], and mice treated with Ad*NEU3* or Ad*LacZ*, as measured by quantitative reverse transcription-polymerase chain reaction. The primers used were the forward primer (5'-GAACGTGAAGCCCATCGAGGAC-3') and the reverse primer (5'-CTGGAGCACCTTGGCGAACA-3') for PPAR γ , as well as the forward primer (5'-CCCAGTGTCTACTCTGGTGA-3') and the reverse primer (5'-CTGTGTTTGGAAATAACTTGCAG-3') for fetuin. The relative amount of mRNA was calculated with β -actin mRNA as the invariant control. Vertical axes represent the fold change in mRNA levels compared with the control mice and those treated with Ad*LacZ*, respectively. The bars represent the fold change of PPAR γ and fetuin relative to the mean expression in the controls \pm SD ($n = 5$). Similar representative results were obtained from 3 or more experiments. * $P < .05$, ** $P < .01$, # $P < .0005$, assessed by unpaired t test.

contents of glycogen and triglycerides suggest increased hepatic insulin sensitivities in C57BL/6 mice. Hepatic *NEU3* overexpression also improves glucose tolerance in 2 types of insulin-resistant mice: C57BL/6 mice on a high-fat diet and KKAY mice.

The present study clearly demonstrates that hepatic *NEU3* expression increased tyrosine phosphorylation of IRS1 in the basal and insulin-stimulated states, thereby leading to enhanced insulin sensitivity *in vivo*. In contrast, *NEU3* overexpression did not affect insulin-stimulated tyrosine phosphorylation of IRS2 or the insulin receptor in the liver. TLC analysis in this study demonstrated increased levels of GM1; in contrast, GM3 was markedly reduced in the livers of Ad*NEU3* mice. Modification of gangliosides regulates intracellular insulin signaling through direct modulation of insulin receptor tyrosine kinase in the microdomain component of hepatocyte plasma membranes [8–10]. These results suggest that hepatic *NEU3* overexpression improves insulin sensitivity and glucose tolerance through modification of the ganglioside composition, possibly via accelerated degradation of GM3.

This study demonstrates that hepatic *NEU3* overexpression enhances hepatic PPAR γ expression, increases triglyceride accumulation, and induces hyperlipidemia. Hyperlipidemia and fatty liver are thought to be part of the metabolic syndrome, and are clinically associated with

hyperglycemia, hyperinsulinemia, and insulin resistance [25]. Therefore, our finding that the beneficial effects of hepatic *NEU3* overexpression on glucose homeostasis are associated with hyperlipidemia and fatty liver is somewhat surprising. However, several recent studies on animal models with hepatic modification of Akt, PTEN, PGC-1 and ANGPTL4 suggest that some signaling pathways that reduce blood glucose and improve insulin sensitivity can simultaneously induce hyperlipidemia and fatty liver [20,26–28].

PPAR γ expression in the liver is low compared with that in adipose tissues [29]; however, hepatic expression of PPAR γ is functionally enhanced in a number of obesity models [30,31]. In addition, liver-specific disruption of PPAR γ in obese (ob/ob) mice prevents hepatic steatosis but increases peripheral adiposity and decreases insulin sensitivity in muscle and fat [32]. Synthetic agonists of PPAR γ , thiazolidinediones, were shown to reduce hepatic glucose production and increase glycogen synthesis in diabetic animal models [33]. Mice lacking adipose tissue showed increased insulin sensitivity with thiazolidinedione administration, suggesting that thiazolidinediones can enhance insulin sensitivity independently of adipose tissues [30].

We recently reported that adenovirus-mediated expression of PPAR γ -2 in the liver induces acute hepatic steatosis,

markedly decreasing peripheral adiposity, increasing energy expenditure, and improving systemic insulin sensitivity [34]. Although there were no differences in peripheral adiposity and energy expenditure between AdNEU3 mice and AdLacZ mice in this study, the AdNEU3 mice had a phenotype of hepatic steatosis and improved systemic insulin sensitivity with enhanced PPAR γ expression in liver. Thus, hepatic PPAR γ plays important roles not only in the development of liver steatosis but also systemic insulin sensitivity. Increased PPAR γ expression in the livers of AdNEU3 mice might be one of the mechanisms underlying increased systemic insulin sensitivity in vivo. This is the first report, to our knowledge, of hepatic *NEU3* expression increasing hepatic PPAR γ expression. Further investigation is needed to elucidate the molecular mechanism by which hepatic *NEU3* expression increases hepatic PPAR γ expression and triglyceride accumulation in the liver.

Fetuin is a natural inhibitor of the insulin-stimulated insulin receptor tyrosine kinase [35,36]. Fetuin KO mice demonstrate increased basal and insulin-stimulated phosphorylation of insulin receptor and the downstream signaling molecules mitogen-activated protein kinase and Akt in liver and skeletal muscle [37]. Glucose and insulin tolerance tests in fetuin KO mice indicate significantly enhanced glucose clearance and insulin sensitivity. Thus, fetuin is a natural regulator of the insulin sensitivity in liver. Reduced expression of fetuin in the AdNEU3 mice fed standard diets and KKAY mice might contribute to improved insulin sensitivity in these mice.

We previously demonstrated *NEU3* transgenic mice to have an insulin-resistant, diabetic phenotype [16]. In contrast, this study demonstrates that *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance in C57BL/6 mice fed standard diet and in 2 types of insulin-resistant mice, C57BL/6 mice on a high-fat diet and KKAY mice. Recent accumulating evidence provides evidence that hepatic expression of PPAR γ regulates systemic insulin sensitivity [30,32,34]. Increased hepatic expression of PPAR γ in the AdNEU3 mice improves insulin sensitivity and glucose tolerance. In contrast, decreased PPAR γ expression in the liver might contribute to the insulin resistance and glucose intolerance of the *NEU3* transgenic mice.

The *NEU3* transgene was expressed in a wide range of tissues, but most prominently in muscles, pancreas, and heart in the transgenic mice. Chronically elevated *NEU3* expression in muscle was speculated to contribute to in vivo insulin resistance because intracellular insulin signaling was reduced in muscles of transgenic mice [16]. In contrast, this study demonstrates that acute expression of *NEU3* in the liver improves insulin sensitivity. These discrepancies might be partially explained by the time (acute or chronic) and tissue (liver or skeletal muscle) differences in *NEU3* overexpression. Further studies are needed to investigate the difference in molecular mechanism to regulate systemic

insulin sensitivity between the AdNEU3 mice and the *NEU3* transgenic mice.

In conclusion, *NEU3* overexpression in the liver improves insulin sensitivity and glucose tolerance, possibly through the expression of PPAR γ and fetuin, and the modification of the ganglioside composition in healthy mice and 2 types of insulin-resistant mice, mice on a high-fat diet and KKAY mice. Our findings also provide further evidence that *NEU3* is an important regulator of insulin sensitivity and glucose tolerance, making it a potential therapeutic target in type 2 diabetes mellitus.

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Regular Article

Reliability and validity of the Japanese version of the World Health Organization-Five Well-Being Index in the context of detecting depression in diabetic patients

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Abstract

The present study had two aims. The first was to evaluate the reliability and the validity of the Japanese version of the World Health Organization (WHO)-Five Well-Being Index (WHO-5-J) as a brief well-being scale. The second was to examine the discriminatory validity of this test as a screening tool for current depressive episodes in diabetic patients. A sample of 129 diabetic patients completed the WHO-5-J. Of these, 65 were also interviewed by psychiatrists to assess whether they had any current depressive episodes according to DSM-IV. The internal consistency was evaluated using Cronbach's alpha, the Loevinger coefficient of homogeneity, and factor analysis. The external concurrent validity was evaluated by correlations with the external scales potentially related to subjective well-being. Discriminatory validity was evaluated using receiver operating characteristic (ROC) analysis. Cronbach's alpha and the Loevinger coefficient were estimated to be 0.89 and 0.65, respectively. A factor analysis identified only one factor. The WHO-5-J was significantly correlated with a number of major diabetic complications, depression, anxiety, and subjective quality of life. ROC analysis showed that the WHO-5-J can be used to detect a current depressive episode (area under curve: 0.92; 95% confidence interval: 0.85–0.98). A cut-off of <13 yielded the best sensitivity/specificity trade-off: sensitivity, 100%; specificity, 78%. The WHO-5-J was thus found to have a sufficient reliability and validity, indicating that it is a useful instrument for detecting current depressive episodes in diabetic patients.

Key words depressive episode, diabetes, screening, validity, well-being.

INTRODUCTION

There is a high prevalence of depression among patients with diabetes.¹ Recent studies suggest that depression has a significantly adverse effect on diabetes in terms of decreased glycemic control, a reduction in the quality of life, and an increased health-care

expenditure.² However, depression frequently goes undetected and it remains untreated.³ It has been demonstrated that primary care patients with major or minor depressive disorders respond well to psychotherapy and/or treatment with antidepressants.^{4,5} Therefore, a brief and rapid instrument for detecting depression in primary care settings may play an important role in the improvement of diabetes care.

One candidate for such an instrument is the World Health Organization (WHO)-Five Well-Being Index (WHO-5). It is a short version of the WHO Well-Being Scale, which was initially developed to evaluate the quality of care for diabetic patients.^{6,7} The WHO-5 is a self-administered five-item scale. Each item assesses

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the degree of positive well-being during the past 2 weeks on a six-point Likert scale graded from 0 (at no time) to 5 (all of the time), and the total score ranges from 0 to 25, with high scores thereby indicating an increased sense of well-being. The scale has been translated into various languages (<http://www.who-5.org/>) and, in the West, validation data have been published in the context of various health conditions, including depressive disorders,⁸⁻¹¹ anxiety disorders,⁸ cognitive impairment and dementia,¹² and psychiatric disorders,¹² in addition to the health-related quality of life.¹³ Discriminatory validity as a screening tool for depressive disorders has also been examined.⁹⁻¹¹ However, no validation data have yet been published in the East, including Japan.

The present study had two aims: to evaluate the reliability and validity of the Japanese version of the WHO-5 (WHO-5-J) as a brief well-being scale, and to examine its discriminatory validity as a screening tool for a current depressive episodes in diabetic patients.

METHODS

Adaptation of the WHO-5 for use in Japan

Using a state-of-the-art procedure for test translation,¹⁴ the original English version of the WHO-5 was adapted to Japanese in collaboration with the European WHO office as follows: two independent forward- and two independent back-translations, linguistic panels, pilot testing, and formal assessment of the reliability and validity of the index.

The WHO-5 was translated into Japanese by two bilingual translators who worked independently to produce two different Japanese versions. These versions were then back-translated and discussed by a panel of experts until agreement was reached on the most suitable items to be included in the final version. The final version was tested in a pilot study, which confirmed a high level of item acceptability and comprehension. The data regarding the reliability and validity are shown in the present study. The WHO-5-J can be obtained from the website <http://www.who-5.org>.

Subjects

The participants were recruited from 197 outpatients with diabetes who received regular treatment at the Diabetes and Metabolism Unit, Tohoku University Hospital. All patients had been diagnosed to have either type 1 or type 2 diabetes mellitus according to the criteria of the American Diabetes Association.¹⁵ Of these patients, a total of 129 were regarded as eligible for the study. These consisted of 71 men and 58 women

who gave their written informed consent, had no explicit dementia (Mini-Mental State ≥ 20), and completed the WHO-5-J as well as a set of questionnaires concerning demographic, social, and health-related variables. The most recent clinical data regarding diabetes were collected from the patients' medical records. All data collection was completed in November 2004. Subject characteristics are given in Table 1.

Approval of the research protocol was obtained from the Ethics Committee of Tohoku University Graduate School of Medicine and Tohoku University Hospital.

Table 1. Description of the 129 diabetic patients who completed the WHO-5-J

| Variables | Mean \pm SD | Range |
|------------------------------|------------------|------------|
| Age (years) | 53.6 \pm 10.4 | 25-73 |
| Sex (Female %) | 45.0 | |
| Educational level (years) | 19.8 \pm 3.2 | 15-34 |
| Type of diabetes (Type 1 %) | 16.3 | |
| Use of insulin (%) | 55.8 | |
| Duration of diabetes (years) | 10.9 \pm 10.2 | 0.1-40 |
| FBS (mg/dL) | 149.3 \pm 50.5 | 66.0-503.0 |
| HbA1c (%) | 7.0 \pm 1.4 | 4.0-12.0 |
| BMI (g/cm ²) | 24.5 \pm 4.4 | 17.0-47.2 |
| Major complication (%) | 43.4 | |
| Neuropathy (%) | 24.8 | |
| Retinopathy (%) | 30.2 | |
| Nephropathy (%) | 13.2 | |
| No. complications | | |
| 0 | 56.6 | |
| 1 | 25.6 | |
| 2 | 10.9 | |
| 3 | 7.0 | |
| SDS score | 37.6 \pm 9.6 | 22-63 |
| STAI score, state | 39.6 \pm 10.6 | 20-67 |
| STAI score, trait | 41.5 \pm 11.9 | 21-74 |
| MMSE score | 28.2 \pm 2.1 | 20-30 |
| SF-36 subscale | | |
| Physical functioning | 82.3 \pm 21.2 | 0-100 |
| Social functioning | 83.2 \pm 22.9 | 12.5-100 |
| Role functioning, physical | 76.8 \pm 37.6 | 0-100 |
| Role functioning, emotional | 78.1 \pm 38.4 | 0-100 |
| Mental health | 70.2 \pm 21.7 | 4-100 |
| Vitality | 62.9 \pm 22.9 | 5-100 |
| Pain | 71.8 \pm 25.8 | 0-100 |
| General health perceptions | 49.7 \pm 21.0 | 0-97 |
| WHO-5-J score | 15.5 \pm 6.1 | 0-25 |

BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-39, Short-Form 36 Health Survey questionnaire; STAI, State-Trait Anxiety Inventory; WHO-5-J, Japanese version of the World Health Organization-Five Well-Being Index.

Measurements

Sociodemographic variables included sex, age, and educational level. Educational levels were assessed by determining the age when schooling was completed. The assessed clinical variables regarding diabetes included the type of diabetes, the duration of diabetes, fasting blood sugar (FBS), glycosylated hemoglobin (HbA1c), body mass index (BMI), and major diabetic complications (retinopathy, neuropathy, and nephropathy).

Zung's Self-Rating Depression Scale (SDS),¹⁶ the State-Trait Anxiety Inventory (STAI),¹⁷ the Mini-Mental State Examination (MMSE),¹⁸ and the Short-Form 36 Health Survey questionnaire (SF-36)¹⁹ were all used as external scales that were potentially related to subjective well-being.

The SDS is an internationally used 20-item self-administered depression scale. Each item is rated on a four-point Likert scale, with a total score ranging from 20 to 80 and high scores indicating increased depression. The Japanese version was developed by Fukuda and Kobayashi²⁰ and has been well validated by Kitamura *et al.*²¹ The STAI comprises 20-item trait and 20-item state anxiety scales. Each item is rated on the four-point Likert scale, with a total score ranging from 20 to 80 for each scale and high scores indicating increased anxiety. The Japanese version was validated by Nakazato and Mizuguchi.²² The MMSE is a brief test of several cognitive functions, with a total score ranging from 0 to 30. A score of 23 or less indicates a cognitive disorder and has a high degree of validity and reliability in detecting cognitive impairment.¹⁸ The Japanese version was validated by Mori *et al.*²³ The SF-36 consists of eight subscales for subjective health-related quality of life, with scores on each subscale ranging from 0 to 100 and high scores indicating a high quality of life. The Japanese version was developed and validated by Fukuhara *et al.*^{24,25} and is widely used in studies on health-related quality of life among Japanese populations.

Subjects with SDS scores ≥ 40 were selected to undergo an interview with psychiatrists, who were blind to the results of the questionnaires, in order to diagnose any current major or minor depressive episodes using the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I).²⁶ The Japanese version of SCID-I was developed by Takahashi.²⁷ Minor depressive episodes were defined as clinically relevant depressive syndromes, not fulfilling the rigorous diagnostic criteria for major depressive episode, but containing two–four symptoms of DSM-IV-defined major depressive episode, one of which must be either a depressed mood or anhedonia (loss of interest or plea-

sure). Almost the same number of subjects who scored < 40 on the SDS were recruited to undergo the same interview.

Statistics

For the evaluation of internal consistency, Cronbach's alpha, the Loevinger coefficient, and a factor analysis were used. Cronbach's alpha is a measure of how well the subsets of the items can be substituted for each other. A high alpha (> 0.9) may suggest a high level of item redundancy; therefore, alpha should fall within the range 0.7–0.9. The Loevinger coefficient of homogeneity was used as part of the Mokken analysis.²⁸ The Loevinger coefficient is an analysis of unidimensionality that tests the extent to which an extra item fits into the structure established by the other items of a scale. According to the Mokken analysis, homogeneity and unidimensionality are acceptable at a Loevinger coefficient of ≥ 0.40 , while a coefficient of 0.30–0.39 is considered to be barely acceptable.²⁸

The Loevinger coefficient is thought to be a better indicator of internal consistency than Cronbach's alpha because, in contrast to Cronbach's alpha, it is independent of the number of items in the scale.^{7,29} A factor analysis was performed as a standard principal component analysis with orthogonal varimax rotation of the components. If a substantial shift between the first and the second eigenvalues could be observed, then unidimensionality is indicated.³⁰

To evaluate the external concurrent validity, the Spearman correlation coefficient was used to correlate the WHO-5-J with health-related variables potentially related to subjective well-being. For the evaluation of discriminatory validity as a screening tool for current depressive episodes, the area under the curve (AUC), sensitivity, and specificity were calculated using a receiver operating characteristic (ROC) analysis. To evaluate the post-test probability, the positive predictive value (PPV) and negative predictive value (NPV) were calculated using a 2×2 contingency table. For group comparisons, Student's *t*-test, the Wilcoxon *U*-test, or χ^2 test were used where applicable. All statistical analyses were performed using SPSS version 11.5 (SPSS, Chicago, IL, USA) and the SAS system, version 9.1.3 (SAS Institute, Cary, NC, USA). Statistical significance was established at $P = 0.05$.

RESULTS

Internal consistency

Cronbach's alpha was found to be 0.89 and the Loevinger coefficient of homogeneity was 0.65. A factor

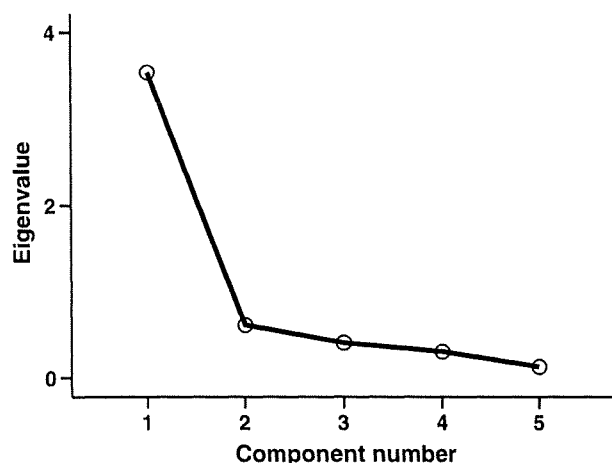


Figure 1. Scree plot for the components of the Japanese version of the World Health Organization-Five Well-Being Index.

Table 2. Factor matrix of the Japanese version of the WHO-5

| Item | Factor |
|---|--------|
| Item 1 I have felt cheerful and in good spirits | 0.91 |
| Item 2 I have felt calm and relaxed | 0.91 |
| Item 3 I have felt active and vigorous | 0.83 |
| Item 4 I woke up feeling fresh and rested | 0.80 |
| Item 5 My daily life has been filled with things that interest me | 0.74 |

WHO, World Health Organization.
Principle component analysis was used.

analysis identified only one factor when considering eigenvalues >1.0 (Fig. 1, Table 2). The first factor explained 70.8% of the variance. These findings thus indicate that the WHO-5-J has an adequate internal consistency and that the total score has a sufficient degree of statistical validity.

External concurrent validity

No significant differences in the WHO-5-J total scores were observed between the male and female subjects (male vs female [mean \pm SD], 15.8 ± 6.0 vs 15.1 ± 6.3 , $t = 0.59$, $P = 0.57$). Neither type of diabetes (type 1 vs type 2, 13.9 ± 5.1 vs 15.8 ± 6.3 , $t = 0.46$, $P = 0.14$) nor status of insulin use (use vs no use, 15.9 ± 6.0 vs 15.1 ± 6.2 , $t = 0.73$, $P = 0.47$) made differences in the WHO-5-J. Table 3 shows correlations between the WHO-5-J and the demographic and health-related variables potentially related to subjective well-being.

Table 3. Correlations between the WHO-5-J and external scales related to subjective well-being

| | Spearman correlation coefficients | <i>P</i> |
|-------------------------------|-----------------------------------|----------|
| Age | 0.23 | 0.01 |
| Education level | 0.03 | 0.77 |
| Duration of diabetes | 0.10 | 0.24 |
| FBS | -0.01 | 0.88 |
| HbA1c | 0.03 | 0.78 |
| BMI | -0.10 | 0.25 |
| Number of major complications | -0.21 | 0.02 |
| SDS score | -0.68 | 0.00 |
| STAI score, state | -0.73 | 0.00 |
| STAI score, trait | -0.74 | 0.00 |
| MMS score | 0.11 | 0.21 |
| SF-36 subscale | | |
| Physical functioning | 0.43 | 0.00 |
| Social functioning | 0.40 | 0.00 |
| Role functioning, physical | 0.43 | 0.00 |
| Role functioning, emotional | 0.51 | 0.00 |
| Mental health | 0.70 | 0.00 |
| Vitality | 0.72 | 0.00 |
| Pain | 0.39 | 0.00 |
| General health perceptions | 0.43 | 0.00 |

BMI, body mass index; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-36, Short-Form 36 Health Survey questionnaire; STAI, State-Trait Anxiety Inventory; WHO-5-J, Japanese version of the WHO-Five Well-Being Index.

The WHO-5-J total score was significantly correlated with age, the number of major complications, and the total scores on the SDS, the STAI, both the state and trait scales, and each subscale of the SF-36. No significant correlations were found between the WHO-5-J total score and educational level, duration of diabetes, FBS, HbA1c, BMI, or the total score on the MMSE.

External discriminatory validity

Table 4 shows the characteristics of the 65 diabetic patients who were interviewed by psychiatrists in order to assess whether they had a current major or minor depressive episode according to the DSM-IV criteria. Seven patients were diagnosed to have a current major depressive episode, while three patients were diagnosed to have a current minor depressive episode. In accordance with our expectations, the patients with current major or minor depressive episodes had a lower mean score on the WHO-5-J as well as higher mean scores on the SDS and the STAI (both the state

Table 4. Description of the 65 diabetic patients who were interviewed for psychiatric diagnosis of a major depressive episode

| | DE (-) | DE (+) | <i>P</i> |
|------------------------------|--------------|---------------|----------|
| <i>n</i> | 55 | 10 | |
| Age (years) | 53.9 ± 10.4 | 49.7 ± 14.0 | 0.39 |
| Sex (Female, %) | 47.3 | 60.0 | 0.51 |
| Educational level (years) | 19.8 ± 3.2 | 18.9 ± 2.2 | 0.29 |
| Type of diabetes (Type 1%) | 16.4 | 20.0 | 0.67 |
| Use of insulin | 50.9 | 50.0 | 1.00 |
| Duration of diabetes (years) | 9.9 ± 10.0 | 5.8 ± 7.5 | 0.15 |
| FBS (mg/dL) | 146.1 ± 38.5 | 179.0 ± 118.3 | 0.10 |
| HbA1c (%) | 6.8 ± 1.2 | 6.8 ± 2.1 | 0.95 |
| BMI (g/cm ²) | 23.4 ± 3.5 | 24.6 ± 4.3 | 0.40 |
| Major complication | 34.6 | 50.0 | 0.48 |
| SDS score | 37.8 ± 9.3 | 51.2 ± 6.1 | 0.00 |
| STAI, state | 37.1 ± 9.9 | 48.9 ± 6.2 | 0.00 |
| STAI, trait | 39.5 ± 10.6 | 57.4 ± 9.3 | 0.00 |
| MMSE | 28.2 ± 2.0 | 28.7 ± 1.5 | 0.41 |
| SF-36 subscale | | | |
| Physical functioning | 82.0 ± 21.4 | 71.5 ± 24.5 | 0.23 |
| Social functioning | 84.7 ± 21.9 | 56.9 ± 24.3 | 0.01 |
| Role functioning, physical | 79.7 ± 35.0 | 47.2 ± 47.5 | 0.08 |
| Role functioning, emotional | 83.7 ± 33.7 | 33.3 ± 44.1 | 0.01 |
| Mental health | 76.0 ± 19.2 | 39.6 ± 17.7 | 0.00 |
| Vitality | 63.4 ± 24.3 | 35.0 ± 13.0 | 0.00 |
| Pain | 74.6 ± 25.3 | 56.3 ± 26.4 | 0.08 |
| General health perceptions | 50.8 ± 21.3 | 34.6 ± 21.5 | 0.06 |
| WHO-5-J score | 16.7 ± 5.8 | 6.9 ± 3.5 | 0.00 |

BMI, body mass index; DE, major or minor depressive episode; FBS, fasting blood sugar; HbA1c, hemoglobin A1c; MMSE, Mini-Mental State Examination; SDS, Zung's Self-Rating Depression Scale; SF-39, Short-Form 36 Health Survey questionnaire; STAI, State-Trait Anxiety Inventory; WHO-5-J, Japanese version of the World Health Organization-Five Well-Being Index.

Statistical analyses were performed using Student's *t*-test, Wilcoxon *U*-test, or χ^2 test.

and trait scales) and lower mean scores on several subscales of the SF-36 (social functioning, emotional role functioning, mental health, and vitality), than patients without a current depressive episode.

Figure 2 shows the ROC curve for the detection of a current major or minor depressive episode according to the WHO-5-J total score. The AUC was estimated to be 0.92 (95% confidence interval [95% CI], 0.85–0.98), which was significantly different from 0.5 ($P < 0.001$). A cut-off of <13 yielded the best sensitivity/specificity trade-off: sensitivity, 100%; specificity, 78.2%; PPV, 45.5%; and NPV, 100%. When associated with a current major depressive episode alone, similar results were obtained: AUC, 0.90; 95% CI, 0.81–0.98; $P < 0.001$; sensitivity, 100%; specificity, 74.1%; PPV, 31.8%; and NPV, 100%.

DISCUSSION

The present report is the first study to evaluate the reliability and validity of the WHO-5 in an Asian

region. Similar to the original version, evaluated in Europe,^{8–10,12,13} the WHO-5-J was found to have adequate reliability and validity for Japanese diabetic patients.

In line with the findings of previous studies,^{12,13} the scale showed a significant correlation with different indicators of health-related variables including depression (SDS), anxiety (STAI), and subjective quality of life (SF-36). It should also be noted that the WHO-5-J score significantly decreased as the number of major complications increased. Recent studies have indicated that depression has an adverse effect on glycemic control, thus increasing the risk of major diabetic complications, and thereby leading to further reductions in the quality of life among diabetic patients.^{2,31} We have reported elsewhere that the presence or absence of major diabetic complications, particularly those of neuropathy, is significantly associated with depression after controlling for potential confounding factors (S. Yoshida *et al.*, unpub. data). Therefore, the WHO-5-J might be sensitive to decreased subjective well-being in

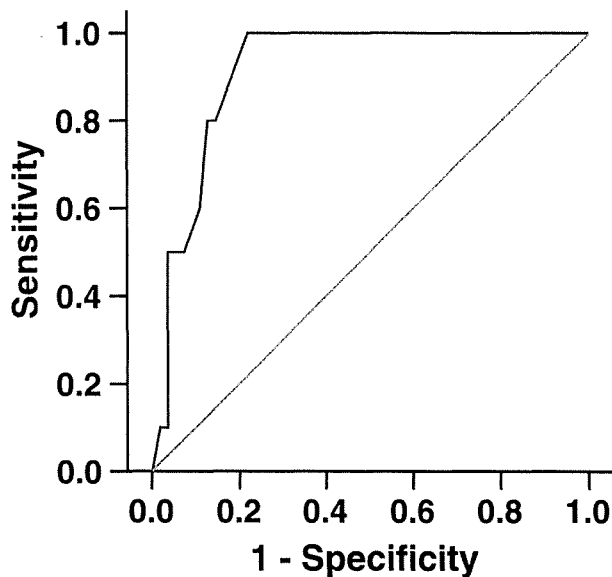


Figure 2. Receiver operating characteristics curve for the Japanese version of the World Health Organization-Five Well-Being Index in association with a current depressive episode as diagnosed according to DSM-IV criteria. The area under the curve was estimated at 0.92 (95% confidential interval: 0.85–0.98), which was significantly different from 0.5 ($P < 0.001$).

patients with major diabetic complications who are prone to develop depression and thus demonstrate a reduction in the quality of life.

However, no significant correlation was observed between the WHO-5-J and cognitive function. The present subjects are comparatively younger and have a higher cognitive functioning than those of a previous study that showed the WHO-5 score to significantly decrease as the MMSE score decreased in a sample of 254 elderly from the general population.¹² This might yield a ceiling effect and conceal the relationship between the cognitive function and subjective well-being. In contrast, the scores on the WHO-5-J significantly decreased as age decreased. In the present study, a decreased age was significantly associated with increases in both state and trait anxiety (state, $r = -0.22$, $P = 0.013$; trait, $r = -0.25$, $P = 0.005$). Younger patients with diabetes might be more likely to feel anxiety regarding an image of his or her future than older patients, and such anxiety might be reflected by the WHO-5-J.

The results of an ROC analysis, in addition to those of comparisons of WHO-5-J scores between the patients with and without a current depressive episode, indicate that the WHO-5-J has a sufficient discrimina-

tory validity as a screening tool for the detection of a current depressive episode. A standard cut-off point of <13 (WHO)³² had an excellent sensitivity/specificity trade-off. Sensitivity of 100% means that all subjects with depressive episodes were detected by this cut-off criteria (NPV, 100%). However, the specificity of 78% indicates that a considerable number of positive screening subjects had no current depressive episodes (PPV, 45%). Therefore, as Henkel *et al.* suggested,^{9,10} this scale might be recommended for use as the first-step screening tool followed by the second-step screening tool such as the Major Depression Inventory to confirm depressive episodes.

The present study had several limitations. First, our sample population may not have been adequately representative of diabetic patients within primary care settings. Because the study population had relatively good glycemic control, the prevalence of depressive episodes in diabetic patients within primary care might have been underestimated. Second, the sample size was relatively small and only 65 patients underwent a diagnostic interview by psychiatrists. To generalize the findings more appropriately, it is necessary to conduct a collaborative multi-institutional study in a larger sample. Third, discriminatory validity for the detection of psychiatric disorders other than depression was not examined in the present study, although it is likely that such psychiatric disorders would influence both the subjective well-being and quality of care of diabetic patients. However, it is evident that depression is highly prevalent and it also has a significantly adverse effect on diabetes. The present study indicates that the WHO-5-J might therefore be a potentially useful screening modality for detecting existing, but unrecognized depression in diabetic patients.

CONCLUSIONS

The WHO-5-J was found to have sufficient reliability and validity as a brief well-being scale. This scale might be a useful instrument for the detection of current major or minor depressive episodes in diabetic patients. A cut-off point of <13 might be recommended for use in the screening of depression.

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Milnacipran, a serotonin and norepinephrine reuptake inhibitor, induces appetite-suppressing effects without inducing hypothalamic stress responses in mice

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Nonogaki K, Nozue K, Kuboki T, Oka Y. Milnacipran, a serotonin and norepinephrine reuptake inhibitor, induces appetite-suppressing effects without inducing hypothalamic stress responses in mice. *Am J Physiol Regul Integr Comp Physiol* 292: R1775–R1781, 2007. First published January 11, 2007; doi:10.1152/ajpregu.00527.2006.—Milnacipran, a selective serotonin (5-HT) and norepinephrine (NE) reuptake inhibitor, increases extracellular 5-HT and NA levels equally in the central nervous system. Here, we report that systemic administration of milnacipran (20–60 mg/kg) significantly suppressed food intake after fasting in C57BL/6J mice. The appetite-suppressing effects of milnacipran were sustained for 5 h. Neither SB242084, a selective 5-HT_{2C} receptor antagonist, nor SB224289, a selective 5-HT_{1B} receptor antagonist, reversed the appetite-suppressing effects of milnacipran. Milnacipran suppressed food intake and body weight in wild-type mice and in A^y mice, which have ectopic expression of the agouti protein. Moreover, milnacipran significantly increased hypothalamic proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) mRNA levels, while having no effect on hypothalamic neuropeptide Y, ghrelin, corticotropin-releasing hormone (CRH), and suppressor of cytokine signaling-3 mRNA levels. Interestingly, milnacipran did not increase plasma corticosterone and blood glucose levels, whereas fenfluramine, which inhibits 5-HT reuptake and stimulates 5-HT release, significantly increased plasma corticosterone and blood glucose levels in association with increased hypothalamic CRH mRNA levels. The appetite-suppressing effects of milnacipran had no effects on food intake in food-restricted, wild-type mice and A^y mice. On the other hand, fenfluramine suppressed food intake in food-restricted wild-type mice, but it had no effects in food-restricted A^y mice. These results suggest that inhibition of 5-HT and NA reuptake induces appetite-suppressing effects independent of 5-HT_{2C} and 5-HT_{1B} receptors, and increases hypothalamic POMC and CART gene expression without increasing plasma corticosterone and blood glucose levels in mice.

5-hydroxytryptamine; norepinephrine; food intake; corticosterone; glucose

BRAIN SEROTONIN (5-HYDROXYTRYPTAMINE; 5-HT) systems contribute to regulate mood, feeding, autonomic outflow, and the hypothalamic-pituitary-adrenal (HPA) axis through complex neural mechanisms. 5-HT drugs, such as m-chlorophenylpiperazine (mCPP), a 5-HT_{2C/1B} receptor agonist, and fenfluramine, which inhibits 5-HT reuptake and stimulates 5-HT release, suppress feeding via 5-HT_{2C} receptors and/or 5-HT_{1B} receptors and the central melanocortin (MC) pathway (12, 16, 27, 28, 29). These drugs are also likely to stimulate the HPA axis and the hypothalamic-sympathetic adrenal medullary axis (3, 4, 9, 14, 19, 20, 26).

Milnacipran, a selective 5-HT and norepinephrine (NE) reuptake inhibitor that increases extracellular 5-HT and NE

levels equally in the central nervous system, was originally prescribed to treat depression (1, 18, 24). Milnacipran has no effect on dopamine reuptake and does not interact with any known neurotransmitter receptors, especially noradrenergic, muscarinic, and histaminergic receptors, unlike tricyclic antidepressants (1). Milnacipran is also recommended as a treatment for binge eating in bulimia nervosa (6). The effects of milnacipran on appetite, obesity, plasma corticosterone, and glucose levels, and the expression of hypothalamic neuropeptide genes that are involved in regulating feeding and energy homeostasis, however, are unknown.

To determine whether milnacipran suppresses appetite and increases blood glucose and plasma corticosterone levels, we investigated the effects of milnacipran on food intake in relation to 5-HT_{2C} and 5-HT_{1B} receptors and on blood glucose and plasma corticosterone levels compared with fenfluramine. We also examined the effects of milnacipran on the gene expression of hypothalamic neuropeptides that are involved in regulating feeding and on food intake and body weight in A^y mice. A^y mice display hyperphagia due to ectopic expression of agouti protein, which interferes with the binding of endogenous ligands to MC-4 and MC-3 receptors (5, 8, 17). Hyperphagic A^y mice are responsive to mCPP and fenfluramine (23).

MATERIALS AND METHODS

Animals and drug treatment. Four-week-old male C57BL/6J mice, A^y mice, and wild-type mice were purchased from Japan CLEA. Mice were individually housed in cages with free access to water and chow pellets in a light- (lights on: 0800, lights off: 2000) and temperature (20–22°C) -controlled environment. Animals were acclimatized to the laboratory environment for 1 wk before the experiment. Drugs were administered between 1000 and 1200, as described previously (22, 23). Milnacipran was a kind gift from Asahi Kasei Pharma, (Tokyo, Japan). SB242084 dihydrochloride, SB22489 hydrochloride, and CP94253 hydrochloride were purchased from Sigma Chemical (Tokyo, Japan). Milnacipran and CP94253 hydrochloride were dissolved in saline. SB242084 was dissolved in distilled water and suspended in saline. SB224289 was suspended in saline.

In *experiments 1* and *2*, separate groups of 5-wk-old C57BL/6J mice were deprived of food for 23 h. The following morning, the animals were injected intraperitoneally with saline or milnacipran (3–60 mg/kg) 30 min before food presentation. Intake of chow pellets was measured every hour for the next 1 h (*experiment 1*) or 6 h (*experiment 2*).

In *experiment 3*, 5-wk-old C57BL/6J mice were deprived of food for 23 h. The following morning, animals were injected intraperitoneally with saline, SB242084 (2 mg/kg), or SB224289 (5 mg/kg).

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