Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 56 (2007) 420–429
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

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

Shinsuke Yoshizumi[a], Susumu Suzuki[a,d,*], Masashi Hirai[a,c], Yoshinori Hinokio[a,c], Tetsuya Yamada[a], Takahiro Yamada[a], Uiko Tsunoda[a], Hiroyuki Aburatani[b], Kazunori Yamaguchi[c,d], Taeko Miyagi[c,d], Yoshitomo Oka[a]

[a]*Department of Metabolism and Diabetes, Tohoku University School of Medicine, Sendai 980-8574, Japan*
[b]*Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 153-8904, Japan*
[c]*Department of Biochemistry, Miyagi Prefectural Cancer Center, Natori, Miyagi 981-1293, Japan*
[d]*Core Research for Evolutional Science and Technology Program (CREST), Japan Science and Technology Corporation, Saitama 332-0012, Japan*

Received 6 May 2006; accepted 26 October 2006

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.

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

* Corresponding author. Department of Metabolism and Diabetes, Tohoku University School of Medicine, Sendai 980-8574, Japan.
E-mail address: ssuzuki@mail.tains.tohoku.ac.jp (S. Suzuki).

0026-0495/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2006.10.027

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 (AdNEU3) 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 AdNEU3 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 $CHCl_3/CH_3OH$ (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 μg 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% $CaCl_2$ (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-H_2SO_4.

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

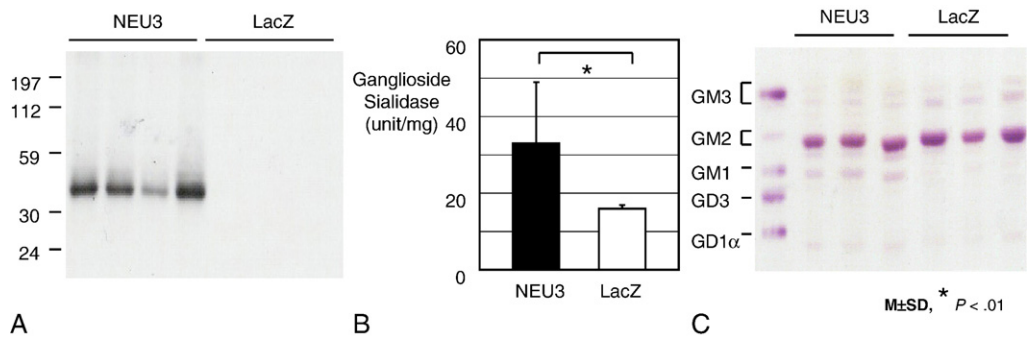

Fig. 1. Hepatic *NEU3* overexpression and ganglioside composition changes in the livers: Western blotting of NEU3 (A), ganglioside-specific sialidase activity (B), and TLC analysis of gangliosides (C). C57BL/6 male mice fed standard diets were injected with 4×10^8 plaque-forming units of adenovirus-containing 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 μg) 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 ± 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.

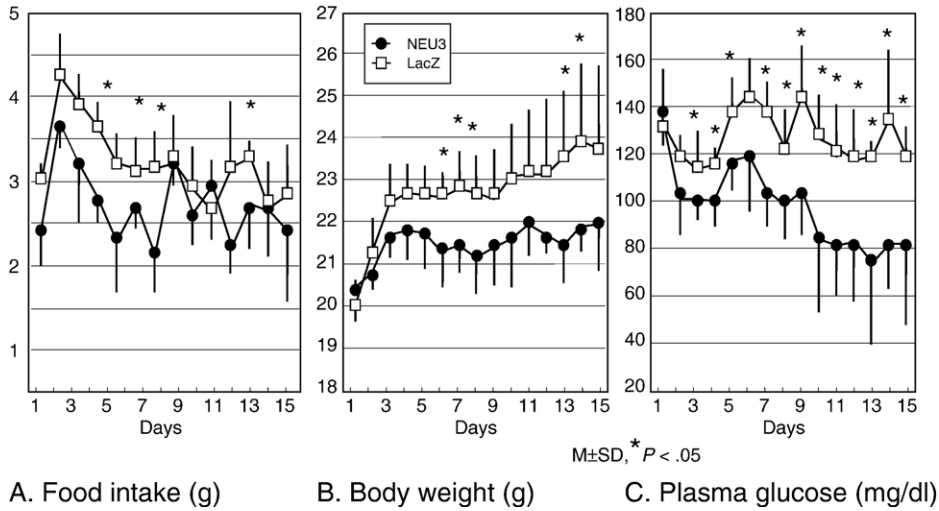

Fig. 2. Hepatic *NEU3* expression reduced food feeding (A), body weights (B), and blood glucose concentrations (C) in C57BL/6 mice fed standard diets. Food feeding (A), body weights (B), and blood glucose levels (C) in the ad libitum–fed state were measured every day after adenoviral administration in 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 ± 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* overexpression significantly improves insulin sensitivity in C57BL/6 mice fed standard diets.

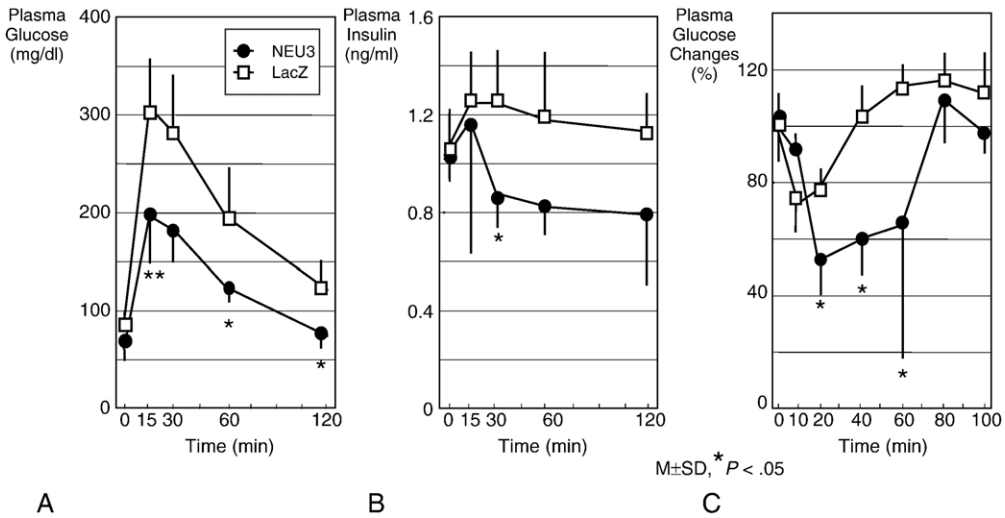

Fig. 3. Hepatic *NEU3* expression improved glucose tolerance and insulin sensitivity in C57BL/6 mice fed standard diets. A and B, Glucose tolerance test. AdLacZ (squares, n = 4) or AdNEU3 mice (circles, n = 4) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose 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 ± SD. *$P < .05$, assessed by unpaired t test.

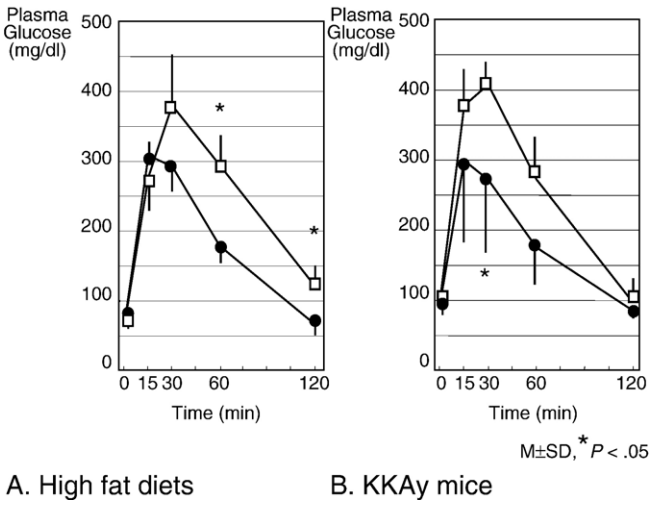

Fig. 4. Hepatic *NEU3* overexpression improved glucose tolerance tests in C57BL/6 mice fed high-fat diets (A) and KKAy mice (B). A and B, glucose tolerance test. AdLacZ (squares, n = 4) or AdNEU3 mice (circles, n = 4) were fasted 16 hours before intraperitoneal injection with glucose (2 g/kg). Blood glucose levels were determined. Significant difference from the AdLacZ value is shown. Data are mean ± 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 differences 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

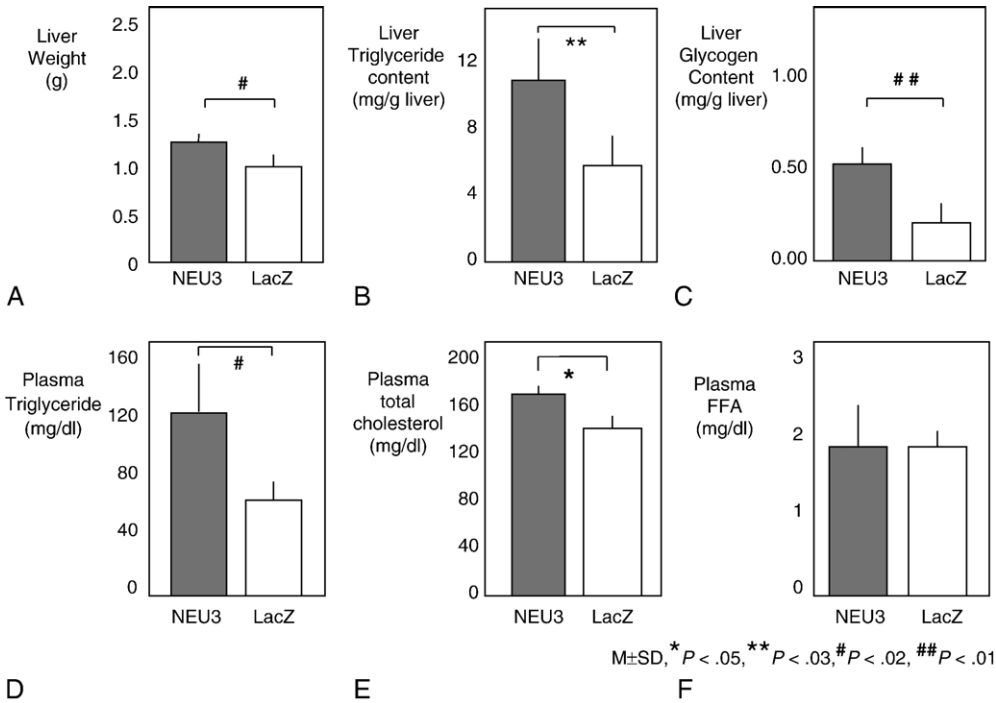

Fig. 5. Hepatic *NEU3* overexpression increased liver weights (A), hepatic contents of triglyceride (B) and glycogen (C), and plasma triglyceride (D) and total cholesterol concentrations (E), but not FFA (F) in C57BL/6 mice fed standard diets. AdLacZ (gray, n = 8) or AdNEU3 mice (white, n = 8) were killed 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 ± 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

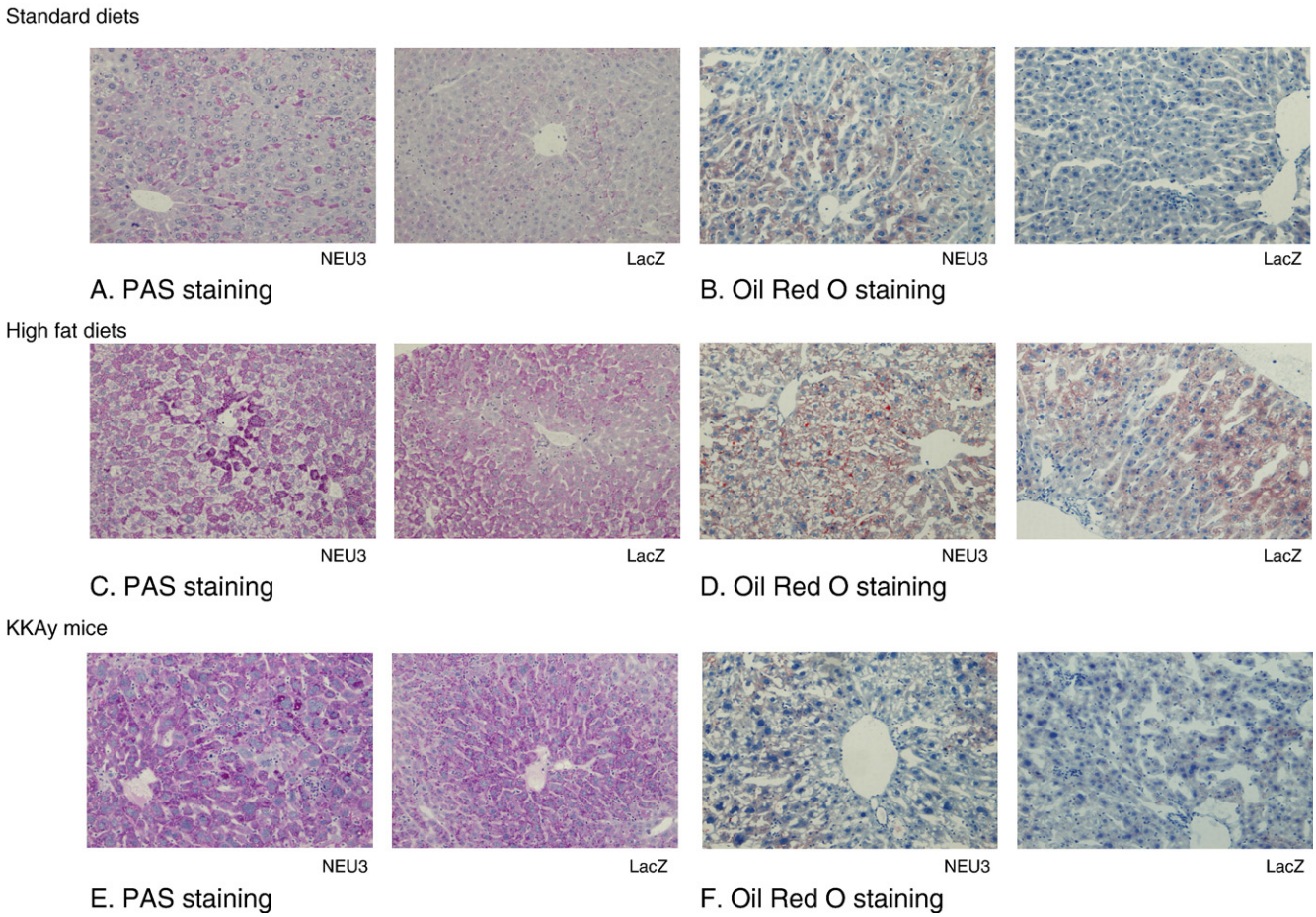

Fig. 6. Hepatic glycogen and fat contents were increased in AdNEU3 mice. Mice were killed after a 16-hour fast on day 7 after adenoviral injection, and livers were removed and subjected to histologic examination. Histologic findings with PAS (A) and Oil Red O staining (B) of the liver from the standard 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 (PPARγ), whereas hepatic overexpression of *NEU3* significantly enhanced hepatic PPARγ 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

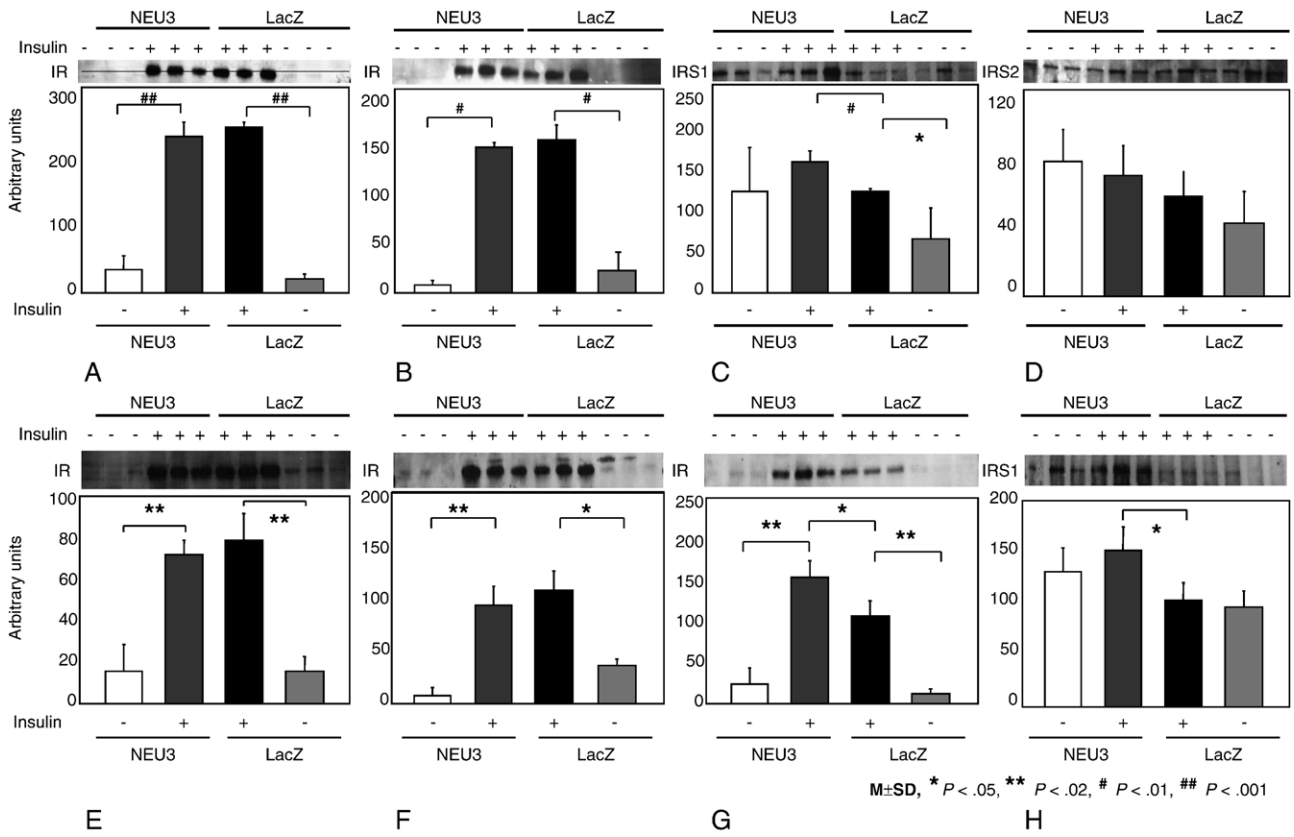

Fig. 7. Insulin-induced tyrosine phosphorylation of the insulin receptor (A, E), IRS1 (C, H), and IRS2 (D) in the livers, and tyrosine phosphorylation of the insulin receptor in skeletal muscles (B, F) and white adipose tissues (G) of C57BL/6 mice on standard (A-D) or high-fat diets (E-H). On day 4 after adenovirus injection, the mice that were fasted for 16 hours received an intravenous injection of 100 μL 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 ± SD. $^{*}P < .05$, $^{**}P < .02$, $^{\#}P < .01$, $^{\#\#}P < .001$, assessed by unpaired t test.

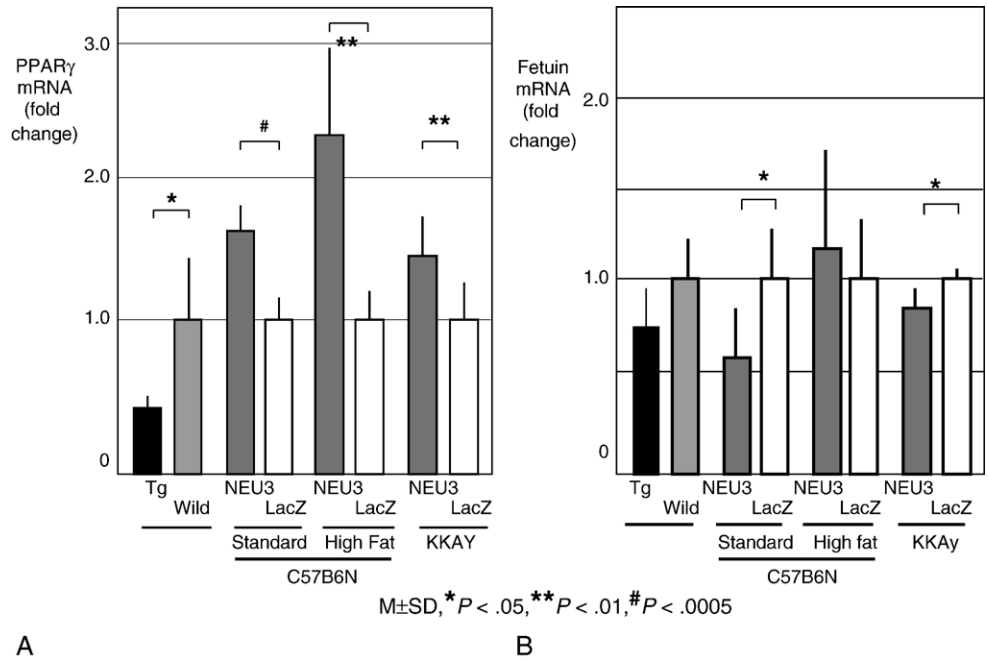

Fig. 8. The gene expression of PPARγ (A) and fetuin (B) in the livers from *NEU3* transgenic and wild C57BL/6 mice and AdNEU3- or AdLacZ-treated C57BL/6 mice on standard or high-fat diets and AdNEU3- or AdLacZ-treated KKAy mice. The relative mRNA levels of PPARγ and fetuin in the livers from *NEU3*-transgenic mice (Tg) and wild C57BL/6 mice [16], and mice treated with AdNEU3 or AdLacZ, 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 PPARγ, as well as the forward primer (5′-CCCAGTGTCTACTCTGGTGGA-3′) and the reverse primer (5′-CTGTGTTTGGAATAACTTGCAG-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 AdLacZ, respectively. The bars represent the fold change of PPARγ and fetuin relative to the mean expression in the controls ± 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 AdNEU3 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 PPARγ 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].

PPARγ expression in the liver is low compared with that in adipose tissues [29]; however, hepatic expression of PPARγ is functionally enhanced in a number of obesity models [30,31]. In addition, liver-specific disruption of PPARγ in obese (ob/ob) mice prevents hepatic steatosis but increases peripheral adiposity and decreases insulin sensitivity in muscle and fat [32]. Synthetic agonists of PPARγ, 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 PPARγ-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 PPARγ expression in liver. Thus, hepatic PPARγ plays important roles not only in the development of liver steatosis but also systemic insulin sensitivity. Increased PPARγ 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 PPARγ expression. Further investigation is needed to elucidate the molecular mechanism by which hepatic NEU3 expression increases hepatic PPARγ 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 PPARγ regulates systemic insulin sensitivity [30,32,34]. Increased hepatic expression of PPARγ in the AdNEU3 mice improves insulin sensitivity and glucose tolerance. In contrast, decreased PPARγ 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 PPARγ 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.

Acknowledgments

This study was supported by Grants-in-Aid for Basic Research Program CREST (Core Research for Evolutional Science and Technology) type from the Japan Science and Technology Agency and Grants-in-Aid for Scientific Research (70216399, 16390259, 16659236) to S. Suzuki from the Ministry of Education, Science, Sports and Culture of Japan.

We gratefully acknowledge the expert assistance of Chitose Suzuki, Takako Suganami, and Setsuko Moriya.

References

[1] Hakomori S, Yamamura S, Handa AK. Signal transduction through glyco (sphingo) lipids. Introduction and recent studies on glyco (sphingo) lipid-enriched microdomains. Ann N Y Acad Sci 1998;845:1-10.
[2] Rampersaud AA, Oblinger JL, Ponnappan RK, Burry RW, Yates AJ. Gangliosides and growth factor receptor regulation. Biochem Soc Trans 1999;27:415-22.
[3] Hakomori S. Traveling for the glycosphingolipid path. Glycoconj J 2000;17:627-47.
[4] Ikonen E, Vainio S. Lipid microdomains and insulin resistance: is there a connection? Sci STKE 2005;268:e3.
[5] Hakomori S. Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 2002;99:225-32.
[6] Allende ML, Proia RL. Lubricating cell signaling pathways with gangliosides. Curr Opin Struct Biol 2002;12:587-92.
[7] Miljan EA, Bremer EG. Regulation of growth factor receptors by gangliosides. Sci STKE 2002;160:RE15.
[8] Nojiri H, Stroud M, Hakomori S. A specific type of ganglioside as a modulator of insulin-dependent cell growth and insulin receptor tyrosine kinase activity. Possible association of ganglioside-induced inhibition of insulin receptor function and monocytic differentiation induction in HL-60 cells. J Biol Chem 1991;266:4531-7.
[9] Tagami S, Inokuchi Ji J, Kabayama K, Yoshimura H, Kitamura F, Uemura S, et al. Ganglioside GM3 participates in the pathological conditions of insulin resistance. J Biol Chem 2002;277:3085-92.
[10] Tsuruoka T, Tsuji T, Nojiri H, Holmes EH, Hakomori S. Selection of a mutant cell line based on differential expression of glycosphingolipid, utilizing anti-lactosylceramide antibody and complement. J Biol Chem 1993;268:2211-6.
[11] Yamashita T, Hashiramoto A, Haluzik M, Mizukami H, Beck S, Norton A, et al. Enhanced insulin sensitivity in mice lacking ganglioside GM3. Proc Natl Acad Sci U S A 2003;100:3445-9.
[12] Kabayama K, Sato T, Kitamura F, Uemura S, Kang BW, Igarashi Y, et al. TNFalpha-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. Glycobiology 2005;15:21-9.

[13] Wada T, Yoshikawa Y, Tokuyama S, Kuwabara M, Akita H, Miyagi T. Cloning, expression, and chromosomal mapping of a human ganglioside sialidase. Biochem Biophys Res Commun 1999;261:21-7.
[14] Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S, et al. Molecular cloning and characterization of a plasma membrane–associated sialidase specific for gangliosides. J Biol Chem 1999;274:5004-11.
[15] Saltiel AR, Pessin JE. Insulin signaling in microdomains of the plasma membrane. Traffic 2003;4:711-6.
[16] Sasaki A, Hata K, Suzuki S, Sawada M, Wada T, Yamaguchi K, et al. Overexpression of plasma membrane–associated sialidase attenuates insulin signaling in transgenic mice. J Biol Chem 2003;278:27896-902.
[17] Folch J, Lees M, Sloane Stanley GH, Stanley H. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497-509.
[18] Lo S, Russell JC, Taylor AW. Determination of glycogen in small tissue samples. J Appl Physiol 1970;28:234-6.
[19] Ogihara T, Shin BC, Anai M, Katagiri H, Inukai K, Funaki M, et al. Insulin receptor substrate (IRS)-2 is dephosphorylated more rapidly than IRS-1 via its association with phosphatidylinositol 3-kinase in skeletal muscle cells. J Biol Chem 1997;272:12868-73.
[20] Ono H, Shimano H, Katagiri H, Yahagi N, Sakoda H, Onishi Y, et al. Hepatic Akt activation induces marked hypoglycemia, hepatomegaly, and hypertriglyceridemia with sterol regulatory element binding protein involvement. Diabetes 2003;52:2905-13.
[21] Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y, Miyagi T. Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation. J Biol Chem 2000;275:8007-15.
[22] Hasegawa T, Carnero CF, Wada T, Itoyama Y, Miyagi T. Differential expression of three sialidase genes in rat development. Biochem Biophys Res Commun 2001;280:726-32.
[23] Chien J-L, Hogan EL. Novel pentahexosyl ganglioside of the globo series purified from chicken muscle. J Biol Chem 1983;258:10727-30.
[24] Nakamura K, Hashimoto Y, Yamakawa T, Suzuki A. Genetic polymorphism of ganglioside expression in mouse organs. J Biochem (Tokyo) 1988;103:201-8.
[25] Yki-Jarvinen H, Westerbacka J. The fatty liver and insulin resistance. Curr Mol Med 2005;5:287-95.
[26] Stiles B, Wang Y, Stahl A, Bassilian S, Lee WP, et al. Liver-specific deletion of negative regulator Pten results in fatty liver and insulin hypersensitivity. Proc Natl Acad Sci U S A 2004;101:2082-7.
[27] Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, et al. PGC-1 promotes insulin resistance in liver through PPAR-alpha–dependent induction of TRB-3. Nat Med 2004;10:530-4.
[28] Xu A, Lam MC, Chan KW, Wang Y, Zhang J, Hoo RL, et al. Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. Proc Natl Acad Sci U S A 2005;102:6086-91.
[29] Fajas L, Auboeuf D, Raspé E, Schoonjans K, Lefebvre AM, Saladin R, et al. The organization, promoter analysis, and expression of the human PPAR gene. J Biol Chem 1997;272:18779-89.
[30] Burant CF, Sreenan S, Hirano K, Tai TAC, Lohmiller J, Lukens J, et al. Troglitazone action is independent of adipose tissue. J Clin Invest 1997;100:2900-8.
[31] Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, et al. Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. J Clin Invest 2000;106:1221-8.
[32] Matsusue K, Haluzik M, Lambert G, Yim SH, Gavrilova O, Ward JM, et al. Liver-specific disruption of PPAR in leptin-deficient mice improves fatty liver but aggravates diabetic phenotypes. J Clin Invest 2003;111:737-47.
[33] Kim SY, Kim HI, Park SK, Im SS, Li T, Cheon HG, et al. Liver glucokinase can be activated by peroxisome proliferator–activated receptor-gamma. Diabetes 2004;53:S66.
[34] Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, Imai J, et al. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. Science 2006;312:1656-9.
[35] Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, et al. Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol Endocrinol 1993;7:1445-55.
[36] Kalabay L, Chavin K, Lebreton JP, Robinson KA, Buse MG, Arnaud P. Human recombinant alpha 2-HS glycoprotein is produced in insect cells as a full length inhibitor of the insulin receptor tyrosine kinase. Horm Metab Res 1998;30:1-6.
[37] Mathews ST, Singh GP, Ranalletta M, Cintron VJ, Qiang X, Goustin AS, et al. Improved insulin sensitivity and resistance to weight gain in mice null for the Ahsg gene. Diabetes 2002;51:2450-8.