

Increased Systemic Glucose Tolerance with Increased Muscle Glucose Uptake in Transgenic Mice Overexpressing RXR γ in Skeletal Muscle

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Abstract

Background: Retinoid X receptor (RXR) γ is a nuclear receptor-type transcription factor expressed mostly in skeletal muscle, and regulated by nutritional conditions. Previously, we established transgenic mice overexpressing RXR γ in skeletal muscle (RXR γ mice), which showed lower blood glucose than the control mice. Here we investigated their glucose metabolism.

Methodology/Principal Findings: RXR γ mice were subjected to glucose and insulin tolerance tests, and glucose transporter expression levels, hyperinsulinemic-euglycemic clamp and glucose uptake were analyzed. Microarray and bioinformatics analyses were done. The glucose tolerance test revealed higher glucose disposal in RXR γ mice than in control mice, but insulin tolerance test revealed no difference in the insulin-induced hypoglycemic response. In the hyperinsulinemic-euglycemic clamp study, the basal glucose disposal rate was higher in RXR γ mice than in control mice, indicating an insulin-independent increase in glucose uptake. There was no difference in the rate of glucose infusion needed to maintain euglycemia (glucose infusion rate) between the RXR γ and control mice, which is consistent with the result of the insulin tolerance test. Skeletal muscle from RXR γ mice showed increased Glut1 expression, with increased glucose uptake, in an insulin-independent manner. Moreover, we performed *in vivo* luciferase reporter analysis using *Glut1* promoter (*Glut1*-Luc). Combination of RXR γ and PPAR δ resulted in an increase in *Glut1*-Luc activity in skeletal muscle *in vivo*. Microarray data showed that RXR γ overexpression increased a diverse set of genes, including glucose metabolism genes, whose promoter contained putative PPAR-binding motifs.

Conclusions/Significance: Systemic glucose metabolism was increased in transgenic mice overexpressing RXR γ . The enhanced glucose tolerance in RXR γ mice may be mediated at least in part by increased Glut1 in skeletal muscle. These results show the importance of skeletal muscle gene regulation in systemic glucose metabolism. Increasing RXR γ expression may be a novel therapeutic strategy against type 2 diabetes.

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Introduction

The skeletal muscle, known as the largest organ in the human body, plays an important role in exercise, energy expenditure, and

glucose metabolism. It is a major site of glucose disposal [1,2]. In type 2 diabetic subjects, glucose uptake in the skeletal muscle is impaired [2]. Blood glucose is taken up by the skeletal muscle via insulin-dependent and independent glucose transporters (Glut4

and Glut1, respectively) [3], where it is converted into glycogen. Increasing capacity of glucose uptake in the skeletal muscle is considered beneficial for the prevention and treatment of type 2 diabetes [3,4]. On the other hand, glucose metabolism in the skeletal muscle may affect the whole body metabolism; the skeletal muscle-specific inactivation of *Glut4* has resulted in defect in insulin action in the adipose tissue (glucose uptake) and liver (suppression of gluconeogenesis) [5]. Thus, identification of the molecular mechanisms involved in skeletal muscle glucose metabolism should help clarify the pathophysiology of diabetes.

Nuclear receptors are part of a large superfamily of transcription factors that includes receptors for steroids, retinoic acid, and thyroid hormones [6]. RXRs are heterodimeric partners of many nuclear receptors, such as retinoic acid receptors (RARs), thyroid hormone receptors (TRs), liver X receptors (LXRs), peroxisome proliferator activated receptors (PPARs), and RXRs themselves [6]. Among RXR heterodimer partners, activation of PPAR δ in the skeletal muscle increases insulin sensitivity [7]. The RXR subfamily consists of RXR α , RXR β , and RXR γ [6,8]. Although RXR γ is preferentially expressed in the skeletal muscle, its functional role is poorly understood. We have recently found that expression of retinoid X receptor γ (RXR γ) is changed in the skeletal muscle under nutritional conditions; RXR γ mRNA expression is down-regulated by fasting and recovered by refeeding [9]. In an attempt to explore the role of RXR γ in the skeletal muscle, we established transgenic mice overexpressing RXR γ in the skeletal muscle (RXR γ mice) and found that they exhibit increased triglyceride contents in the skeletal muscle as a result of increased expression of sterol regulatory element binding protein 1c (SREBP1c), a transcriptional master regulator of lipogenesis [9]. Indeed, RXR γ has been shown to enhance SREBP1c gene expression in C2C12 myocytes *in vitro* at least in part by heterodimerization with LXR [9]. On the other hand, we also found that blood glucose levels are lower in RXR γ mice than in control mice [9]. These observations, taken together, suggest that RXR γ plays a critical role in glucose and lipid metabolism in the skeletal muscle. However, the molecular mechanism involved in RXR γ regulation of glucose metabolism in the skeletal muscle and how it affects systemic glucose metabolism are poorly understood.

Here, we demonstrate enhanced glucose metabolism with increased Glut1 expression and glucose uptake in RXR γ mice. This study suggests that activation of the skeletal muscle RXR γ is a novel therapeutic strategy to treat or prevent type 2 diabetes.

Methods

Animals

C57BL6 mice were purchased from Charles River Japan (Yokohama, Japan). Generation of RXR γ mice under the control of the human α -actin promoter was described previously [9]. They were allowed free access to food (CRF-1; Charles River) and water, unless otherwise stated. All animal experiments were approved by Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (approval ID: No. 0090041).

Blood analysis

Serum samples were obtained from mice, when fed *ad libitum*. Serum glucose levels were measured by the blood glucose test meter (Glutest PRO R; Sanwa-Kagaku, Nagoya, Japan). Serum concentrations of insulin were determined by the enzyme-linked immunosorbent assay (ELISA) kits (Morinaga Institute of Biological Science, Inc., Yokohama, Japan).

Glucose and insulin tolerance tests

For glucose tolerance test, D-glucose (1 mg/g of body weight, 10% (w/v) glucose solution) was administered by intraperitoneal injection after an overnight fast. For insulin tolerance test, human insulin (Humulin R; Eli Lilly Japan K.K., Kobe, Japan) was injected intraperitoneally (0.75 mU/g of body weight), when fed *ad libitum*.

Hyperinsulinemic-euglycemic clamp studies

We analyzed as described previously [10–12] with slight modification. Two days before the clamp studies, a catheter was inserted into the right jugular vein for infusion under general anesthesia with sodium pentobarbital. Studies were performed on mice under conscious and unstressed conditions after a 4-h fast. The clamp study began with a prime (4 mg/kg for 5 min) of [6,6-²H] glucose (Cambridge Isotope Laboratories, Inc, Andover, MA) followed by continuous infusion at a rate of 0.5 mg/kg per minute for 2 hr to assess the basal glucose turnover. After the basal period, hyperinsulinemic-euglycemic clamp was conducted for 120 min with a primed/continuous infusion of human insulin (25 mU/kg prime for 5 min, 5 mU/kg/min infusion) and variable infusion of 20% glucose to maintain euglycemia (approximately 100 mg/dl). The 20% glucose was enriched with [6,6-²H] glucose to approximately 2.5% as previously described [12]. To determine the enrichment of [6,6-²H]glucose in plasma at basal and insulin stimulated state, samples were deproteinized with trichloroacetic acid and derivatized with *p*-aminobenzoic acid ethyl ester. The atom percentage enrichment of glucose_{m+2} was then measured by high-performance liquid chromatography with LTQ-XL-Orbitrap mass spectrometer (Thermo Scientific, CA). The glucose_{m+2} enrichment was determined from the *m/z* ratio 332.2: 330.2. The hepatic glucose production was calculated by using the rate of infusion of [6,6-²H]glucose over the atom percent excess in the plasma minus the rate of glucose being infused. The insulin-stimulated whole-body glucose uptake was calculated by adding the total glucose infusion rate plus the hepatic glucose production [12].

Quantitative real-time PCR. Quantitative real-time PCR was performed as described [9]. Total RNA was prepared using Sepazol (Nacalai Tesque, Kyoto, Japan). cDNA was synthesized from 5 μ g of total RNA using Superscript II reverse transcriptase (Invitrogen Inc., Carlsbad, CA) with random primers. Gene expression levels were measured with an ABI PRISM 7700 using SYBR Green PCR Core Reagents (Applied Biosystems, Tokyo, Japan). The primers used were as follows, RXR γ : Fw: 5'-CAC-

Table 1. Body and dissected tissue weight and blood glucose level in RXR γ mice (4-3 line).

	Wild-type	RXR γ
Body weight (g)	26.4 \pm 0.3	26.5 \pm 0.4
Epididymal fat mass (g)	0.138 \pm 0.004	0.156 \pm 0.090
Gastrocnemius muscle weight (g)	0.282 \pm 0.004	0.238 \pm 0.008**
Liver weight (g)	1.290 \pm 0.029	1.321 \pm 0.033
Glucose (mg/dL) basal	170.0 \pm 9.4	145.6 \pm 4.9*
Glucose (mg/dL) fasting	74.4 \pm 1.7	67.0 \pm 0.6**

Mice were males 12 weeks of age. The number of animals used was 6 for both wild-type control and RXR γ mice.

* $P < 0.05$;

** $P < 0.01$, compared with wild-type control.

Values are the means \pm SE. These samples were also used in Fig 2A, B, and D. doi:10.1371/journal.pone.0020467.t001

Table 2. Body and dissected tissue weight and blood glucose level in RXR γ mice (5-3 line).

	Wild-type	RXR γ
Body weight (g)	36.0 \pm 1.8	31.9 \pm 1.2
Epididymal fat mass (g)	1.030 \pm 0.191	0.861 \pm 0.134
Gastrocnemius muscle weight (g)	0.305 \pm 0.016	0.214 \pm 0.020**
Liver weight (g)	1.375 \pm 0.119	1.320 \pm 0.066
Glucose (mg/dL) basal	142.5 \pm 8.6	117.3 \pm 8.2
Glucose (mg/dL) fasting	67.2 \pm 1.6	53.3 \pm 2.5**

Mice were males 38 weeks of age. The number of animals used was 6 for both wild-type control and RXR γ mice.

** $P < 0.01$, compared with wild-type control.

Values are the means \pm SE.

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CCTGGAGGCCTATACCA-3', Rv: 5'-AAACCTGCCTGG-CTGTTCC-3', Glut1: Fw: 5'-CCAGC TGGGA ATCGT CGTT-3', Rv: 5'-CAAGT CTGCA TTGCC CATGAT-3', Glut4: Fw: 5'-TCTGTGGGTGGCATGATCTCT-3', Rv: 5'-GCCCT-TTTCCTTCCCAACC-3', glucose phosphate isomerase 1: Fw: 5'-AGCGCTTCAACAACCTTCAGCT-3', Rv: 5'-CAGAATAT-GCCCATGGTTGGT-3', phosphoglycerate mutase 1: Fw: 5'-TCCTGAAACATCTGGAAGGTATCTC-3', Rv: 5'-CAGTG-GGCAGAGTGATGTTGAT-3', fructose biphosphatase 2: Fw: 5'-TGAATGCAATCCTGTGGCC-3', Rv: 5'-TGGTTGCCA-TACCTCCTGCT-3', pyruvate dehydrogenase kinase isoenzyme 1: Fw: 5'-GGACTTCTATGCGCGCTTCT-3', Rv: 5'-CTGA-

CCCGAAGTCCAGGAAC-3', glycogen synthase 2: Fw: 5'-AG-GATCATTCCAGAGGAACCGC-3', Rv: 5'-CCAGTCCAGGA-GATCTGAGAGC-3'.

Tissue sampling for analysis of Glut1 and Glut4 protein levels

Skeletal muscles were homogenized in ice-cold buffer containing 250 mmol/L sucrose, 20 mmol/L 2-[4-(2-hydroxyethyl)-1-piperadiny] ethansulfonic acid (HEPES) (pH 7.4), and 1 mmol/L EDTA, and centrifuged at 1200 g for 5 minutes. The supernatant was centrifuged at 200 000 g for 60 minutes at 4°C [13]. The resulting pellet was solubilized in Laemmli sample buffer containing dithiothreitol. Samples were subjected to Western blotting as described [14]. Antibodies used were those against Glut1 (#07-1401, Millipore, Temecula, CA), and Glut4 (SC-1606, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Muscle incubation and glucose transport

Glucose transport was measured as described [15]. Mice were fasted overnight and killed. The extensor digitorum longus (EDL) muscles were rapidly removed, each of which was mounted on the incubation apparatus and preincubated in Krebs-Ringer bicarbonate (KRB) buffer containing 2 mmol/l pyruvate for 30 min. The muscles were then incubated in KRB buffer in the absence or presence of 50 mU/ml insulin for 10 min. The buffers were kept at 37°C throughout the experiment and gassed continuously with 95% O₂ and 5% CO₂. Immediately after incubation, the muscle was transferred to KRB buffer containing 1 mmol/l 2-[³H]-deoxy-d-glucose (1.5 μ Ci/ml) and 7 mmol/l d-[¹⁴C]-mannitol (0.3 μ Ci/ml).

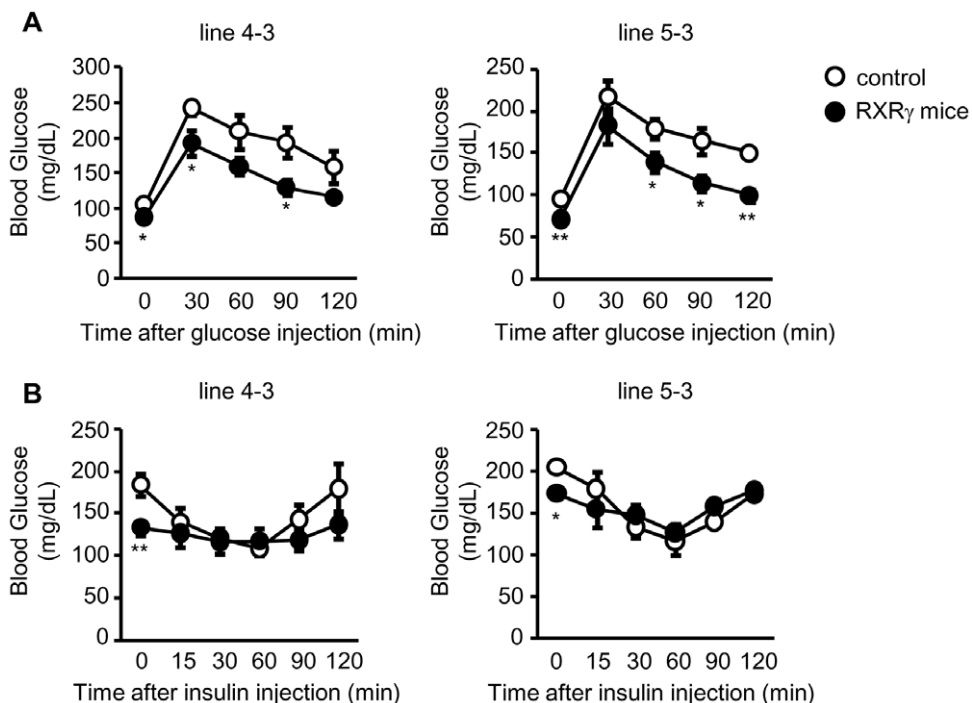


Figure 1. Glucose tolerance and insulin tolerance tests on RXR γ mice. (A, B) In A and B, male mice, 5 months of age, were used. The number of animals used was 6 for both control (open circles) and RXR γ mice (filled circles) of line 4-3, and 5 for both control (open circles) and RXR γ (filled circles) mice of line 5-3. * $P < 0.05$ and ** $P < 0.01$ compared with respective control.

doi:10.1371/journal.pone.0020467.g001

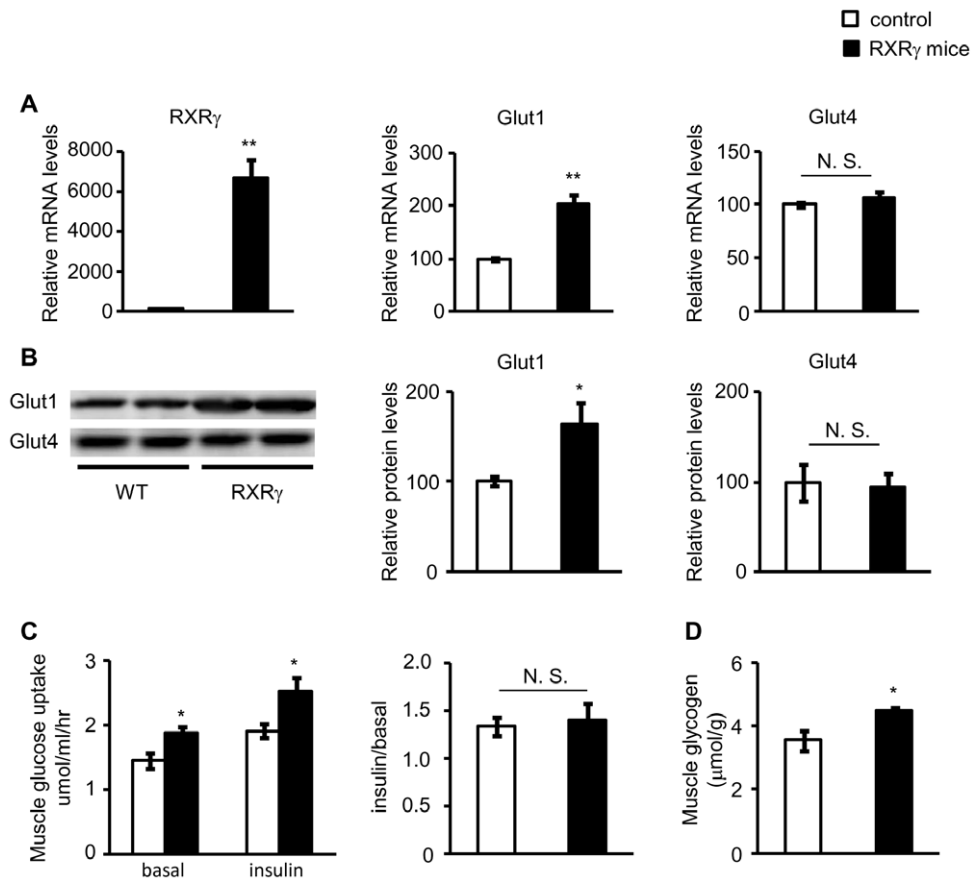


Figure 2. Levels of Glut 1 and Glut4, and glucose uptake in the skeletal muscle of RXR γ mice. (A) Gene expressions of RXR γ , Glut1, and 4 were examined by quantitative real-time PCR. The value for wild-type (littermates of line 4-3) mice was set at 100, and relative values are shown. (B) Protein levels of Glut1 and Glut4 were examined by Western blotting. Results of relative densitometric signal for Glut1 and 4 are shown. (C) Glucose uptake in the absence or presence of insulin and (D) glycogen content were increased in the skeletal muscle of RXR γ mice. Ratio of enhanced glucose uptake in the presence of insulin (insulin/basal) was similar in control and RXR γ mice. In A, B and D, the same samples were used. Mice were males of 12 weeks of age. The number of animals was 6 for both control (open bars) and RXR γ (filled bars) mice. These samples were also used in Table 1. In C, mice were males of 24–27 weeks of age. The number of animals was 6 for both control (open bars) and RXR γ (filled bars) mice. * $P < 0.05$ and ** $P < 0.01$ compared with respective control. N. S., not significant. doi:10.1371/journal.pone.0020467.g002

Measurement of skeletal muscle glycogen

The skeletal muscle glycogen content was measured as glycosyl units after acid hydrolysis [16]. The skeletal muscle samples were minced, 50 mg of which was added to 1 ml of 0.3 M perchloric acid and homogenized on ice. One ml of 1 N HCl was added and incubated for 2 h at 100°C, thereafter, 1 ml of 1 N NaOH was added at room temperature. Glucose content was examined using the F-kit glucose (Roche Diagnostics, Mannheim, Germany).

Plasmids

The coding region of mouse RXR γ and PPAR δ cDNA were subcloned into a mammalian expression plasmid, pCMX [9]. The 1.5-kb 5'-flanking region of the mouse Glut1 promoter was obtained by PCR with mouse genomic DNA (RefSeqs number: NC_000070). The PCR primers used were: Fw: 5'-GTGGTG-CGCGCCTGTAGTCC-3' and Rv: 5'-GGCGCACTCCACG-GATGCCG-3'. The fragment was subcloned into the pGL3-basic luciferase vector (Promega Corporation, Madison, WI). The promoter regions used were: -1500 to +75, -647 to +75, and -152 to +75, counting the transcription start site as +1.

Electroporation and *in vivo* luciferase reporter analysis

In vivo electroporation was performed according to the modified method of Aihara and Miyazaki [17]. Under the pentobarbital anesthesia (30 mg/kg), bilateral quadriceps muscles from C57BL/6

Table 3. Body weight, blood glucose and plasma insulin levels in RXR γ mice in clamp study.

		Wild-type	RXR γ
	Body weight (g)	24.1 \pm 1.2	23.4 \pm 0.3
Basal period	Glucose (mg/dL)	141.8 \pm 15.3	106.6 \pm 5.5*
	Insulin (ng/mL)	0.55 \pm 0.08	0.44 \pm 0.07
Clamp period	Glucose (mg/dL)	92.5 \pm 3.8	96.7 \pm 3.6
	Insulin (ng/mL)	2.60 \pm 0.20	2.38 \pm 0.33

Mice were males 13–16 weeks of age. The number of animals used was 6 for wild-type control mice and 7 for RXR γ mice.

* $P < 0.05$, compared with wild-type control.

Values are the means \pm SE. These mice were also used in Fig 3.

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mice (male, 12 weeks of age) were injected with 80 μ g of plasmid DNA (25 μ l) by using a 29-gauge needle attached to a 0.5-ml insulin syringe (Terumo Corporation, Tokyo, Japan). Square-wave electrical pulses (160 V/cm) were applied six times with an electrical pulse generator (CUY21EDIT, Nepa Gene Co. Ltd., Chiba, Japan) at a rate of one pulse per second, with each pulse being 20 ms. in duration. The electrodes were a pair of stainless steel needles inserted into the quadriceps muscles and fixed 5 mm apart. Seven days after gene delivery, the muscles were removed and subjected to analysis. Frozen muscle tissues were homogenized in ice-cold passive lysis buffer from Promega. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was reserved for luciferase assay using Promega's dual luciferase assay kit. The luciferase activity was calculated as the ratio of

firefly to Renilla (internal control) luciferase activity and represented as the average of triplicate experiments.

Computer-based DNA sequence motif search

We used MATCH software [18] (BIOBASE GmbH, Wolfenbuettel, Germany) to investigate transcriptional binding sites in the mouse *Glut1* promoter. We investigated mouse genome in the region of -1500 to +100 relative to transcription start of *Glut1* (Chr.4 11878131(+)).

cDNA microarray analysis

RNA was isolated from the skeletal muscle of sex- and age-matched RXR γ mice (line 4-3) and non-transgenic control mice

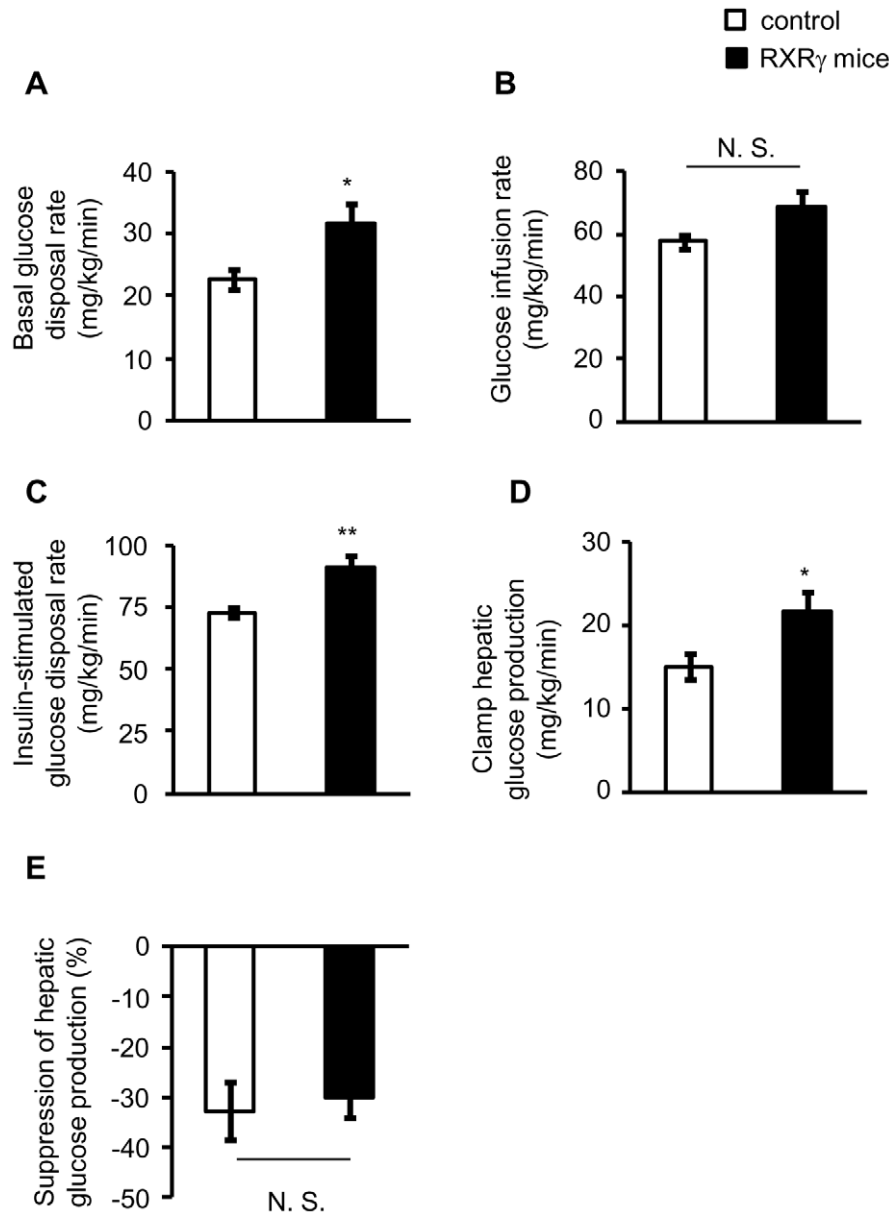


Figure 3. Hyperinsulinemic-euglycemic clamp test in RXR γ mice, fed a chow diet. (A) Basal glucose disposal rate, (B) glucose infusion rate needed to maintain euglycemia, (C) insulin-stimulated glucose disposal rate and (D) clamp hepatic glucose production (hepatic glucose production during the clamp period) (E) suppression of hepatic glucose production during the clamp period in RXR γ mice. Male mice, 13~16 weeks of age, were used. The number of animals used was 6 for control mice (open bars) and 7 for RXR γ mice (filled bars). * $P < 0.05$ and ** $P < 0.01$ compared with respective control. N. S., not significant. doi:10.1371/journal.pone.0020467.g003

(females at 4 months of age, five samples from each group were combined). Each of the combined samples was hybridized to the Affymetrix MG430 microarray, which contains 45,102 genes, including expressed sequence tags (ESTs), and analyzed with the software Affymetrix Gene Chip 3.1. Of the 45,102 genes including ESTs analyzed, 8,054 (non-transgenic control mice) and 8,083 (transgenic) were expressed at a substantial level (absolute call is present and average difference is above 150). In order of fold changes in gene expression levels in skeletal muscle from RXR γ mice relative to control mice, genes whose expression was increased more than 2^{0.4}-fold in RXR γ mice were listed (Dataset S1). Fold changes were calculated as an indication of the relative change of each transcript represented on the probe array. Differentially expressed genes were identified using the following criteria; 'absolute call' is present, and 'average difference' was above 250. 'Absolute call', which was calculated with this software using several markers, is an indicator of the presence or absence of each gene transcript. The 'average difference' value is a marker of the abundance of each gene, obtained by comparing the intensity of hybridization to 20 sets of perfectly matched 25-mer oligonucleotides relative to 20 sets of mismatched oligonucleotides using Affymetrix Gene Chip 3.1 software. All data of microarray is MIAME compliant and that the raw data has been deposited in a MIAME compliant database (GEO), whose accession number is GSE28448.

Gene Ontology Analysis

We used DAVID v6.7 [19] for gene ontology (GO) analysis. DAVID is a web application providing a comprehensive set of functional annotation tools to understand the biological meaning behind a large list of genes. Functional Annotation Clustering of DAVID was applied to the genes whose gene expression increased in RXR γ mice. Our GO analysis produced 62 GO terms from gene sets from RXR γ mice with increased expression compared to the wild-type mice, under the condition of $P < 0.05$ (P value from Fisher's Exact Test). The obtained GO terms contained many similar functional concepts. In order to group similar GO terms,

we applied the Functional Annotation Clustering tool provided by DAVID [19,20]. Twenty-three clusters were produced with genes showing increased expression. We showed one GO term of the lowest P value of all the members of an individual cluster.

Transcriptional factor binding sites analysis

We used MATCH software [18] with BKL TRANSFAC 2010.3 Release (BIOBASE GmbH, Worfenbuettel, Germany) to investigate transcriptional binding sites in the promoter regions of genes. The F-Match algorithm compares the number of sites found in a query sequence set against the background set. It is assumed, if a certain transcription factor (or factor family), alone or as a part of a *cis*-regulatory module, plays a significant role in the regulation of the considered set of promoters, then the frequency of the corresponding sites found in these sequences should be significantly higher than expected by random chance. We investigated the mouse genome in the region of -1000 to +100 relative to the transcription start of an individual gene. Statistical hypothesis testing was evaluated against housekeeping genes of mice. We investigated promoter regions of 15 genes: that is 14 'glucose metabolic process' genes with increased expression in RXR γ mice, plus *Glut1*. In the GO analysis, *Glut1* was categorized as a 'transporter' not 'glucose metabolic process' gene.

Statistical analysis

Statistical analysis was performed using the Student's t test and analysis of variance (ANOVA) followed by Scheffe's test. Data were expressed as the mean \pm SE. $P < 0.05$ was considered statistically significant.

Results

Increased glucose metabolism in RXR γ mice

In our previous study, we established two lines of RXR γ mice (named lines 4-3 and 5-3) with similar expression levels of the RXR γ transgene and protein specifically in the skeletal muscle [9]. There was no significant difference in body weight, adipose tissue,

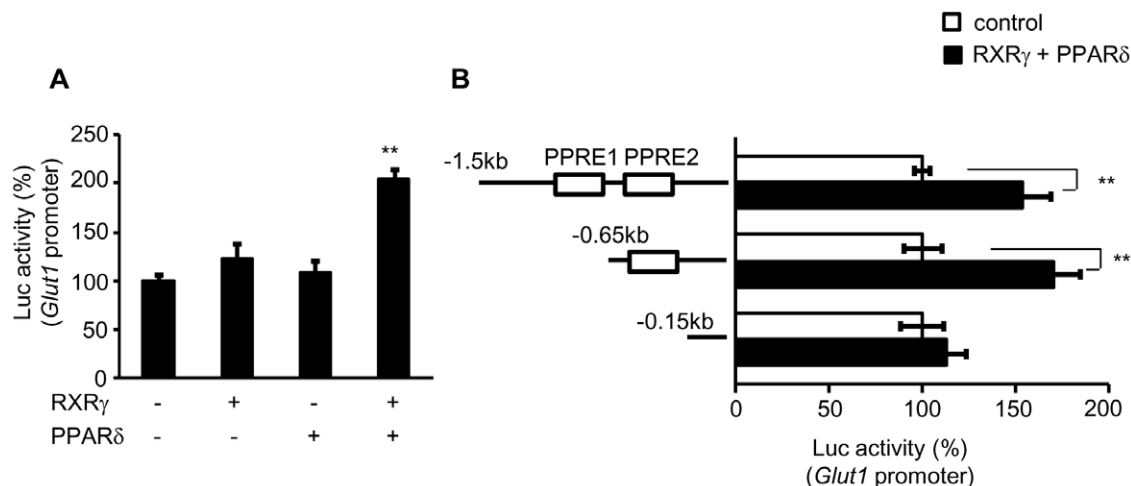


Figure 4. Transient transfection-reporter assay of the effect of RXR γ on *Glut1* promoter. (A) *Glut1*-Luc plasmid, with or without RXR γ and/or PPAR δ expression vectors, was transfected into the quadriceps muscle of C57BL6 mice. Activation of the luciferase reporter gene was measured in relative light units and normalized to dual luciferase activity. Mean values from experiments ($n = 5$) are shown as fold induction, where the activity in the absence of RXR γ is the reference value (set at 100). (B) Schematic representations of serial deletion of *Glut1* promoter constructs are shown in the figure. Squares denote the putative PPAR/RXR binding sites. Open bars; *Glut1*-Luc without RXR γ and PPAR δ expression vectors, and filled bars; *Glut1*-Luc with RXR γ and PPAR δ expression vectors. The activity in the absence of RXR γ and PPAR δ in each experiment for different *Glut1*-Luc construct in the reference value (set at 100). ** $P < 0.01$, compared with the value of wild-type promoter in the absence of RXR γ /PPAR δ . doi:10.1371/journal.pone.0020467.g004

and liver weight between RXR γ and control mice in both lines 4-3 and 5-3, although the skeletal muscle weight was slightly lower in RXR γ mice than in control mice (Tables 1 and 2). In this study, blood glucose in RXR γ mice was lower than in control mice (fasting state, $P < 0.01$ in lines 4-3 and 5-3; basal state, $P < 0.05$ in line 4-3 and $P = 0.06$ in line 5-3), which is consistent with our previous report [9].

To elucidate the role of the skeletal muscle RXR γ in systemic glucose metabolism, we performed glucose and insulin tolerance tests in RXR γ mice. The glucose tolerance test revealed increased glucose disposal in RXR γ mice relative to control mice (Fig. 1A). On the other hand, there was no significant difference in the insulin-induced hypoglycemic response between genotypes (Fig. 1B). These observations suggest that RXR γ mice have a higher capacity for glucose disposal with no change in insulin sensitivity.

Increased Glut1 expression and glucose uptake in the skeletal muscle of RXR γ mice

As both lines of RXR γ mice showed increased glucose metabolism, for the following experiments, we only utilized the line 4-3 of RXR γ mice (hereafter just RXR γ mice). Interestingly, mRNA expression of *Glut1* was increased in the skeletal muscle from RXR γ mice relative to control mice ($P < 0.01$), whereas that of *Glut4* was unchanged (Fig. 2A). We also observed that Glut1 is significantly increased in RXR γ mice at the protein level ($P < 0.05$), with no significant difference in Glut4 between genotypes

(Fig. 2B). Consistently, we also found increased glucose uptake in the skeletal muscle from RXR γ mice relative to control mice ($P < 0.05$), which was not further enhanced in the presence of insulin (Fig. 2C). We also observed increased glucose glycogen content in the skeletal muscle from RXR γ mice relative to control mice ($P < 0.05$, Fig. 2D).

Increased basal glucose disposal rate of RXR γ mice

To gain further insight into the glucose metabolism in RXR γ mice, we performed a hyperinsulinemic-euglycemic clamp study. Plasma insulin concentrations during the basal period were similar between genotypes (Table 3). The basal glucose disposal rate was significantly increased in RXR γ mice than in the controls ($P < 0.05$, Fig. 3A), supporting that an insulin-independent increase in glucose uptake occurred, as observed in Fig. 2C. Meanwhile, we observed that the rate of glucose infusion needed to maintain euglycemia (glucose infusion rate) was similar between genotypes (Fig. 3B), which is consistent with the result of the insulin tolerance test (Fig. 1B). On the other hand, insulin-stimulated glucose disposal rate was higher in RXR γ mice than in the controls ($P < 0.01$, Fig. 3C), which probably reflects the increased basal glucose disposal rate (Fig. 3A). Also, the clamp hepatic glucose production (hepatic glucose production during the clamp period) was higher in RXR γ mice than in the controls ($P < 0.05$, Fig. 3D). Meanwhile, hepatic glucose production was similarly suppressed by insulin in both genotypes (Fig. 3E). Together, these data support the idea that Glut1, an insulin-independent glucose transporter, is involved in the increased glucose disposal in the skeletal muscle of RXR γ mice.

Activation of the *Glut1* promoter by combination of RXR γ and PPAR δ in the skeletal muscle *in vivo*

To examine whether RXR γ directly activates mRNA expression of *Glut1*, we performed the *in vivo* luciferase reporter analysis

Table 4. Gene Ontology Analysis.

GO ID	GO Term	P value
GO:0060537	muscle tissue development	6.91E-05
GO:0006461	protein complex assembly	6.78E-05
GO:0008104	protein localization	1.18E-04
GO:0051146	striated muscle cell differentiation	6.06E-04
GO:0030334	regulation of cell migration	8.04E-04
GO:0006886	intracellular protein transport	0.002367379
GO:0019220	regulation of phosphate metabolic process	0.00312153
GO:0048514	blood vessel morphogenesis	0.00172443
GO:0045859	regulation of protein kinase activity	0.00563927
GO:0006915	apoptosis	0.007513423
GO:0006006	glucose metabolic process	0.002958786
GO:0006917	induction of apoptosis	0.005257921
GO:0042981	regulation of apoptosis	0.014518039
GO:0043066	negative regulation of apoptosis	0.022995692
GO:0006633	fatty acid biosynthetic process	0.012664127
GO:0032956	regulation of actin cytoskeleton organization	0.027565775
GO:0043388	positive regulation of DNA binding	0.021966247
GO:0006469	negative regulation of protein kinase activity	0.045894089
GO:0016477	cell migration	0.045248711
GO:0030521	androgen receptor signaling pathway	0.027402985
GO:0055003	cardiac myofibril assembly	0.020081231
GO:0000165	MAPKKK cascade	0.032045331
GO:0046825	regulation of protein export from nucleus	0.027402985

738 genes up-regulated in RXR γ mice compared with wild-type mice by microarray (Listed in Dataset S1) were classified into GO functional annotations, as described in Methods.

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Table 5. List of 'glucose metabolic process' genes.

Gene Symbol	Gene description
Atf3	Activating transcription factor 3
Bpgm	2-3-bisphosphoglycerate mutase
Dcxr	Dicarbonyl L-xylulose reductase
Fbp2	Fructose bisphosphatase 2
Gbe1	Glucan branching enzyme 1
Gpd1l	Glycerol-3-phosphate dehydrogenase 1-like
Gpi1	Glucose phosphate isomerase 1
Gys2	Glycogen synthase 2
Igf2	Insulin-like growth factor 2
Mat2b	Methionine adenosyltransferase II, beta
Nisch	Nischarin; an imidazoline receptor
Pdk1	Pyruvate dehydrogenase kinase isoenzyme 1
Pgam1	Phosphoglycerate mutase 1
Pgd	Phosphogluconate dehydrogenase
Pgm2l1	Phosphoglucomutase 2-like 1
Phkb	Phosphorylase kinase beta

Up-regulated genes in RXR γ mice in the microarray, classified as 'glucose metabolic process' genes by GO analysis, as described in Method. Genes are listed in alphabetic order of gene symbol. Glut1, which appeared in the up-regulated list in the microarray (Dataset S1), is not included in this list, as it was classified 'transporter' in the GO analysis.

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using *Glut1* promoter (*Glut1*-Luc). In this study, the activity of *Glut1*-Luc was marginally enhanced by RXR γ alone (Fig. 4A). Moreover, we found no significant activation of the *Glut1*-Luc by PPAR δ , an important regulator of glucose as well as lipid metabolism in the skeletal muscle [7]. Interestingly, combination of RXR γ and PPAR δ resulted in significant increase in *Glut1*-Luc activity in the skeletal muscle *in vivo* ($P < 0.01$) (Fig. 4A). Motif search analysis revealed two putative PPAR-responsive elements (PPRE1 and PPRE2) ($-657/-645$ and $-520/-508$, respectively) in the mouse *Glut1* promoter. A series of deletion mutant analysis showed that combination of RXR γ and PPAR δ activates the regions of $-1500/+75$, and $-647/+75$, but not the region of $-152/+75$ in the *Glut1* promoter (Fig. 4B), suggesting that the second putative PPRE ($-520/-508$) is involved in the RXR γ /PPAR δ -induced *Glut1* promoter activation.

Microarray and bioinformatics analyses of up-regulated gene in RXR γ mice

In order to gain insight into the gene expression change in RXR γ mice, we performed microarray analysis. As shown in Dataset S1, 738 genes were up-regulated in the analysis. As expected, *Glut1* expression was increased in the microarray data. Also, SREBP1c expression, which we previously reported [9], was increased in the microarray data. Using the data, we performed GO analysis to determine if genes, up-regulated in RXR γ mice,

are associated with particular biological processes. Our GO analysis revealed genes with increased expression in the RXR γ mice in various categories (Table 4), including 'glucose metabolic process' genes and 'fatty acid biosynthetic process' genes, which indicated that overexpression of RXR γ affects the expression of many genes. The up-regulated genes categorized as glucose metabolism genes in GO term are listed in Table 5. Among them, we confirmed enhanced gene expression by quantitative real time PCR (Fig. 5), supporting the microarray data reliable. Moreover, we calculated the ratio of putative transcription factor binding motifs in glucose metabolism genes, which were up-regulated in RXR γ mice. In the sample, several motifs showed statistical significance (Table 6) ($P < 0.05$), including PPAR responsive elements. These data suggest that glucose metabolism genes up-regulated in RXR γ mice are possible target genes of the RXR γ and PPAR heterodimer.

Discussion

RXR γ is a nuclear receptor-type transcription factor that is expressed abundantly in the skeletal muscle and is regulated by nutritional conditions. Treatment of obese and diabetic mice with RXR pan-agonists (agonists for all the RXR isoforms) has improved glucose metabolism in mice [21–23], suggesting the beneficial effect of RXR on diabetes. However, which RXR isoform(s) are involved and where they work to improve diabetes

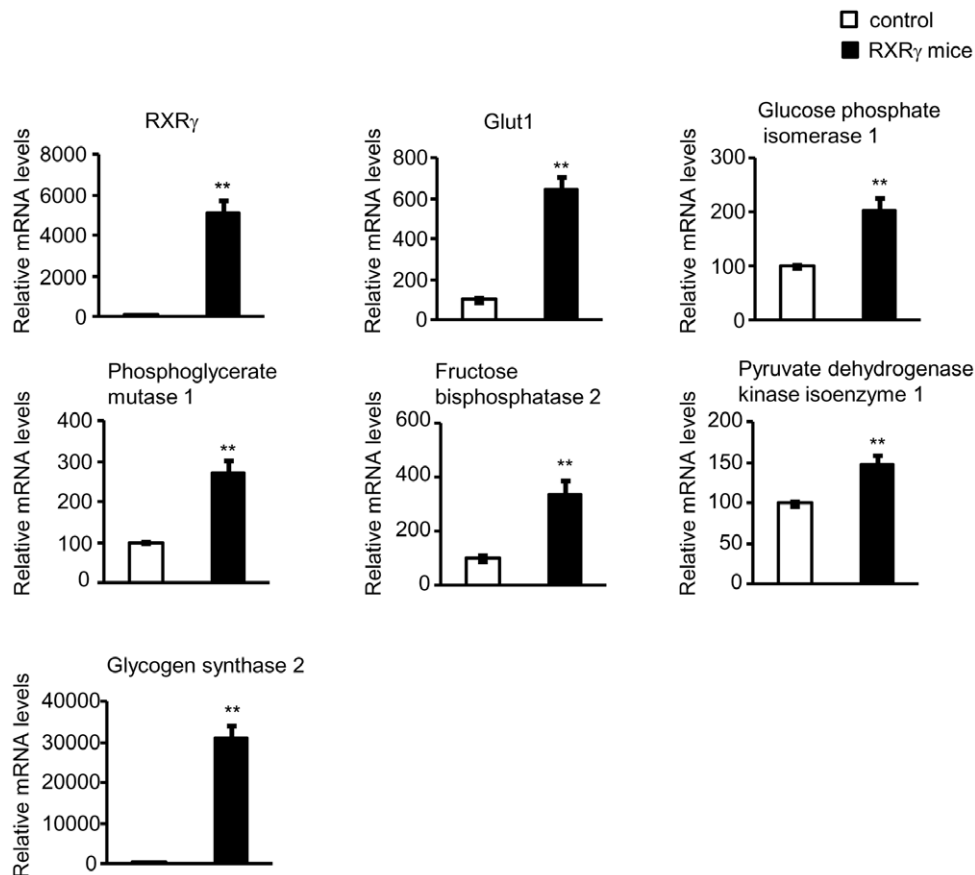


Figure 5. Levels of 'glucose metabolic process' gene expression in the skeletal muscle of RXR γ mice. Representative gene expressions of 'glucose metabolic process genes' analyzed by microarray and GO analysis (Table 5) were examined by quantitative real-time PCR. The value for wild-type (littermates of line 4-3) mice was set at 100, and relative values are shown. Mice were females of 4 months of age. The number of animals was 6 for both control (open bars) and RXR γ (filled bars) mice. These samples were also used in microarray analysis (Dataset S1). * $P < 0.05$ and ** $P < 0.01$ compared with respective control. N. S., not significant. doi:10.1371/journal.pone.0020467.g005

Table 6. Possible transcription factor binding sites in the 'glucose metabolic process' gene up-regulated in RXR γ mice.

Transcription factors	Matrix name	P-value
WT1, WT1 -KTS, WT1 I, WT1 I -KTS	WT1_Q6	6.98E-04
HNF-4, HNF-4alpha	HNF4ALPHA_Q6	0.0013
PPARalpha	PPARA_01	0.0017
c-Myb, c-Myb-isoform1	VMYB_02	0.0026
PPARalpha, PPARdelta, PPARgamma	PPAR_DR1_Q2	0.003
CART1, CART1, CART1	CART1_01	0.0036
COUP-TF1, COUP-TF2, HNF-4, HNF-4alpha	COUP_DR1_Q6	0.0036
CP2, CP2-isoform1	CP2_02	0.0055
NRF1-isoform1, NRF1-isoform2, NRF1-xbb1	TCF11_01	0.0057
SZF1	SZF11_01	0.0057
PPARgamma	PPARG_01	0.0057
COUP-TF1, COUP-TF2, HNF-4, HNF-4alpha, HNF4gamma	DR1_Q3	0.0057
BRCA1, BRCA1	BRCA_01	0.0065
HNF-3beta	HNF3B_01	0.007
Pax-2b, pax2	PAX2_01	0.007
C/EBPalpha, C/EBPbeta(LAP), C/EBPbeta(p20), C/EBPbeta(p20), C/EBPbeta(p34), C/EBPbeta(p35), C/EBPgamma, cebpe, CRP3, NF-IL6-1, NF-IL6-3	CEBP_Q3	0.007
MITF, MITF-M1, tcfec, TFEA, TFEA-xbb1, TFEA-xbb2, tfeb, tfeb-isoform1	TFE_Q6	0.007
AP-2alpha, AP-2beta, AP-2gamma	AP2_Q6_01	0.0077
Pax-4a, Pax-4c, Pax-4d, Pax4	PAX4_04	0.0079
c-Myc, deltaMax, max, max-isoform1, max-isoform2, N-Myc	MYC_MAX_03	0.0093

The mouse genome in the region of -1000 to +100 relative to the transcription start of an individual gene classified as glucose metabolism gene by GO analysis (Table 5), was analyzed. Statistical hypothesis testing was evaluated against housekeeping genes of mice. Matrices names are based on the MATCH software (see **Methods**), which are listed in *P*-value order. Indicated binding sites of transcription factors appeared significantly more frequent occurrence in the promoter sets. doi:10.1371/journal.pone.0020467.t006

has not been addressed. Here, we investigated glucose metabolism in RXR γ mice.

We demonstrated increased glucose metabolism in RXR γ mice relative to control mice, although there was no significant difference in the insulin-induced hypoglycemic effect between genotypes, based on insulin tolerance test and glucose clamp analysis. Because insulin level is similar between RXR γ mice and control mice, it is unlikely that increased insulin secretion is responsible for increased glucose tolerance in RXR γ mice. On the other hand, mRNA and protein expression of Glut1 is increased in the skeletal muscle from RXR γ mice relative to control mice, with increased glucose uptake and glycogen content. Because transgenic overexpression of Glut1 in the skeletal muscle has resulted in increased glucose uptake [24], glycogen content [25] and lowering of blood glucose [24], it is likely that enhanced glucose tolerance in RXR γ mice is mediated at least in part by increased Glut1 in the skeletal muscle.

As RXR γ is a nuclear receptor-type transcription factor that heterodimerizes with many nuclear receptors [6], we examined whether the *Glut1* gene is directly regulated by RXR γ . In an *in vivo* luciferase reporter analysis, combination of RXR γ and PPAR δ activates the *Glut1* promoter activity, which is diminished by deletion of the putative PPREs. It is, therefore, RXR γ /PPAR δ may activate *Glut1* expression in the skeletal muscle *in vivo*. In this regard, we found that overexpression of RXR γ or PPAR δ alone or both in C2C12 myocytes *in vitro* does not induce *Glut1* gene expression (unpublished data). This may be because C2C12 cells lack other factor(s) that are present in the skeletal muscle *in vitro* and are required to activate *Glut1* transcription. Whether the increased *Glut1* gene in RXR γ mice is mediated by RXR γ /PPAR δ should be

confirmed by additional experiments using PPAR δ knockout mice. Meanwhile, the transgene expression level in RXR γ mice was very high, and appeared to be beyond the physiological level. Nonetheless, if RXR γ can be enhanced in skeletal muscle, an increase in glucose metabolism can be expected.

We recently demonstrated that in addition to glycogen content, RXR γ mice exhibit increased triglyceride in the skeletal muscle as a result of increased SREBP1c gene expression [9]. In obese and diabetic subjects, intramuscular lipid is high with insulin resistance [26]. Moreover, in athletes, the skeletal muscle is also high in lipid but with high insulin sensitivity, which is known as the athlete paradox [27,28]. A partial explanation is an increased fatty acid load in obese and diabetic subjects, but not in athletes; increased intramuscular lipotoxic fatty acid metabolites, such as diacylglycerol and ceramide, cause insulin resistance in skeletal muscles [29,30]. Taken together with our previous report [9], this study demonstrates that RXR γ mice do not develop insulin resistance despite high intramuscular lipids, and appear to be protected against obesity-induced lipotoxicity in the skeletal muscle. Depositing glucose as both glycogen and triglycerides in the skeletal muscle may be effective in enhancing glucose metabolism.

Microarray analysis showed that various genes are up-regulated in the skeletal muscle of RXR γ mice. As RXR γ can heterodimerize several nuclear receptors, it is not surprising that expression of many genes was up-regulated by RXR γ overexpression. Concerning glucose metabolism genes, in the skeletal muscle of RXR γ mice, the expression levels of genes, such as glucose phosphate isomerase 1 and phosphoglycerate mutase 1 (stimulate glycolysis or gluconeogenesis), fructose biphosphatase 2 (stimulates gluconeogenesis), pyruvate dehydrogenase kinase

isoenzyme 1 (suppresses glycolysis), glycogen synthase 2 (stimulates glycogen synthesis)[31,32], were markedly increased (Fig. 5), suggesting the anabolic reaction of glucose. This is consistent with the observation that the glycogen content of skeletal muscle is higher in RXR γ mice (Fig. 2C). It is of note that glycogen synthase 2 is a liver-type enzyme, not a skeletal muscle-type enzyme [32]. In addition, usually, gluconeogenesis is not considered to be a major metabolic pathway in skeletal muscle. Chronic transgenic overexpression of RXR γ may have caused a super-physiological gene expression change in the skeletal muscle. Meanwhile, our bioinformatics analysis showed that glucose metabolism genes up-regulated in RXR γ mice contain several transcription factor binding motifs including PPRE in their promoter region. These observations support the concept that the RXR γ /PPAR heterodimer contributes to activation of these gene sets, although this needs to be confirmed by further experiments.

In summary, we demonstrated enhanced glucose metabolism with increased Glut1 expression and glucose uptake in RXR γ mice. This study suggested that activation of the skeletal muscle RXR γ is a novel therapeutic strategy to treat or prevent type 2 diabetes.

References

- Zurlo F, Larson K, Bogardus C, Ravussin E (1990) Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J Clin Invest* 86: 1423–1427.
- DeFronzo RA (1988) Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM. *Diabetes* 37: 667–687.
- Marshall BA, Hansen PA, Ensor NJ, Ogden MA, Mueckler M (1999) GLUT-1 or GLUT-4 transgenes in obese mice improve glucose tolerance but do not prevent insulin resistance. *Am J Physiol* 276: E390–E400.
- Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, et al. (2007) The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. *Proc Natl Acad Sci U S A* 104: 12587–12594.
- Kim JK, Zisman A, Fillmore JJ, Peroni OD, Kotani K, et al. (2001) Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin Invest* 108: 153–160.
- Shulman AI, Mangelsdorf DJ (2005) Retinoid x receptor heterodimers in the metabolic syndrome. *N Engl J Med* 353: 604–615.
- Lee CH, Olson P, Hevener A, Mehl I, Chong LW, et al. (2006) PPARdelta regulates glucose metabolism and insulin sensitivity. *Proc Natl Acad Sci U S A* 103: 3444–3449.
- Szanto A, Narkar V, Shen Q, Uray IP, Davies PJ, et al. (2004) Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ* 11: S126–S143.
- Kamei Y, Miura S, Suganami T, Akaike F, Kanai S, et al. (2008) Regulation of SREBP1c gene expression in skeletal muscle: role of retinoid X receptor/liver X receptor and forkhead-O1 transcription factor. *Endocrinology* 149: 2293–2305.
- Suzuki R, Tobe K, Aoyama M, Inoue A, Sakamoto K, et al. (2004) Both insulin signaling defects in the liver and obesity contribute to insulin resistance and cause diabetes in *Irs2(-/-)* mice. *J Biol Chem* 279: 25039–25049.
- Uno K, Katagiri H, Yamada T, Ishigaki Y, Ogihara T, et al. (2006) Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. *Science* 312: 1656–1659.
- Erion DM, Yonemitsu S, Nie Y, Nagai Y, Gillum MP, et al. (2009) SirT1 knockdown in liver decreases basal hepatic glucose production and increases hepatic insulin responsiveness in diabetic rats. *Proc Natl Acad Sci U S A* 106: 11288–11293.
- Tanaka S, Hayashi T, Toyoda T, Hamada T, Shimizu Y, et al. (2007) High-fat diet impairs the effects of a single bout of endurance exercise on glucose transport and insulin sensitivity in rat skeletal muscle. *Metabolism* 56: 1719–1728.
- Ito A, Suganami T, Miyamoto Y, Yoshimasa Y, Takeya M, et al. (2007) Role of MAPK phosphatase-1 in the induction of monocyte chemoattractant protein-1 during the course of adipocyte hypertrophy. *J Biol Chem* 282: 25445–25452.
- Fujii N, Ho RC, Manabe Y, Jessen N, Toyoda T, et al. (2008) Ablation of AMP-activated protein kinase alpha2 activity exacerbates insulin resistance induced by high-fat feeding of mice. *Diabetes* 57: 2958–2966.
- Lowry OH, Passonneau JV (1972) A flexible system of enzymatic analysis. Academic Press London 1–291.
- Aihara H, Miyazaki J (1998) Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol* 16: 867–870.
- Kel AE, Gossling E, Reuter I, Cheremushkin E, Kel-Margoulis OV, et al. (2003) MATCH: A tool for searching transcription factor binding sites in DNA sequences. *Nucleic Acids Res* 31: 3576–3579.
- Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4: 44–57.
- Carbon S, Ireland A, Mungall CJ, Shu S, Marshall B, Lewis S (2009) AmiGO: online access to ontology and annotation data. *Bioinformatics* 25: 288–289.
- Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, et al. (1997) Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* 386: 407–410.
- Davies PJ, Berry SA, Shipley GL, Eckel RH, Hennuyer N, et al. (2001) Metabolic effects of rexinoids: tissue-specific regulation of lipoprotein lipase activity. *Mol Pharmacol* 59: 170–176.
- Shen Q, Cline GW, Shulman GI, Leibowitz MD, Davies PJ (2004) Effects of rexinoids on glucose transport and insulin-mediated signaling in skeletal muscles of diabetic (db/db) mice. *J Biol Chem* 279: 19721–19731.
- Marshall BA, Ren JM, Johnson DW, Gibbs EM, Lillquist JS, et al. (1993) Germ-line manipulation of glucose homeostasis via alteration of glucose transporter levels in skeletal muscle. *J Biol Chem* 268: 18442–18445.
- Ren JM, Marshall BA, Gulve EA, Gao J, Johnson DW, et al. (1993) Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 268: 16113–16115.
- Goodpaster BH, Wolf D (2004) Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes. *Pediatr Diabetes* 5: 219–226.
- Stannard SR, Johnson NA (2004) Insulin resistance and elevated triglyceride in muscle: more important for survival than "thrifty" genes? *J Physiol* 554: 595–607.
- Goodpaster BH, He J, Watkins S, Kelley DE (2001) Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* 86: 5755–5761.
- Schenk S, Horowitz JF (2007) Acute exercise increases triglyceride synthesis in skeletal muscle and prevents fatty acid-induced insulin resistance. *J Clin Invest* 117: 1690–1698.
- Liu L, Zhang Y, Chen N, Shi X, Tsang B, et al. (2007) Upregulation of myocellular DGAT1 augments triglyceride synthesis in skeletal muscle and protects against fat-induced insulin resistance. *J Clin Invest* 117: 1679–1689.
- Kaslow HR, Lesikar DD, Antwi D, Tan AW (1985) L-type glycogen synthase. Tissue distribution and electrophoretic mobility. *J Biol Chem* 260: 9953–9956.
- Salway JG (1999) *Metabolism at a glance*. London: Blackwell Science Ltd.

Supporting Information

Dataset S1 List of genes up-regulated in RXR γ mice compared with wild-type control mice by microarray. (XLS)

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