Putative PPAR Target Genes Express Highly in Skeletal Muscle of Insulin-Resistant MetS Model SHR/NDmc-cp Rats

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Summary It is known that insulin resistance in skeletal muscle induces subsequent metabolic diseases such as metabolic syndrome (MetS). However, which genes are altered in the skeletal muscle by development of insulin resistance in animal models has not been examined. In this study, we performed microarray and subsequent real-time RT-PCR analyses using total RNA extracted from the gastrocnemius muscle of the MetS model, spontaneously hypertensive corpulent congenic (SHR/NDmc-cp) rats, and control Wistar Kyoto (WKY) rats. SHR/NDmc-cp rats displayed overt insulin resistance relative to WKY rats. The expression of many genes related to fatty acid oxidation was higher in SHR/NDmc-cp rats than in WKY rats. Among 18 upregulated genes, putative peroxisome proliferator responsive elements were found in the upstream region of 15 genes. The protein expression of ACOX2, an upregulated gene, and peroxisome proliferator-activated receptor (PPAR) G1, but not of PPARG2, PPARG1A or PPARG3, was higher in the gastrocnemius muscle of SHR/NDmc-cp rats than that in WKY rats. These results suggest that insulin resistance in the MetS model, SHR/NDmc-cp rats, is positively associated with the expression of fatty acid oxidation-related genes, which are presumably PPARs' targets, in skeletal muscle.

Key Words insulin resistance, skeletal muscle, fatty acid oxidation, PPAR, SHR/NDmc-cp rats

Skeletal muscle is responsible for approximately 75% of insulin-dependent glucose incorporation. A reduction in skeletal muscle glucose incorporation leads to the development of insulin resistance and subsequent development of metabolic diseases such as type 2 diabetes (1). In particular, insulin resistance with obesity in humans is frequently associated with hypertension and hyperlipidemia, as well as with hyperglycemia. Abnormal values of more than two of the following parameters—fasting

blood glucose, lipids and blood pressure—in combination with obesity is generally called metabolic syndrome (MetS). It is known that subjects with MetS have subsequent higher incidences of cardiovascular diseases (CVD) and associated death (2). Therefore, maintaining insulin sensitivity in skeletal muscle by controlling dietary habits is important for preventing the onset and progression of MetS and related diseases.

The main mechanism of glucose uptake in skeletal muscle is via glucose transporter (GLUT) 4, which is translocated from intracellular storage vesicles to the plasma membrane in an insulin-dependent manner (3). Insulin-induced translocation of GLUT4 is signaled by the insulin receptor substrate-phosphoinositide 3-kinase pathway and by the AMP activated protein kinase pathway (4). Skeletal muscle glucose is stored as glycogen by dephosphorylated glycogen synthase via the insulin signal. These findings indicate that the insulin signal in skeletal muscle enhances glucose incorporation independently of expression changes in metabolic genes such as glycogen synthesis- and fatty acid synthesis-related genes.

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Abbreviations: *Acox1*, acyl-Coenzyme A oxidase 1, palmitoyl; *Acs1L*, acyl-CoA synthetase long-chain family member 1; ChIP, chromatin immunoprecipitation; *CPT1B*, carnitine palmitoyltransferase-1b; CVD, cardiovascular diseases; *Cyts*, cytochrome c, somatic; GLUT, glucose transporter; MetS, metabolic syndrome; PPAR, peroxisome proliferator-activated receptor; *PPARGC1A*, peroxisome proliferator-activated receptor (PPAR) γ co-activator-1 α ; PPRE, peroxisome proliferator response elements; SHR/NDmc-cp, spontaneously hypertensive corpulent congenic; WKY, Wistar Kyoto; ZDF, Zucker diabetic fatty.

Recently, many studies have demonstrated that fatty acid oxidation in the skeletal muscle is associated with insulin resistance in this muscle. Indeed, it has been reported in a human study that physical activity by subjects with moderate insulin resistance ameliorated the insulin resistance and reduced the expression of genes related to fatty acid oxidation including carnitine palmitoyltransferase-1b (*CPT1B*) and peroxisome proliferator-activated receptor (PPAR) γ co-activator-1 α (*PPARGC1A*, generally called PGC-1 α) in skeletal muscle (5). Furthermore, a recent human study has demonstrated that the expression of fatty acid oxidation-related genes such as *PPARGC1A*, *CPT1B* and *PPARA* (generally called PPAR α), which is one of the nuclear receptors that activate fatty acid oxidation, in MetS subjects was positively associated with moderate insulin resistance, but not with severe insulin resistance (6). It is very likely that moderate insulin resistance induces fatty acid oxidation in skeletal muscle using fatty acids as an energy source. In contrast, development of severe insulin resistance and associated type 2 diabetes from moderate insulin resistance may suppress the fatty acid oxidation-related genes. In animal studies, it has been reported that insulin-resistant Zucker diabetic fatty (ZDF) rats, which unlike humans rapidly develop type 2 diabetes with pronounced hyperglycemia, did not show higher expression of genes related to fatty acid oxidation compared with the control animals (7, 8). In addition, an 8-wk high-fat diet intake induced moderate insulin resistance without changes in fatty acid oxidation-related genes in skeletal muscle of C57BL/6J mice (9). These animal studies' results do not correlate with the human studies. Whether development of insulin resistance is associated with experimental changes in a series of fatty acid oxidation-related genes as well as other genes in skeletal muscle in models resembling humans should be examined.

In the present study, we examined by microarray analysis whether the expression of genes related to fatty acid oxidation as well as other genes in skeletal muscle differed between spontaneously hypertensive corpulent congenic (SHR/NDmc-cp) rats, an animal model of development of MetS with obesity, hyperlipidemia, hyperglycemia and hypertension based on insulin resistance accompanied by excessive eating (10), and the normal model, Wistar Kyoto (WKY) rats. Furthermore, to investigate whether the upregulated genes are regulated in the same fashion, we searched for putative peroxisome proliferator response elements (PPREs), which are important transcription factors for regulating fatty acid oxidation and other fatty acid metabolism (11), in the promoter/enhancer region of upregulated genes in skeletal muscle of SHR/NDmc-cp rats.

MATERIALS AND METHODS

Animals. Five-week-old male SHR/NDmc-cp rats ($n=6$) and WKY rats ($n=5$), which are control rats (10, 12), were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in cages under controlled conditions (temperature $23\pm2^{\circ}\text{C}$; humidity $55\pm5\%$; 12-h

light/dark cycle). Rats were fed a laboratory diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and had free access to food and water throughout the study. Non-fasting plasma samples at 18 wk of age were collected from the tail vein using a capillary tube containing heparin-lithium (Terumo, Co., Ltd., Tokyo, Japan) between 9:30 am and 10:00 am. Rats were then killed by decapitation between 10:00 am and 12:00 pm, and gastrocnemius muscles were collected (13). The experimental procedures used in the study conformed to the guidelines of the Animal Usage Committee of the University of Shizuoka.

Microarray analysis. Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi (14). For microarray analysis, we used total RNA from three animals of each group. Total RNA (300 ng) was converted to complementary RNA (cRNA) and labeled with biotin using an Ambion WT Expression kit (Life Technologies, Carlsbad, CA), a GeneChip WT terminal labeling kit and an Eukaryotic poly-A RNA Control kit (Affymetrix, Santa Clara, CA) according to the manufacturers' instructions. The biotin-labeled cRNA (10 μg) was hybridized to Rat 1.0 ST arrays (Affymetrix), washed and analyzed with GeneChip Scanner 3000 (Affymetrix) as previously reported (15). Variable spots detected by an algorithm in the Partec software (Ryoka System, Tokyo, Japan) in all plates were defined as non-expressed genes and were removed. The signals were normalized by the global median normalization method based on the Partec algorithm. Data are represented in base 2 logarithms ($\log_2$), and only the signals in the microarray with differences between two groups that were greater than two after the $\log_2$ transformation are mentioned. The upregulated or downregulated genes with significant differences between the two groups, as calculated by Student's *t*-test ($p<0.05$), were subjected to further analyses. Among the 1,477 gene signals detected in the microarray, we detected 42 upregulated genes and 33 downregulated genes in the SHR/NDmc-cp rats compared with the WKY rats. We focused on the upregulated genes, which were classified into nine groups based on their functions, namely energy/metabolism, signal transduction, transcription/chromatin, structural proteins, immune response, transport of nutrients/ions, stress, others, and unknown. Each gene was classified under a category based on gene ontology (GO) annotations and PubMed research. The GO annotations and the PubMed ID for each gene are indicated in Table 3. Genes related to nutrient metabolism were classified into the energy/metabolism category. Genes related to G-protein coupled receptors, membrane receptors and cascades of phosphorylation signals under these receptors were classified into the signal transduction category. Transcriptional factors and transcriptional co-factors were classified into the transcription/chromatin category. Genes related to transport of nutrients and ions into the cells were classified into the transport of nutrients/ions category. Genes related to elimination of reactive oxygen species and oxidized substances were classified into the stress category.

Table 1. Sequences of oligonucleotide primers used for real-time RT-PCR.

Gene	Sequence
Acyl-CoA oxidase 2, branched chain (<i>Acox2</i>)	5'-TGTGAGCTATGCCTTCCACTT-3' 5'-GCCTTCATACCAGTGCTCAAT-3'
Asparagine synthetase (glutamine-hydrolyzing) (<i>Asns</i>)	5'-TTCTGGCTGTGTGTTTTCAGAAG-3' 5'-GGGCTCCACTTTTAGGAAGG-3'
Acyl-CoA synthetase medium-chain family member 5 (<i>Acsm5</i>)	5'-CTTGCGTTTTTGTGCATGAG-3' 5'-TTATTGGGAACCTGCAGAGAG-3'
Pantothenate kinase 2 (<i>Pank2</i>)	5'-GACCTTGCCAGAGCAACTTT-3' 5'-TTGCCGACAAATACAACCTG-3'
Acyl-CoA thioesterase 2 (<i>Acot2</i>)	5'-TTGAAGAAGCCGTGAACCTACC-3' 5'-GCCATTGATGACAACAGCAG-3'
Phosphoserine aminotransferase 1 (<i>Psat1</i>)	5'-GGAATGGATCAAGAACAATGG-3' 5'-CGGAATGTTTCATCTGCTTC-3'
Nudix (nucleoside diphosphate linked moiety X)-type motif 7 (<i>Nudt7</i>)	5'-TCCCACCTTGTCCTTAGTAAGT-3' 5'-CAGGTGCCCTTCTCAGCTT-3'
GDP-D-glucose phosphorylase 1 (<i>RDG1560277</i>)	5'-CTAGGATATCCAACCTCAGGTTGC-3' 5'-CGTAGACAAAGTCGGGGATG-3'
Melanocortin 5 receptor (<i>Mc5r</i>)	5'-TTCGACTCCATGATCTGCAT-3' 5'-ATGTACCTGTCCACCGCAAT-3'
Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (<i>Pla2g7</i>)	5'-TCAAGCCCTAAGTGAAGACCA-3' 5'-TGGAATTCGGCAGAGTTGAT-3'
Activating transcription factor 5 (<i>Atf5</i>)	5'-CTGCTCACACCGACTCTTCA-3' 5'-GTGAAGACAGGCACCAAGG-3'
Smooth muscle alpha-actin (<i>Acta2</i>)	5'-CTCCCAGCACCATGAAGATT-3' 5'-TCCACATTTGTTGGAAGGTAGAC-3'
Interferon-induced protein with tetratricopeptide repeats 1 (<i>Ifit1</i>)	5'-CTTTGCTGAAATGCCACGTA-3' 5'-GGATCACGAGAGCCATAAAGA-3'
Catalase (<i>Cat</i>)	5'-TTCATCAGGGATGCCATGT-3' 5'-TGATGGAGAGACTCTGGACAAA-3'
Glycoprotein, alpha-galactosyltransferase 1,3 (<i>Gta1p</i>)	5'-GCTCTGGGACTGGTTCAATC-3' 5'-AAGTGCCTTCCCACACAATC-3'
Receptor (chemosensory) transporter protein 4 (<i>Rtp4</i>)	5'-GGACCCTGCACTTGGATAAG-3' 5'-CATCTGGAACACTGGAACCTG-3'
Similar to interferon-inducible GTPase (<i>RGD1309362</i>)	5'-TACACCGCTCAATGTTGCTG-3' 5'-TCTCCACCACCCCAATTTTA-3'
Similar to hypothetical protein MGC29761 (<i>RGD1306233</i>)	5'-AAGAAAACCCCAAGAGTGC-3' 5'-TTTCTCTGGGGCAGGCTTA-3'
General transcription factor IIB (<i>Gtf2b</i>)	5'-TACAGGAGCGGCAAGTTTTGA-3' 5'-ATTGCGTGGGAGGTTGATTCT-3'

RNA analysis. Total RNA samples (250 ng) from SHR/NDmc-cp rats ($n=6$) and WKY rats ($n=5$) were converted into cDNA by reverse transcription (RT) using SuperScript™ III reverse transcriptase (Life Technologies) according to the manufacturer's instructions. To quantitatively estimate mRNA levels, PCR amplification was performed on the Light Cycler 480 System (Roche Diagnostics, Basel, Switzerland) as described previously (15). The primer sequences used are listed in Table 1.

Western blot analysis. Total protein was extracted from the gastrocnemius muscle using RIPA buffer and the soluble supernatants were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blot analysis was performed as previously reported (13). The primary antibodies were A-TUBULIN (Sigma-Aldrich, St. Louis, MO), ACOX2 (Proteintech Group, Inc., Chicago, USA), PPARA (Santa Cruz Biotechnology, Santa Cruz, CA), PPARD (generally called PPAR δ ; Santa Cruz Biotechnology) and PPARG (generally called PPAR γ ; Cell Signaling Technology). Positive signals were detected by chemiluminescence (Western Lightning ECL Pro; PerkinElmer, Waltham, MA) using a Chemidog XRS plus instrument (Bio-Rad, Hercules, CA).

Searching for putative PPRES in the upstream region of the upregulated genes. Putative PPRES in the -5000 bp to -1 bp promoter/enhancer region of upregulated genes were searched for with the web program PPRESearch (<http://www.classicus.com/PPRE/>) (16, 17). Search criteria were two mismatches, 80% binding efficiency, one directed repeat and zero flanking match. We searched both sense and antisense sequences.

Statistical analysis. Data are expressed as mean $\pm$ standard error of the mean (SE). Significant differences between two groups were analyzed by Student's t -test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Body weight and blood biochemical parameters in WKY and SHR/NDmc-cp rats

We previously reported the body weight and concentrations of blood glucose, insulin and triacylglycerol in SHR/NDmc-cp rats, WKY rats and streptozotocin-treated rats (13). In this study, we now reanalyze the results from SHR/NDmc-cp rats and WKY rats using Student's t -test (Table 2).

Gene expression profiles of gastrocnemius muscles of WKY and SHR/NDmc-cp rats

We performed microarray analysis of total RNA from gastrocnemius muscles of WKY and SHR/NDmc-cp rats. We identified 14 upregulated genes classified as energy/metabolism, six upregulated genes in the signal transduction category, two upregulated genes in the transcription/chromatin category, one upregulated gene in the structural protein category, three upregulated genes in the immune response category, three upregulated genes in the transport of nutrients/ions category, two upregulated genes in the stress/apoptosis category, and 11 upregulated genes in the other or unknown category (Table 3).

Table 2. Body weight and blood biochemical parameters in WKY and SHR/NDmc-cp rats.

	WKY	SHRcp
Body weight (g)	402 $\pm$ 7.88	603 $\pm$ 23.00**
Plasma triglyceride (mg/dL)	64 $\pm$ 14.22	946 $\pm$ 230.36**
Plasma glucose (mg/dL)	172 $\pm$ 17.56	326 $\pm$ 99.91**
Plasma insulin (ng/mL)	2.10 $\pm$ 0.75	14.01 $\pm$ 0.29**

Values are indicated as mean $\pm$ SD of 5–6 animals.

** $p < 0.01$ by Student's t -test.

Real-time RT-PCR of the upregulated genes and PPAR protein expression in gastrocnemius muscles of the WKY and SHR/NDmc-cp rats

We performed real-time RT-PCR on the abovementioned upregulated genes in gastrocnemius muscles of the WKY and SHR/NDmc-cp rats. Among the 42 genes tested, the expression of 18 genes—*Acox2*, *Asns*, *Acsn5*, *Pank2*, *Acot2*, *Psat1* and *Nudt7* in the energy/metabolism category, *RGD1560277*, *Mc5r* and *Pla2g7* in the signal transduction category, *Atf5* in the transcription/chromatin category, *Acta2* in the structural protein category, *Ifit1* in the immune response category, *Cat* in the stress category, *Ggta1p*, *Rtp4*, *RGD1309362* and *RGD1306233* in the other or unknown category—was significantly higher in the SHR/NDmc-cp rats than in the WKY rats (Fig. 1A). The expression of *Rdh16* ($p=0.051$) in the energy/metabolism category, *Pld5* ($p=0.084$) in the signal transduction category, *Slc7a5* ($p=0.060$) in the transport of nutrients/ions category, and *Cym* ($p=0.061$) in the other category tended to be higher in the SHR/NDmc-cp rats than in the WKY rats, but the difference was not significant (data not shown). The expression of other upregulated genes as determined by the microarray analysis did not differ between the two groups. We also tested the expression of the fatty acid oxidation-related genes, acyl-coenzyme A oxidase 1, palmitoyl (*Acox1*), acyl-CoA synthetase long-chain family member 1 (*Acs1l*), *Cpt1b*, cytochrome c, somatic (*Cyts*) and *Ppargc1a*. The expression of *Acox1* ($p=0.059$) and *Acs1l* ($p=0.065$) tended to be higher in the SHR/NDmc-cp rats than in the WKY rats, but the difference was not significant (data not shown). Furthermore, the protein levels of ACOX2 were significantly higher in the SHR/NDmc-cp rats than in the WKY rats (Fig. 1B).

The gene expression of PPARs (*Ppara*, *Ppard*, *Pparg1* and *Pparg2*) did not differ between the SHR/NDmc-cp rats and the WKY rats (data not shown). The protein level of PPARG1, but not PPARA, PPARD or PPARG2, was higher in the SHR/NDmc-cp rats than in the WKY rats (Fig. 1C).

Putative PPRES search in the upregulated genes

Next, we searched for putative PPRES with the web-software PPRESearch (16, 17) in the upstream region (-5000 bp to -1 bp) of the 18 upregulated genes confirmed by real-time RT-PCR. We found putative PPRES in *Acox2*, *Asns*, *Acsn5*, *Pank2*, *Psat1* and *Nudt7* in the

Table 3. Upregulated genes in skeletal muscle of SHR/NDmc-cp rats compared with WKY rats.

Gene function	Gene symbol	Gene name	UniGene ID	Ratio	p	PubMed ID	Gene ontology annotation biological process
Energy/ metabolism	<i>Scd1</i>	Stearoyl-coenzyme A desaturase 1	Rn.1023	6.19	0.002	22211186	fatty acid biosynthetic process
	<i>Acox2</i>	Acyl-CoA oxidase 2, branched chain	Rn.10622	3.64	0.000	14728675	lipid metabolic process
	<i>Asns</i>	Asparagine synthetase (glutamine-hydrolyzing)	Rn.11172	3.49	0.000	23403946	cellular amino acid metabolic process
	<i>Chst5</i>	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5	Rn.151213	3.09	0.035	21440637	carbohydrate metabolic process
	<i>Adipoq</i>	Adiponectin, C1Q and collagen domain containing	Rn.24299	2.79	0.024	15655035	—
	<i>Agpat2</i>	1-acylglycerol-3-phosphate O-acyltransferase 2	Rn.55456	2.73	0.002	21873652	positive regulation of cytokine production
	<i>Acsm5</i>	Acyl-CoA synthetase medium-chain family member 5	Rn.35367	2.70	0.002	23087649	lipid metabolic process
	<i>Ndufv3</i>	NADH dehydrogenase (ubiquinone) flavoprotein 3	Rn.6452	2.49	0.006	9344673	transport
	<i>Pank2</i>	Pantothenate kinase 2	Rn.91999	2.43	0.001	17242360	—
	<i>Rdh16</i>	Retinol dehydrogenase 16 (all- <i>trans</i>)	Rn.94108	2.27	0.001	11967490	retinoid metabolic process
	<i>Acot2</i>	Acyl-CoA thioesterase 2	Rn.37524	2.09	0.012	21212523	very-long-chain fatty acid metabolic process
	<i>Psat1</i>	Phosphoserine aminotransferase 1	Rn.100813	2.06	0.005	12633500	L-serine biosynthetic process
	<i>Prps1</i>	Phosphoribosyl pyrophosphate synthetase 1	Rn.9761	2.04	0.019	20380929	purine base metabolic process
	<i>Nudt7</i>	Nudix (nucleoside diphosphate linked moiety X)-type motif 7	Rn.99447	2.03	0.036	18799520	coenzyme A catabolic process
Signal transduction	<i>RGD1560277</i>	GDP-D-glucose phosphorylase 1		2.83	0.016	21507950	—
	<i>Ppp2r4</i>	Protein phosphatase 2A activator, regulatory subunit 4	Rn.24600	2.42	0.002	23298795	negative regulation of phosphoprotein phosphatase
	<i>Mc5r</i>	Melanocortin 5 receptor	Rn.9673	2.34	0.016	20654690	signal transduction
	<i>Pla2g7</i>	Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	Rn.90768	2.29	0.021	21281786	—
Transcription/ chromatin	<i>Ptpn11</i>	Protein tyrosine phosphatase-like A domain containing 1	Rn.767	2.19	0.048	10747961	—
	<i>Pld5</i>	Phospholipase D family, member 5	Rn.214167	2.02	0.042	23915343	—
	<i>Atf5</i>	Activating transcription factor 5	Rn.229186	2.64	0.001	12213205	regulation of transcription from RNA polymerase II promoter
	<i>Fhl2</i>	Four and a half LIM domains 2	Rn.3849	2.26	0.017	22633286	osteoblast differentiation
Structural protein	<i>Acta2</i>	Smooth muscle alpha-actin (Acta2)	Rn.3114	2.97	0.005	8106541	apoptosis
Immune response	<i>Ifit1</i>	Interferon-induced protein with tetratricopeptide repeats 1	Rn.48677	3.53	0.001	21642987	—
	<i>Thrsf12a</i>	Tumor necrosis factor receptor superfamily, member 12a	Rn.105040	2.23	0.028	23760533	substrate-bound cell migration, cell attachment to substrate
	<i>Ly6c</i>	Ly6-C antigen	Rn.40119	2.09	0.018	22213571	—
	<i>Aqp7</i>	Aquaporin 7	Rn.11111	2.76	0.026	17077387	transport
Transport of nutrients/ions	<i>Slc7a5</i>	Solute carrier family 7 (amino acid transporter light chain, L system), member 5	Rn.32261	2.32	0.007	24586861	transport
	<i>Kcnmb1</i>	Potassium large conductance calcium-activated channel, subfamily M, beta member 1	Rn.10820	2.26	0.039	16356551	response to calcium ion
Stress	<i>Cat</i>	Catalase	Rn.3001	2.63	0.010	15879174	response to reactive oxygen species
	<i>Gstt3</i>	Glutathione S-transferase, theta 3	Rn.92581	2.17	0.006	12038961	glutathione metabolic process
Other	<i>Ggta1p</i>	Glycoprotein, alpha-galactosyltransferase 1.3	Rn.916	3.50	0.005	1559713	—
	<i>Slh</i>	Sarcophilin	Rn.16457	3.12	0.004	24358354	regulation of calcium ion transport
	<i>Cym</i>	Chymosin	Rn.48676	3.02	0.003	1455180	—
	<i>Ehd3</i>	EH-domain containing 3	Rn.229018	2.95	0.034	12121420	—
	<i>Rtp4</i>	Receptor (chemosensory) transporter protein 4	Rn.4244	2.28	0.032	18836069	detection of chemical stimulus involved in sensory perception
Unknown	<i>RGD1309362</i>	Similar to interferon-inducible GTPase	Rn.160023	3.50	0.008		—
	<i>RGD1306233</i>	Similar to hypothetical protein MGC29761	Rn.94715	2.86	0.025		—
	<i>Tmem128</i>	Transmembrane protein 128	Rn.41433	2.61	0.048		—
	<i>Ccdc92</i>	Coiled-coil domain containing 92	Rn.32270	2.39	0.006		—
	<i>RGD1311723</i>	Similar to KIAA1731 protein	Rn.160168	2.30	0.033		—
	<i>LOC681292</i>	Hypothetical protein LOC681292		2.12	0.013		—

Fig. 1. RT-PCR of upregulated genes in microarray analysis and PPARs protein expression in gastrocnemius muscle. (A) mRNA levels of upregulated genes in microarray. Values were normalized against general transcription factor IIb (*Gtf2b*) ($n=5, 6$). (B) Protein levels of ACOX2. (C) Protein levels of PPARs. Quantification of protein levels was performed by normalization against A-TUBULIN ($n=5$). Values are mean $\pm$ SE. Statistical analysis was performed using a two-sided unpaired Student's *t*-test. ** $p<0.01$, * $p<0.05$, SHR/NDmc-cp (SHRcp) vs. WKY rats.

energy/metabolism category, *RGD1560277* and *Mc5r* in the signal transduction category, *Atf5* in the transcription/chromatin category, *Acta2* in the structural protein category, *Cat* in the stress category, *Gsta1p*, *Rtp4*, *RGD1306233* and *RGD1309362* in the other or unknown categories (Table 4). Among the 18 upregulated genes confirmed by real-time RT-PCR, putative PPRES were found in the upstream region of 15 genes.

DISCUSSION

In this study, we found that the expression of many genes related to energy/metabolism, particularly fatty acid oxidation-related genes, such as *Acox2*, *Asns*, *Acsn5*, *Pank2*, *Acot2*, *Psat1* and *Nudt7*, and other genes including *Ifit1*, *Cat* and *Pla2g7*, was higher in gastrocnemius muscle of the insulin-resistant MetS animal model, SHR/NDmc-cp rat, than in that of the normal model, WKY rat. In particular, we found that ACOX2 protein

expression was significantly higher in the SHR/NDmc-cp rats than in the WKY rats. Our results demonstrate for the first time that the expression of genes related to fatty acid oxidation in the skeletal muscle is higher in the insulin-resistant MetS animals than in the control animals. It has been reported that *Acox2* (18) and *Nudt7* (19), *Ifit1* (20), and *Cat* (21), are regulated by PPARs. In addition, it has been reported that the subtypes and/or cascades of *Acsn5* (18), *Pank2* (22) and *Acot2* (23) in the energy/metabolism category, and *Pla2g7* (24) in the signal transduction category are targeted by PPARs. Furthermore, most genes (15 out of the 18 upregulated genes confirmed by real-time RT-PCR) possess putative PPRES in their promoter/enhancer region. Only in three genes, *Acot2*, *Pla2g7*, and *Ifit1*, did we not find putative PPRES in the promoter/enhancer region. However, as mentioned above, the expression of *Ifit1* and subtypes of *Acot2* and *Pla2g7* is enhanced by PPAR overexpression

Table 4. Putative PPREs in the -5000 bp to -1 bp promoter/enhancer region of the upregulated genes in the SHR/NDmc-cp rats.

Gene function	Gene symbol	Strand	PPRE name	Binding efficiency	PPRE pattern	Gene sequence	Gene position (bp)	PubMed ID
Energy/metabolism	<i>Acox2</i>	sense	PPAR γ (in vivo)	91	AGGCCATAGGTCA	AGGCCTCAGGTCA	-630	1487072
			PPAR α (in vivo)	100	AGGCCATAGGTCA	AGGCCTCAGGTCA	-630	1487072
	<i>Asns</i>	complement	PPAR α (in vitro)	83	ACTGGAAACTCGA	ACTGGACACTCGT	-3424	17650321
	<i>Acsn5</i>	complement	PPAR α (in vitro)	83	ACTGGAAACTCGA	ACAGGAAACACGA	-1288	17650321
		sense	PPAR γ (in vitro)	81	AGGTCAAAAGTTCA	AGGACAAAGTTCC	-1870	17650321
	<i>Pank2</i>	complement	PPAR γ (in vitro)	81	ACTTGAACACTGGA	ACCTCAAACTGGA	-62	17650321
	<i>Psat1</i>	complement	PPAR γ (in vitro)	89	GCTGGAAACTGGA	GGTGGAGACTGGA	-2985	17650321
			PPAR γ (in vivo)	93	ACGGGAAACGGGG	ACGGGAGACAGGG	-3824	9933587
			PPAR α (in vivo)	96	ACGGGAAACGGGG	ACGGGAGACAGGG	-3824	9933587
	<i>Nudt7</i>	sense	PPAR α (in vitro)	83	AGTCAAAAGGTCA	GGCTCAGAGGTCA	-2621	17650321
		complement	PPAR α (in vitro)	83	ACTGGAAACTCGA	ACTCGACACTCGA	-832	17650321
	Signal transduction	<i>RGD1560277</i>	sense	PPAR α (in vitro)	83	AGTCAAAAGGTCA	AGTCAAAAGTTCA	-2304
			PPAR γ (in vitro)	81	AGGTCAAAAGTTCA	AGTCAAAAGTTCA	-2304	17650321
<i>Mc5r</i>		complement	PPAR γ (in vivo)	93	ACGGGAAACGGGG	AAGGGAAAATGGGG	-4464	9933587
	<i>Atf5</i>		PPAR α (in vivo)	96	ACGGGAAACGGGG	AAGGGAAAATGGGG	-4464	9933587
		sense	PPAR α (in vitro)	83	AGTCAAAAGGTCA	AGTCAAAAGGCCA	-1198	17650321
		complement	PPAR γ (in vivo)	91	ACTGGATACCGGA	ACAGGAGACCGGA	-1368	1487072
	<i>Acta2</i>		PPAR α (in vivo)	100	ACTGGATACCGGA	ACAGGAGACCGGA	-1368	1487072
		sense	PPAR γ (in vivo)	93	GGGGCAAAAGGGCA	GGGGCAGGGGGCA	-2027	9933587
			PPAR α (in vivo)	96	GGGGCAAAAGGGCA	GGGGCAGGGGGCA	-2027	9933587
Stress	<i>Cat</i>	sense	PPAR α (in vitro)	83	AGTCAAAAGGTCA	AAATCAAAAGGTCA	-665	17650321
		complement	PPAR γ (in vitro)	81	ACTTGAACACTGGA	AGTTGAAAATGGA	-896	17650321
Other	<i>Ggta1p</i>	complement	PPAR γ (in vivo)	91	ACTGGATACCGGA	ACTGGAGAACCGGA	-2307	1487072
			PPAR α (in vivo)	100	ACTGGATACCGGA	ACTGGAGAACCGGA	-2307	1487072
	<i>Rtp4</i>	sense	PPAR γ (in vivo)	100	AGGGTAAAGTTCA	AGGGGAAAAGTTCT	-4304	7887901
			PPAR α (in vivo)	82	AGGGTAAAGTTCA	AGGGGAAAAGTTCT	-4304	7887901
		complement	PPAR γ (in vivo)	93	ACGGGAAACGGGG	ACGAGAAACGTGG	-2630	9933587
			PPAR γ (in vivo)	93	ACGGGAAACGGGG	ACTGGAAAGGGGG	-1530	9933587
			PPAR α (in vivo)	96	ACGGGAAACGGGG	ACGAGAAACGTGG	-2630	9933587
			PPAR α (in vivo)	96	ACGGGAAACGGGG	ACTGGAAAGGGGG	-1530	9933587
Unknown	<i>RGD1309362</i>	complement	PPAR γ (in vitro)	81	ACTTGAACACTGGA	ACCTGAGACTGGA	-344	17650321
			PPAR γ (in vivo)	93	ACGGGAAACGGGG	CCGGGAGACGGGG	-2331	9933587
			PPAR α (in vivo)	96	ACGGGAAACGGGG	CCGGGAGACGGGG	-2331	9933587
	<i>RGD1306233</i>	sense	PPAR γ (in vivo)	85	AGTCTCTAGTTCA	AGTCTCTGTCGA	-3563	1502166
		complement	PPAR α (in vitro)	83	ACTGGAAACTCGA	ACATGAAACTCGA	-4637	17650321
			PPAR γ (in vitro)	81	ACTTGAACACTGGA	ACATGAAACTCGA	-4637	17650321

Sequence mismatches=2; binding efficiency=80; directed repeats=1; flanking match=0.
Sequences with bold and underline are mismatched.

or PPAR agonist treatment. PPAR is one of the nuclear receptors that regulate fatty acid metabolism in each metabolic tissue and is activated by fatty acids and their metabolites (11). These results indicate that the expression of most genes in skeletal muscle induced by insulin resistance could be regulated by PPARs. It should be noted that we did not find reports on upregulation of genes possessing PPRES. We did not detect significant upregulation of the typical PPRES-possessing fatty acid oxidation-related gene, *Acox1* ($p=0.059$) (25), or of other typical fatty acid oxidation-related genes such as *Acs11* ($p=0.065$) and *Cpt1b* ($p=0.872$). These results indicate that PPAR may target many genes broadly related to energy/metabolism, in particular genes related to fatty acid oxidation. Additionally, our results indicate that the upregulated genes rather than *Acox1*, *Acs11* and *Cpt1b*, may be strongly regulated by PPAR in skeletal muscle of SHR/NDmc-cp rats. However, this issue will be examined in further studies by chromatin immunoprecipitation (ChIP) assays. Additionally, it should be examined in future studies whether PPARs bind to the putative PPRES located in the promoter/enhancer region of these genes in the gastrocnemius muscle of the SHR/NDmc-cp rats by ChIP assay, and whether PPARs transactivate these putative PPRES in skeletal muscle cell lines by reporter assays.

It is still unclear why other insulin-resistant models such as ZDF rats and C57BL/6J mice fed a high-fat diet did not show expression changes in fatty acid oxidation-related genes (7–9). The pathology of the SHR/NDmc-cp rats may have greater similarity to the human skeletal muscle with moderate insulin resistance than to ZDF rats and C57BL/6J mice fed a high-fat diet. The skeletal muscle gene expression profiles should be compared between our current results and human skeletal muscle in various stages of insulin resistance, MetS and type 2 diabetes obtained by biopsy.

In this study, we did not detect significant differences in PPARG or PPARG protein expression between the SHR/NDmc-cp rats and the WKY rats. We showed that the protein expression of PPARG1, but not PPARG2, was higher in the SHR/NDmc-cp rats than in the WKY rats. Previously, it has been suggested that PPARG mainly functions in adipose tissues and macrophages (11). However, a recent study has demonstrated that muscle-specific gene targeting of PPARG in mice induced fat accumulation in the adipose tissues and insulin resistance (26). Additionally, using in situ hybridization it has been shown that the expression of *Pparg*, whose isoforms were not divided in the experiment, was detectable in the skeletal muscle at a level of 5–10% of that in adipose tissues (27). This evidence indicates that PPARG functions in fatty acid oxidation and insulin sensitivity in the skeletal muscle. It has been reported that the expression of PPARG1, but not PPARG2, in human skeletal muscle obtained by a biopsy was positively associated with insulin resistance (28). Moreover, mouse muscle cell line C2C12 cells expressed *Pparg1*, but not *Pparg2* (29). PPARG2 is mainly expressed in adipose tissues, and adipocytes are located in the perivascular

space of the skeletal muscle, but not in the interstitium among muscle bundles, in SHR/NDmc-cp rats (13). Thus, PPARG1 expression in the skeletal muscle of SHR/NDmc-cp rats may be associated with the expression of fatty acid oxidation-related genes and/or PPAR target genes. It should be examined in further studies whether PPARG1 is expressed in the skeletal muscle cells of SHR/NDmc-cp rats, which are associated with the development of insulin resistance in rats. Here, we did not detect significant differences in the expression of *Pparg1a* or *Cpt1b*, which encode typical rate-controlling enzymes of fatty acid oxidation, between SHR/NDmc-cp rats and WKY rats. This may be because of differences in the responsiveness of subtypes or isoforms to each gene. To determine which PPAR subtypes/isoforms regulate the expression of each fatty acid oxidation-related gene, including *Pparg1a* and *Cpt1b*, in the skeletal muscle, in vivo ChIP assays should be performed.

In conclusion, our results show that insulin-resistant SHR/NDmc-cp rats, a MetS model, have a higher expression of a series of fatty acid oxidation-related genes, which are presumably targeted by PPARs, in skeletal muscle.

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