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Comprehensive Messenger Ribonucleic Acid Profiling Reveals That Peroxisome Proliferator-Activated Receptor γ Activation Has Coordinate Effects on Gene Expression in Multiple Insulin-Sensitive Tissues

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ABSTRACT

Peroxisome proliferator-activated receptor γ (PPARγ) agonists, including the glitazone class of drugs, are insulin sensitizers that reduce glucose and lipid levels in patients with type 2 diabetes mellitus. To more fully understand the molecular mechanisms underlying their therapeutic actions, we have characterized the effects of the potent, tyrosine-based PPARγ ligand GW1929 on serum glucose and lipid parameters and gene expression in Zucker diabetic fatty rats. In time-course studies, GW1929 treatment decreased circulating FFA levels before reducing glucose and triglyceride levels. We used a comprehensive and unbiased messenger RNA profiling technique to identify genes regulated either directly or indirectly by PPARγ in epididymal white adipose tissue, interscapular brown adipose tissue, liver, and soleus skeletal muscle. PPARγ activation stimulated the expression of a large number of genes involved in lipogenesis and fatty acid metabolism in both white adipose tissue and brown adipose tissue. In muscle, PPARγ agonist treatment decreased the expression of pyruvate dehydrogenase kinase 4, which represses oxidative glucose metabolism, and also decreased the expression of genes involved in fatty acid transport and oxidation. These changes suggest a molecular basis for PPARγ-mediated increases in glucose utilization in muscle. In liver, PPARγ activation coordinately decreased the expression of genes involved in gluconeogenesis. We conclude from these studies that the antidiabetic actions of PPARγ agonists are probably the consequence of 1) their effects on FFA levels, and 2), their coordinate effects on gene expression in multiple insulin-sensitive tissues. (*Endocrinology* **142:** 1269–1277, 2001)

ADULT-ONSET (TYPE 2) diabetes mellitus afflicts more than 100 million people worldwide, and its prevalence is soaring in Western countries with high fat diets and sedentary lifestyles. Insulin resistance, which is an inability of the body to respond efficiently to insulin, is an early step in the onset of type 2 diabetes. The thiazolidinedione-based glitazones, including Avandia (rosiglitazone) and Actos (pioglitazone), are a promising new class of drug used for the treatment of type 2 diabetes (1–3). These drugs increase insulin sensitivity and lower glucose levels in type 2 diabetics by enhancing glucose metabolism in muscle and decreasing glucose biosynthesis (gluconeogenesis) in liver (1, 4).

The glitazones mediate their therapeutic effects by binding and activating peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor family of ligand-activated transcription factors (1, 2, 5). Recently, potent nonglitazone PPARγ ligands such as the tyrosine analogs GI262570 and GW1929 have also been shown to enhance insulin sensitivity in humans and rodent models of type 2 diabetes (6–8). PPARγ is highly expressed in adipose tissue and plays a pivotal role in fat cell differentiation (1, 9). PPARγ regulates gene expression by binding as a heterodimer with the 9-*cis* retinoic acid receptors (RXRs) to DNA response elements comprised of two copies of the consensus nuclear receptor half-site sequence AGGTCA organized as a direct repeat (DR) and separated by a single nucleotide spacer, a so-called DR-1 motif. PPARγ response elements have been identified in the regulatory regions of several genes involved in fatty acid and carbohydrate metabolism (10). In addition to the synthetic glitazones and tyrosine analogs, PPARγ is activated by various naturally occurring polyunsaturated fatty acids and polyunsaturated fatty acid metabolites (2, 5). Thus, PPARγ may represent a molecular link between fatty acids and insulin sensitivity.

Although the antidiabetic actions of PPARγ agonists are well established, the mechanism underlying the pharmacological activities of these drugs has remained obscure. How does activation of PPARγ, which is highly expressed in fat cells, result in therapeutic effects in muscle and liver? There are several plausible explanations involving either direct or indirect effects on muscle and liver (1, 2). To better understand the mechanism by which PPARγ activation improves insulin sensitivity, we have identified genes regulated by the selective, tyrosine-based PPARγ agonist GW1929 in major insulin-sensitive tissues, including epididymal white adi-

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pose tissue (WAT), interscapular brown adipose tissue (BAT), soleus skeletal muscle, and liver of Zucker diabetic fatty (ZDF) rats. PPARγ-regulated genes were identified using an unbiased and comprehensive messenger RNA (mRNA) profiling technique, called GeneCalling (11). In this technique, complementary DNA (cDNA) fragments representing differentially expressed genes are identified by comparing the length of each fragment against a sequence database. The identities of differentially expressed genes are confirmed by competitive PCR using gene-specific oligonucleotides. Our results demonstrate that PPARγ activation has coordinate effects on genes regulating important metabolic pathways in WAT, BAT, skeletal muscle, and liver.

Materials and Methods

Materials

GW1929 was synthesized by the Medicinal Chemistry Department at Glaxo Wellcome Inc.

Experimental animals and protocols

All procedures performed were in compliance with the Animal Welfare Act and USDA regulations and were approved by the Glaxo Wellcome Inc., institutional animal care and use committee. Animals were housed at 72 F and 50% relative humidity with a 12-h light, 12-h dark cycle, and fed chow diet (Formulab Diet 5008, PMI Feeds, Inc., Richmond, IN). Age-matched (9 weeks) and glucose-matched male ZDF rats (Genetic Models, Inc., Indianapolis, IN) were gavaged twice daily for the indicated periods of time with either vehicle (0.05 M *n*-methylglucamine) or GW1929 (5.0 mg/kg). Glucose, triglycerides, and FFAs were measured as previously described (7).

Biochemical assays

Serum triglyceride and FFA levels were determined using a Technicon AXON automated chemistry analyzer (Bayer Corp., Tarrytown, NY) as previously described (7). Serum triglyceride concentrations were measured using Bayer Corp. reagents and Axon method SM4–2148K94. FFA concentrations were measured using Waco FFA C test kit 990–75401 (Waco, Neuss, Germany). Plasma glucose measurements were made with a glucose analyzer (model 2700, YSI, Inc., Yellow Springs, OH).

GeneCalling differential gene expression methodology

Genes that were differentially expressed in GW1929- *vs.* vehicle-treated ZDF rats were identified by GeneCalling technology essentially as previously described (11) using 96 different pairs of endonucleases and mRNA prepared from WAT, BAT, skeletal muscle, and liver. All genes that were determined to be regulated 1.5-fold or more by treatment were confirmed by competitive PCR using gene-specific oligonucleotides as previously described (11).

Real-time quantitative PCR (RTQ-PCR)

RTQ-PCR was performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA) as previously described (12, 13) with minor modifications. Briefly, the expression levels of selected genes were compared between tissues from either vehicle- or GW1929-treated animals using RNA pooled from each treatment group (three animals per group). Pooled RNA samples were normalized for comparison by determining 18S ribosomal RNA levels by RTQ-PCR. Expression levels of selected genes were determined by generating a seven-point serial standard curve (each point performed in triplicate) using vehicle-treated RNA for each gene, with the final assay concentration ranging from 1.6–100 ng total RNA/25 μl reaction. This curve was used to calculate the amount of target gene mRNA in vehicle and treated sample based on RTQ-PCR performed with 25 ng total RNA/25-μl reaction (reactions performed in quintuplicate). Primer/probe sequences used were as follows: glycerol-3-phosphate acyltransferase: forward, CGAAGGAGGCTGATCGCA; reverse, ATGATAGCGCAGGACTTGCTG; probe, ACCTGGCGGAGCACATTCTCTTC-ACC; ketoacyl-coenzyme A (ketoacyl-CoA) thiolase: forward, ACTCTGCCGACCGTCTGG; reverse, GAACGCAGTGCATATTTATCCTGT; probe, TGCTGCCTTTGCTGTTT-CTCGAATGG; heart fatty acid-binding protein (hFABP): forward, CAAGTCGGTCGTGACACTGG; reverse, CCTGCCCGTCCCACTTC; probe, CGGAGGCAAACTGGTCCATGTGC; peroxisomal enoyl-CoA isomerase: forward, CTTCTTGTGAGGAGTCTTGCCA; reverse, CGCAATCATGAGCTTTGTTACC; probe, TGTCGTGGACTTCACAGCTTTGGCTTT; carnitine/acylcarnitine carrier protein: forward, TTGGGATCCGTGGCTTCTAC; reverse, ATCCCACTGGCAGGAACATC; probe, AGGGACTGCGCTCACTCTCATGCG; long chain enoyl CoA hydratase: forward, CTCCAAGGACACCACAGCGT; reverse, TTGACCACAATGATGACC-TTCC; probe, TGCCGTGGCCGTGGGTCTC; and phosphoenolpyruvate carboxykinase (PEPCK): forward, TGAGGAAGTTTGTGGAAGGCA; reverse, GCCGTCGCAGATGTGAATATACT; probe, TGCCCAGCTGTGCCAGCCA. Results are expressed as the fold change by determining the ratio of calculated units of RNA in treated compared with vehicle groups. ANOVA was used to evaluate statistical significance.

Generation of cDNA probes

cDNA fragments encoding rat pyruvate dehydrogenase kinase 4 (PDK4; 423 bp) or hFABP (397 bp) were amplified by PCR from 250 ng rat heart cDNA (CLONTECH Laboratories, Inc., Palo Alto, CA) using the following PCR primer pairs: PDK4, 5′-CCAATCCACATCGTGTACGTTCC-3′ (coding strand) and 5′-TGCGGAAACAAGAGTCCACACACATTC-3′ (noncoding strand); and hFABP, 5′-ATGGCGGACGCCTTTGTCGGTAC-3′ (coding strand) and 5′-TCACGTTCTCGTAAGTCCGAGTG-3′ (noncoding strand). A 544-bp cDNA fragment encoding a portion of rat PEPCK was amplified by PCR from 250 ng rat liver cDNA (CLONTECH Laboratories, Inc., Palo Alto, CA) using the primer pair 5′-TCTACGAAGCTCTCAGCTGGCAG-3′ (coding strand) and 5′-TTACATCTGGCTGATTCTCTGTTTC-3′ (noncoding strand). Fragments were labeled with [^{32}P]deoxy-CTP using the MegaPrime random primers labeling system according to the manufacturer's protocol (Amersham Pharmacia Biotech, Aylesbury, UK).

RNA preparation and Northern blot analysis

Total RNA from liver, WAT, and soleus muscle was prepared from ZDF rats using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Inc., Gaithersburg, MD), resolved on a 1% agarose/formaldehyde gel, and transferred to a nylon membrane. After UV cross-linking, filters were prehybridized at 68 C in Express-Hyb (CLONTECH Laboratories, Inc.) for 60 min, followed by hybridization to specific ^{32}P-labeled cDNA probes at a concentration of 1×10^6 cpm/ml for 2 h at 68 C. Filters were washed twice in 2 × SSC (standard saline citrate)/0.1% SDS for 20 min, followed by a single wash in 0.1 × SSC/0.1% SDS at 60 C and exposed to storage phosphor screens for several hours. Northern blots were stripped and reprobed with β-actin for normalization. Image analysis and quantitation from the phosphor screen were performed on a Storm optical scanner using the ImageQuant software package (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Genes regulated by the PPARγ agonist GW1929 in insulin-sensitive tissues

To identify genes regulated by a PPARγ agonist, ZDF rats were treated for 7 days with either GW1929 or vehicle alone. GW1929 is a tyrosine-based, nonthiazolidinedione PPARγ agonist that activates PPARγ *in vitro* with a half-maximal effective concentration of about 10 nM. GW1929 is more than 1000-fold selective for PPARγ relative to either PPARα or PPARδ (6, 7). As expected, GW1929 treatment resulted in marked decreases in serum glucose, triglyceride, and FFA levels (Table 1). At the end of the

TABLE 1. Effects of GW1929 on serum parameters in ZDF rats

		Day 0	Day 7
Glucose (mg/dl)	Vehicle	370 ± 36	330 ± 49
	GW1929	380 ± 22	110 ± 10[a]
NEFAs (mEq/liter)	Vehicle	0.73 ± 0.11	0.67 ± 0.15
	GW1929	0.77 ± 0.13	0.25 ± 0.04[a]
Triglycerides (mg/dl)	Vehicle	810 ± 44	770 ± 32
	GW1929	860 ± 45	82 ± 10[a]

ZDF rats (n = 5/group) were treated for 7 days with GW1929 or vehicle alone. Data are the mean ± SE.

[a] $P < 0.05$ compared with vehicle treatment.

treatment period, mRNA was prepared from epididymal WAT, interscapular BAT, liver, and soleus skeletal muscle. Genes whose expression was either increased or decreased in response to GW1929 were identified by GeneCalling mRNA profiling technology (11). Based on the fraction of GeneCalling cDNA fragments that were changed by treatment, we estimate that approximately 10% of all genes were regulated 1.5-fold or more in WAT and BAT, approximately 2% in liver, and approximately 1% in skeletal muscle. These data are consistent with the finding that PPARγ is much more highly expressed in WAT and BAT than in either liver or skeletal muscle. Genes with known function that were identified in this study as regulated 1.5-fold or more by GW1929 are listed in Table 2. As part of the standard GeneCalling analysis, the regulation of each of these genes was confirmed by a competitive PCR reaction using a gene-specific oligonucleotide as previously described (11). In addition, the changes in the expression of a number of these genes were confirmed by either Northern analysis or RTQ-PCR (Table 2). In all cases, the direction in which the gene was regulated (*i.e.* either stimulation or repression) agreed between GeneCalling and Northern or RTQ-PCR analysis. In most cases, we also observed a good quantitative correlation between the data obtained via the GeneCalling technology and either Northern or RTQ-PCR analysis (Table 2 and Figs. 1 and 2).

WAT

GW1929 treatment resulted in a coordinate increase in the expression of a large number of genes involved in glucose and fatty acid homeostasis in WAT. Among these were many genes whose products are required for lipogenesis, including the dihydrolipoamide acyltransferase subunit of pyruvate dehydrogenase (PDH; Table 2, line 32), acetyl-CoA carboxylase (Table 2, line 1), which catalyzes the rate-limiting step in long-chain fatty acid biosynthesis, fatty acid synthase (Table 2, line 2), proteins of the pyruvate/malate cycle (citrate synthase, pyruvate carboxylase, tricarboxylate transport protein, ATP-citrate lyase, cytosolic malate dehydrogenase, and malic enzyme; Table 2, lines 3–7 and 31), and proteins involved in fatty acid desaturation (stearyl-CoA desaturase; Table 2, line 8) and triglyceride biosynthesis (PEPCK, long-chain acyl-CoA synthetase, and glycerol-3-phosphate acyltransferase; Table 2, lines 9, 11, 30). PEPCK and acyl-CoA synthetase were previously shown to be regulated by glitazones and PPARγ (14, 15). Glycogen synthase expression was also markedly increased by GW1929 treatment (Table 2, line 34). The increased expression of these genes is consistent with GW1929 stimulating the storage of glucose in white adipocytes as either triglyceride or glycogen.

GW1929 treatment stimulated the expression of genes involved in different aspects of fatty acid metabolism in WAT, including lipoprotein catabolism (lipoprotein lipase; Table 2, line 24), fatty acid transport (CD36, fatty acid transport protein, and hFABP; Table 2, lines 25–27), and fatty acid oxidation (*e.g.* carnitine palmitoyltransferase I, short-chain and long-chain acyl-CoA dehydrogenases, and monoglyceride lipase; Table 2, lines 12–18 and 20, 21, 23). Several of these genes, including CD36, lipoprotein lipase, and fatty acid transport protein, were previously shown to be regulated by glitazones (15–17). In agreement with a previous study (18), uncoupling protein 3 (UCP3) expression was also increased by PPARγ agonist treatment (Table 2, line 16). Although its precise function is unknown, UCP3 expression increases under physiological conditions that raise FFA levels (19). A recent report showed that overexpression of UCP3 in the skeletal muscle of mice resulted in the dissipation of energy (20). Taken together, these changes in gene expression suggest that increased uptake, storage, and oxidation of fatty acids in WAT contribute to the marked hypolipidemic actions of GW1929. We note that expression of the adipocyte fatty acid-binding protein (aP2), which is directly regulated by PPARγ during adipocyte differentiation and highly expressed in mature fat cells (21, 22), was only modestly (1.6-fold) increased in WAT by GW1929 treatment (Table 2, line 28), whereas expression of hFABP, which is expressed in a wide variety of tissues (23), was dramatically stimulated (Table 2, line 27). Interestingly, GW1929 treatment increased expression of the transcription factor adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1c (ADD1/SREBP1c) 2.2-fold in WAT (Table 2, line 65). ADD1/SREBP1c is a transcription factor that cooperates with PPARγ in promoting adipocyte differentiation *in vitro*. Moreover, ADD1/SREBP1c regulates several genes involved in lipogenesis in mature adipocytes and may play a broad role in mediating the actions of insulin on genes involved in lipid and carbohydrate metabolism (24, 25). GW1929 treatment also induced expression of the rat ortholog of insulin-induced growth response protein (CL-6; Table 2, line 45). Although its function is not known, insulin-induced growth response protein is highly induced in the liver after insulin treatment and is also known to be expressed in cultured fibroblasts and adipocytes (26, 27). The induction of CL-6 suggests that PPARγ activation is potentiating the actions of insulin in WAT.

Expression of several genes was decreased by GW1929 in WAT, including phosphodiesterase I, α-crystalin, and the contrapsin-like and C1 protease inhibitors (Table 2, lines 46–49). We note that tumor necrosis factor-α and leptin, which were previously shown to be inhibited by glitazones (28–30), were not regulated 1.5-fold or more in WAT or other tissues in this study (data not shown). This may reflect differences in the animal model, the treatment regimen, or the PPARγ agonists employed.

TABLE 2. Genes differentially regulated by a PPARγ agonist in insulin-sensitive tissues

Row	Genbank Accession	Gene Description	WAT	BAT	Muscle	Liver	Putative Function
1	j03808	acetyl-coenzyme A carboxylase	4.0	3.1			lipogenesis
2	x13415	fatty acid synthase	6.6	7.2		2.0	
3	◆	mitochondrial citrate synthase	2.5	1.7			
4	l12016	tricarboxylate transport protein	5.6	9.1			
5	j05210	ATP citrate-lyase	3.8	6.7		1.6	
6	af093773	cytosolic malate dehydrogenase	2.7	2.3			
7	m26581	malic enzyme	6.2 *	15			
8	j02585	stearyl-CoA desaturase	4.2	16	3.9	1.7	
9	d90109	long-chain acyl-CoA synthetase	1.9	2.3			
10	ab012933	acyl-CoA synthetase 5		6.0		1.5	
11	af021348	sn-glycerol-3-phosphate acyltransferase	3.0 *	3.5	-1.8 *		
12	af029875	carnitine palmitoyltransferase I	3.0				FA oxidation
13	j05030	short chain acyl-coenzyme A dehydrogenase	3.1	2.1	-1.6		
14	l11276	long chain acyl-CoA dehydrogenase	2.7			-1.5	
15	ai072959	monoglyceride lipase homolog	2.2	4.3			
16	af030163	uncoupling protein-3	4.6	5.1	-4.0		
17	ai104482	peroxisomal 3,2-enoyl-CoA isomerase homolog	1.9 *	3.6	-2.3 *		
18	x97831	carnitine/acylcarnitine carrier protein	2.8 *	2.0	-1.6 *		
19	x61184	mitochondrial 3-2 trans-enoyl-CoA isomerase		-2.0	-2.3	-2.6	
20	d16478	mitochondrial long chain enoyl CoA hydratase	1.8 *	-1.7	-1.8 *	-1.6	
21	d16479	mitochondrial long chain 3-ketoacyl-CoA thiolase	3.6 *		-2.0 *	-1.7	
22	x05341	3-oxoacyl-CoA thiolase			-2.4		
23	m29853	CYPIVB1	5.5	2.6	1.7		
24	l03294	lipoprotein lipase	2.3	2.3			fatty acid transport/storage
25	af072411	fatty acid translocase/CD36	1.9	1.8			
26	u89529	fatty acid transport protein	1.9	2.0	-1.6		
27	m18034	heart fatty acid binding protein	46 *†	13 †	-3.0 *		
28	u75581	adipocyte fatty acid binding protein	1.6		1.8		
29	d78592	glucose-6-phosphatase				-2.4	gluconeogenesis
30	k03243	phosphoenolpyruvate carboxykinase	3.5 *			-3.6 †	gluconeogenesis/lipogenesis
31	u32314	pyruvate carboxylase	3.2	5.1		-1.7	
32	d10655	dihydrolipoamide acetyltransferase	3.3	2.1			glucose metabolism/lipogenesis
33	af034577	pyruvate dehydrogenase kinase 4	2.2	-3.1	-7.5 †		
34	j05446	glycogen synthase	4.1	7.7			glucose metabolism
35	aj223355	mitochondrial dicarboxylate carrier	2.4	4.3			
36	ai059618	UTP-glucose-1-phosphate uridyltransferase-1 homolog	3.3	1.8			
37	x74125	NAD+-isocitrate dehydrogenase	2.3				
38	j04218	glucokinase				2.7 *	
39	u07181	lactate dehydrogenase-B	2.8				
40	m94040	branched chain alpha-keto acid dehydrogenase	1.7	2.5			amino acid metabolism
41	m77694	fumarylacetoacetate hydrolase	3.0	2.0			
42	m33648	HMG-CoA synthase				-1.7	ketogenesis
43	ab004087	lanosterol 14-demethylase	2.0			2.0	cholesterol biosynthesis
44	m95591	hepatic squalene synthetase	2.1			1.5	
45	l13619	insulin-induced growth-response protein	2.3	9.2			other
46	d28560	phosphodiesterase I	-2.4	-2.6			
47	s74229	alpha B-crystallin	-2.0	-4.4			
48	d00753	contraspin-like protease inhibitor	-3.4				
49	aw915763	C1 inhibitor homolog	-1.8	-1.9			
50	m14864	creatine kinase		-9.0			
51	af197916	myoglobin		-7.0			
52	◆	titin		-4.3			
53	x52815	γ-actin				1.7	
54	u31287	alpha-2u globulin			-3.4		
55	aw918792	CIDE-A homolog			-3.2		
56	ab001349	steroidogenic acute regulatory protein	3.3				
57	x68312	Ig rearranged mu-chain C region				2.0	
58	l13039	annexin II	1.8				
59	u68562	heat shock protein 60	2.5	2.9			
60	m99223	calcium transporting ATPase		-6.0			
61	m11670	catalase	2.0	2.8	1.7		
62	x65083	cytosolic epoxide hydrolase	2.9	2.2		-1.6	
63	d00688	monoamine oxidase A	1.9				
64	l00090	angiotensinogen	-2.0	-1.8			
65	l16995	ADD1/SREBP1c	2.2 *				
66	l23874	iron-responsive element-binding protein	2.2	2.3			
67	x67948	channel integral membrane protein 28		-8.0	-1.6		
68	◆	BEC1 homolog		-6.3			
69	x06148	ribosomal protein L5		-2.3			

Genes that were either increased or decreased >1.5-fold in expression by GW1929 treatment in epididymal white adipose tissue (WAT), interscapular brown adipose tissue (BAT), skeletal muscle, and liver are indicated in *red* and *blue*, respectively. The regulation of a number of these genes was verified independently by either RTQ-PCR (*) or Northern blot analysis (†). Regulation of adipocyte fatty acid binding protein expression was determined by Northern blot analysis because its expression levels precluded accurate quantitation by GeneCalling analysis. Genes for which there are no available GenBank Accession Numbers are indicated (◆).

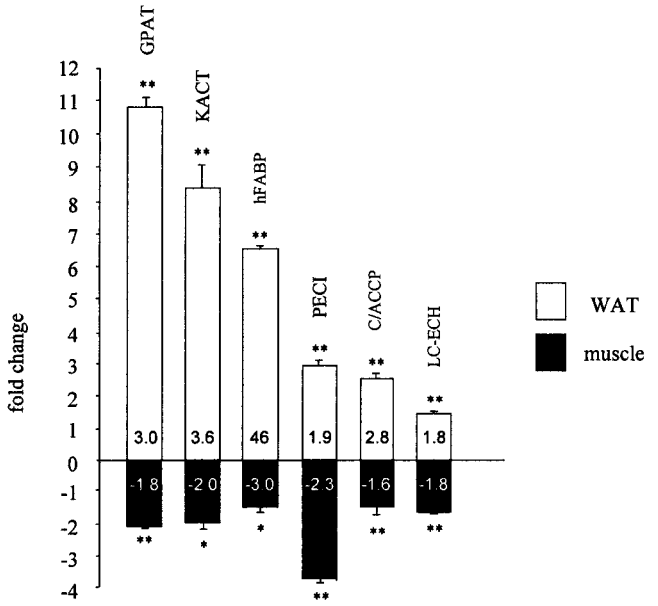

FIG. 1. PPARγ activation differentially regulates gene expression in WAT and muscle. ZDF rats were treated with either GW1929 or vehicle alone for 7 days (n = 3/treatment group), and total RNA was prepared from WAT and soleus skeletal muscle. Levels of glycerol-3-phosphate acyltransferase (GPAT), ketoacyl CoA-thiolase (KACT), hFABP, peroxisomal enoyl-CoA isomerase (PECI), carnitine/acylcarnitine carrier protein (C/ACCP), and long-chain enoyl-CoA hydratase (LC-ECH) mRNA were determined by RTQ-PCR analysis in WAT (□) and muscle (■) and are plotted as fold regulation in GW1929 treatment *vs.* vehicle treatment. *, $P < 0.01$; **, $P < 0.001$ (compared with vehicle treatment). The change in expression (fold regulation) for each of the genes as determined by GeneCalling analysis (Table 2) is superimposed on each of the bars for comparison.

BAT

Overall, the pattern of gene regulation by GW1929 in interscapular BAT was very similar to that seen in WAT, with changes occurring in the expression of genes involved in lipogenesis and fatty acid transport and oxidation (Table 2). These data demonstrate that PPARγ agonists regulate many common genes in BAT and WAT. Only a few genes were differentially regulated by GW1929 in BAT relative to WAT. For example, mitochondrial long chain enoyl-CoA hydratase (Table 2, line 20) and PDK4 (Table 2, line 33), which were modestly up-regulated by GW1929 in WAT, were down-regulated in BAT. The expression of several genes that were unaffected by treatment in WAT was decreased in BAT, including creatine kinase and myoglobin (Table 2, lines 50 and 51). The significance of these differences between adipose tissue depots is unclear. However, as BAT and WAT subserve distinct physiological roles, the finding that there are differences in their gene expression patterns is not surprising.

Liver

In contrast to WAT and BAT, roughly equal numbers of genes were up-regulated and down-regulated in liver by GW1929 (Table 2). Notably, GW1929 treatment resulted in decreases in the expression of PEPCK, pyruvate carboxylase, and glucose-6-phosphatase (Table 2, lines 29–31), which are required for hepatic gluconeogenesis. PEPCK expression was previously shown to be reduced by glitazones in cultured rat hepatocytes (31) and in streptozotocin-treated rats (32). These changes in gene expression suggest a molecular basis for the finding that PPARγ agonists reduce hepatic glucose production *in vivo* (33, 34). GW1929 treatment also decreased the expression of several genes involved in fatty acid oxidation (Table 2, lines 14 and 19–21) and HMG-CoA synthase (Table 2, line 42), which encodes the enzyme responsible for the rate-limiting step in ketogenesis. The formation of ketone bodies is decreased by PPARγ agonists in rodent models of insulin resistance (35). In contrast, GW1929 treatment increased the expression of several genes involved in lipogenesis (Table 2, lines 2, 5, 8, and 10) and increased hepatic expression of glucokinase (Table 2, line 38), which catalyzes a key step in glucose metabolism.

Skeletal muscle

In contrast to the other tissues, most of the genes regulated by GW1929 in skeletal muscle showed decreased expression (Table 2). Notably, expression of PDK4 was decreased about 8-fold by GW1929 treatment (Table 2, line 33). As PDK4 phosphorylates and inactivates the PDH complex, thus inhibiting oxidative glucose metabolism, this finding suggests a molecular basis for increased glucose utilization in muscle of PPARγ agonist-treated animals. In agreement with this observation, PDH activity was recently shown to be reduced in ZDF rats and to be restored by troglitazone treatment (36). GW1929 treatment also resulted in a coordinate repression of 10 genes involved in fatty acid transport and oxidation (Table 2, lines 13, 16–22, 26, and 27), suggesting a decrease in the utilization of fatty acids for energy in the muscle of treated animals. In agreement with a previous study performed with pioglitazone (37), UCP3 expression was decreased by PPARγ agonist treatment (Table 2, line 16). As expression of both PDK4 and UCP3 is stimulated by physiological conditions that increase FFA levels (19, 38), the data suggest that GW1929 treatment decreases fatty acid levels in muscle. Expression of aP2 was modestly increased (1.8-fold) in skeletal muscle (Table 2, line 28). It is not clear whether this reflects changes in aP2 expression in myocytes or in adipocytes present in the soleus muscle tissue. Expression of aP2 can be induced by PPARγ agonists in cultured muscle cells (39, 40), but it is not known whether this *trans*-differentiation occurs *in vivo*. Taken together, the gene expression data suggest an increase in glucose utilization and a decrease in fatty acid utilization in the muscle of PPARγ agonist-treated animals.

GW1929 has opposing effects on gene expression in WAT and muscle

The GeneCalling analysis revealed that many of the same genes involved in fatty acid transport and oxidation showed increased expression in WAT and BAT and decreased expression in skeletal muscle in response to GW1929 treatment (Table 2). We confirmed the differential regulation of several of these genes by RTQ-PCR analysis using RNA derived from WAT or skeletal muscle. As expected, the expression of glycerol-3-phosphate acyltransferase, ketoacyl-CoA-thiolase, hFABP, peroxisomal enoyl-CoA isomerase, carnitine/acylcarnitine carrier protein, and long-chain enoyl-CoA hy-

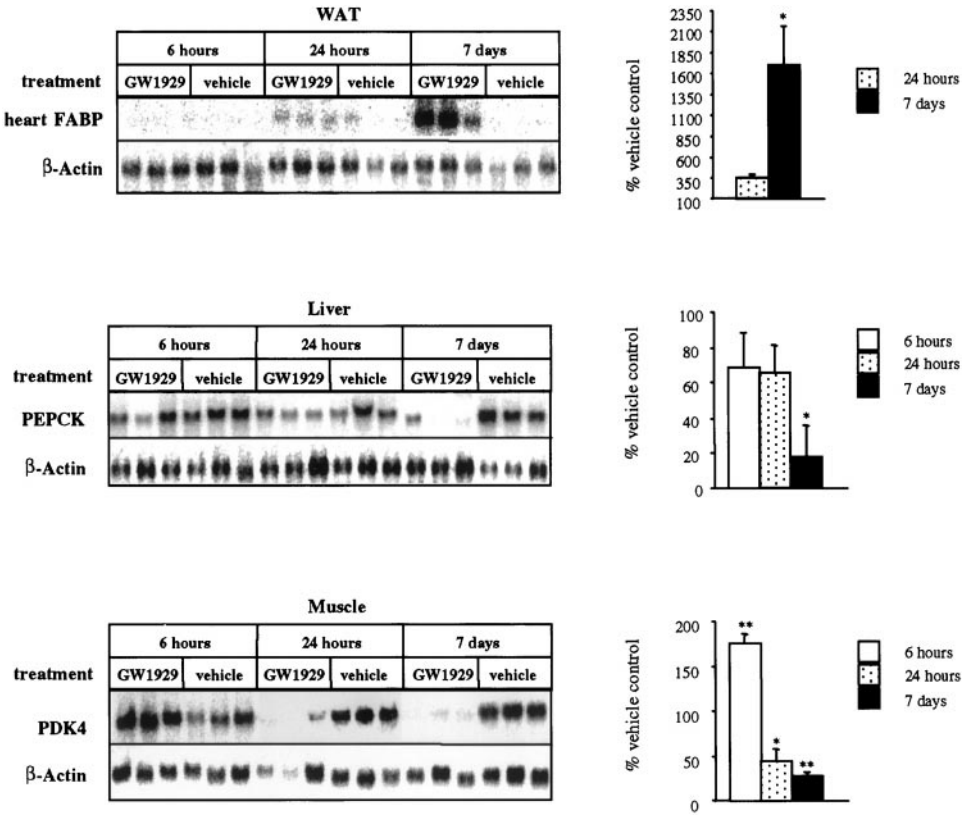

FIG. 2. Time-course study for effects of PPARγ agonist treatment on gene expression in WAT, liver, and muscle. ZDF rats were treated with either GW1929 or vehicle alone for 6 h, 24 h, or 7 days (n = 3/treatment group), and total RNA was prepared from WAT, liver, and soleus skeletal muscle at each of the time points. Northern blot analysis was performed with probes for hFABP (*top*), PEPCK (*middle*), or PDK4 (*bottom*) as indicated. Northern blot data were quantified with a PhosphorImager and normalized using β-actin, and are shown to the *right* of each blot. *, $P < 0.05$; **, $P < 0.01$ (compared with vehicle treatment).

TABLE 3. Time course for effects of GW1929 on serum parameters in ZDF rats

	0	6 h	24 h	7 days
Glucose (% vehicle)	100 ± 2.6	100 ± 2.6	101 ± 0.90	39 ± 16[a]
FFA (% vehicle)	100 ± 12	70 ± 7.9	62 ± 8.4[a]	51 ± 10[a]
Triglycerides (% vehicle)	97 ± 6.6	97 ± 6.7	94 ± 3.7	31 ± 9.5[a]

ZDF rats (n = 3/group) were treated for 6 h, 24 h, or 7 days with GW1929 or vehicle alone. Data are the mean ± SE.

[a] $P < 0.05$ compared with vehicle treatment.

dratase was significantly increased in WAT and decreased in muscle by GW1929 (Fig. 1). The magnitudes of these changes roughly paralleled those seen in the GeneCalling analysis (Fig. 1 and Table 2). These data provide strong evidence that PPARγ activation differentially modulates fatty acid metabolism in WAT and muscle.

Time course for the tissue-specific effects of GW1929

We sought to determine the kinetics with which GW1929 affected serum glucose and lipid levels and gene expression in different insulin-sensitive tissues. ZDF rats were treated with either GW1929 or vehicle alone for 6 h, 24 h, or 7 days to distinguish early effects from secondary effects. Clinical parameters were measured, and total RNA was prepared from WAT, liver, and muscle at each time point. A statistically significant decrease in FFA levels was observed at 24 h, although a more modest decrease was seen at the 6 h point (Table 3). Changes in serum glucose and triglyceride levels were seen only at 7 days (Table 3). These data demonstrate that PPARγ agonist-induced decreases in FFA levels precede those in glucose and triglyceride levels, suggesting that decreases in FFA levels may be important for the insulin-sensitizing actions of PPARγ agonists.

To examine the temporal relationship between changes in serum parameters and gene expression, we performed Northern blot analysis of hFABP expression in WAT, PEPCK expression in liver, and PDK4 expression in skeletal muscle at the 6 h, 24 h, and 7 day points. These genes were chosen based on their high degree of regulation by GW1929 in their respective tissues (Table 2, lines 27, 30, and 33). An increase in hFABP mRNA in WAT was observed at 24 h after treatment (Fig. 2). hFABP expression levels were further increased after 7 days of treatment. Interestingly, we observed a statistically significant change in PDK4 expression as early as 6 h after treatment with GW1929 (Fig. 2). Surprisingly, PDK4 mRNA levels increased in GW1929-treated animals at the 6 h point. The reason for this initial increase in PDK4 expression is not known. However, after 24 h of treatment, PDK4 mRNA levels had fallen to approximately 50% of those in vehicle-treated animals. A further decrease in PDK4 mRNA levels was seen at the 7 day point (Fig. 2). By contrast, no change in liver PEPCK mRNA expression was observed in GW1929-treated rats until the 7 day point (Fig. 2). These data demonstrate that changes in FFA levels in serum, hFABP expression in WAT, and PDK4 expression in muscle are relatively early events after PPARγ activation and precede the decreases in serum triglyceride and glucose levels. However, the time lag between these early events and decreases in serum glucose levels suggests that other factors may be critical for the improvement in insulin sensitization. The time-course results raise the interesting possibility that

the effects of GW1929 on PDK4 expression are due to direct activation of PPARγ in muscle rather than being a secondary effect of PPARγ activation in adipose tissue. However, as PDK4 is known to be regulated by fatty acids (38), we cannot rule out the possibility that regulation of its expression is secondary to effects on FFA levels. In this regard, the most dramatic regulation of PDK4, hFABP, and PEPCK was observed at the 7 day point (Fig. 2), indicating that at least some of the effect of GW1929 on their expression is likely to be indirect.

Discussion

The therapeutic utility of PPARγ agonists in the treatment of insulin resistance and type 2 diabetes is now firmly established. However, the mechanism underlying the glucose- and lipid-lowering actions of PPARγ agonists has remained obscure. In this report we have used an unbiased and comprehensive mRNA profiling technique to systematically identify genes regulated by a potent PPARγ agonist in four insulin-responsive tissues. Most of the PPARγ-regulated genes that were identified in this study fall into key metabolic pathways involved in carbohydrate and/or lipid homeostasis. As might be expected based on its expression levels, we found that PPARγ regulates many more genes in adipose tissue than in either liver or muscle. Nevertheless, the gene expression data reveal that PPARγ activation has coordinate effects on fundamental metabolic pathways in each of these tissues, including glucose and fatty acid metabolism in skeletal muscle and gluconeogenesis in the liver (Fig. 3).

To date, no PPARγ-regulated genes have been identified in muscle that can readily account for the increase in glucose disposal effected by PPARγ ligands. In this report we show that GW1929 treatment results in a marked decrease in PDK4 expression in muscle. PDK4 phosphorylates and inactivates the PDH complex, which catalyzes the first irreversible step in oxidative glucose metabolism. Thus, decreases in PDK4 levels would be expected to result in increases in glucose oxidation. A recent study showed that PDH activity in soleus muscle was reduced in ZDF rats relative to that in their lean littermates and was restored to normal levels by troglitazone treatment (36). PDK activity is increased in response to fatty acids and has been proposed to play a primary role in the development of insulin resistance in obese individuals (41). Notably, PDK4 expression in skeletal muscle has been shown to positively correlate with plasma insulin concentrations and to negatively correlate with insulin-mediated glucose uptake in nondiabetic Pima Indians, a population with a high prevalence of type 2 diabetes associated with obesity (42). Thus, the inhibition of PDK4 expression may represent an important mechanism by which PPARγ agonists enhance glucose utilization in muscle.

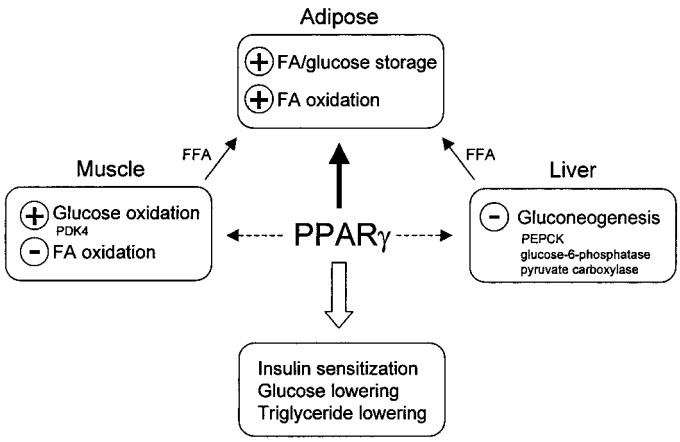

FIG. 3. Model for the physiological actions of PPARγ. PPARγ activation either stimulates (+) or represses (−) key metabolic pathways in adipose, muscle, and liver and promotes a flux of FFA from muscle and liver to adipose. The overall effect is to increase glucose utilization in muscle and to decrease glucose production in the liver. Key genes regulated by PPARγ are indicated. The effects of PPARγ activators are direct (*solid arrow*) on adipose and either direct or indirect (*dashed arrows*) on muscle and liver.

In addition to its effects on PDK4 expression, GW1929 treatment resulted in a coordinate decrease in the expression of a number of genes involved in fatty acid transport and oxidation in muscle. These data are consistent with a decreased reliance on fatty acids and an increased reliance on glucose as an energy source in muscle. Strikingly, expression of these same genes was increased in adipose tissue in response to GW1929, suggesting that PPARγ activation promotes a flux of fatty acids into adipose tissue and away from muscle (Fig. 3). In this regard, two recent studies showed that glitazone treatment decreased triglyceride levels in the skeletal muscle of ZDF rats (36, 43). Repartitioning of fatty acids from muscle to adipose tissue might be expected to enhance insulin sensitivity based on the Randle cycle, in which fatty acids and glucose compete for use as energy substrate in muscle (41). FFAs are also known to stimulate glucose production in the liver (41, 44). Hence, a flux of fatty acids away from liver might also account for the decreases that we observed in the expression of PEPCK, pyruvate carboxylase, and glucose-6-phosphatase (Fig. 3).

Do PPARγ agonists regulate gene expression in muscle and liver by direct receptor activation or by more indirect means, such as altering metabolite levels? In time-course studies, we found that PPARγ agonist-induced decreases in FFA levels preceded drops in glucose and triglyceride levels (Table 3). These data suggest that decreases in FFA levels may be important for the insulin-sensitizing actions of PPARγ agonists. However, the time lag between the decreases in FFA and glucose levels suggests that other factors are likely to be important in insulin sensitization. Interestingly, the expression of PDK4, which we used as a marker of changes in gene expression in muscle, was decreased as early as 24 h posttreatment (Fig. 2). These changes preceded those in serum triglyceride and glucose levels but occurred at roughly the same time as the decreases in FFA levels. Thus, the effects of GW1929 on the expression of PDK4 and other genes in muscle may be secondary to changes in circulating FFA concentrations. It is important to note, however, that our studies do not rule out direct effects of PPARγ agonists on muscle. Several studies have shown that the PPARγ protein is present in human and rodent muscle (45, 46), and troglitazone has been reported to regulate gene expression and enhance glucose utilization in human muscle cultures (40, 47). Moreover, troglitazone was shown to improve insulin sensitivity in mice that had been engineered to lack adipose tissue (48), demonstrating that PPARγ agonists can affect

metabolism in the absence or near absence of fat. Conclusive evidence that PPARγ agonists mediate effects directly on muscle is likely to require gene knockout animals in which the PPARγ gene has been disrupted in specific tissues. We note that in our time-course studies, PEPCK expression in liver was altered only at the 7 day point, suggesting that its regulation may be secondary to changes in FFAs or other metabolites.

In conclusion, we have demonstrated that PPARγ regulates genes involved in key metabolic pathways in adipose tissue, muscle, and liver. In muscle, PPARγ activation decreased the expression of PDK4 and a series of genes involved in fatty acid metabolism. These changes are likely to account in part for the enhancement of glucose utilization mediated by PPARγ agonists. In liver, PPARγ activation reduced the expression of three genes involved in gluconeogenesis, which is likely to account for the effects of PPARγ agonists on hepatic glucose production. Thus, the therapeutic actions of PPARγ agonists represent their coordinate effects in multiple, insulin-sensitive tissues.

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