Peroxisome Proliferator-Activated Receptor Subtype-Specific Regulation of Hepatic and Peripheral Gene Expression in the Zucker Diabetic Fatty Rat

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Fibrates and thiazolidinediones are used clinically to treat hypertriglyceridemia and hyperglycemia, respectively. Fibrates bind to the peroxisome proliferator-activated receptor (PPAR)-α, and thiazolidinediones are ligands of PPAR-γ. These intracellular receptors form heterodimers with retinoid X receptor to modulate gene transcription. To elucidate the target genes regulated by these compounds, we treated Zucker diabetic fatty rats (ZDF) for 15 days with a PPAR-α–specific compound, fenofibrate, a PPAR-γ–specific ligand, rosiglitazone, and a PPAR-α/-γ coagonist, GW2331, and measured the levels of several messenger RNAs (mRNAs) in liver by real-time polymerase chain reaction. All 3 compounds decreased serum glucose and triglyceride levels. Fenofibrate and GW2331 induced expression of acyl-coenzyme A (CoA) oxidase and enoyl-CoA hydratase and reduced apolipoprotein C-III and phosphoenolpyruvate carboxykinase mRNAs. Rosiglitazone modestly increased apolipoprotein C-III mRNA and had no effect on expression of the other 2 genes in the liver but increased the expression of glucose transporter 4 and phosphoenolpyruvate carboxykinase in adipose tissue. We identified a novel target in liver, mitogen-activated phosphokinase phosphatase 1, whose down-regulation by PPAR-α agonists may improve insulin sensitivity in that tissue by prolonging insulin responses. The results of these studies suggest that activation of PPAR-α as well as PPAR-γ in therapy for type 2 diabetes will enhance glucose and triglyceride control by combining actions in hepatic and peripheral tissues.

THE PEROXISOME proliferator-activated receptors (PPARs) are intracellular receptors that heterodimerize with the retinoid X receptor (RXR) to modulate gene transcription. Ligands for either PPAR-γ (eg, thiazolidinediones [TZDs]) or RXR (rexinoids) are effective insulin sensitizers in rodent models of type 2 diabetes,[1-3] and fibrates, which bind to PPAR-α, are used to treat hypertriglyceridemia in humans. PPAR-γ is highly expressed in adipose tissue, with much lower expression in liver and skeletal muscle; while the RXRs are expressed in most tissues.[4,5] PPAR-α is highly expressed in liver,[6] and ligands for either PPAR-α or RXR can regulate hepatic gene expression.[7,8] Because fibrates and TZDs have overlapping physiologic effects, eg, on decreasing triglycerides, and PPAR-α and-γ recognize the same DNA response elements, we wanted to determine if target genes are common or distinct to each subtype. If subsets of regulated genes are found to be specific to PPAR-α or -γ, further therapeutic value might be afforded by combining their activities.

Fibrates and some TZDs decrease plasma triglyceride levels in rodent models of obesity and insulin resistance.[9] At least some of the hypotriglyceridemic effect of fibrates is mediated through the negative regulation of expression of the apolipoprotein C-III (apoC-III) gene in liver.[10-12] ApoC-III associates with lipoprotein lipase (LPL) and represses its activity.[13,14] ApoC-III expression in the liver of rats is unaffected by treatment with rosiglitazone (BRL49653), a potent TZD, and the mechanism for triglyceride lowering probably involves induction of the LPL gene in adipose tissue.[15] Fibrates also promote lipid catabolism in the liver by inducing several peroxisomal genes. Two examples are acyl-coenzyme A (CoA) oxidase (AOX) and the bifunctional enzyme enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), which together catalyze the first 3 steps in the β-oxidation of fatty acids and have well-characterized PPAR response elements (PPREs) in their promoters.[16-18]

TZDs promote glucose removal in skeletal muscle, the major tissue for insulin-induced glucose uptake.[19] The TZD troglitazone also reduces hepatic glucose output in humans, but only at high doses.[20] Whether this is a direct effect on the liver or secondary to changes in the periphery is not clear. A key determinant of glucose production in liver is phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme for gluconeogenesis. PEPCK activity is primarily determined by the level of PEPCK messenger RNA (mRNA). PEPCK mRNA is up-regulated by PPAR-α or -γ agonists in adipose tissue to provide glycerol for triglyceride synthesis and storage; insulin opposes this effect.[21,22] In the liver, PEPCK mRNA is strongly repressed by insulin, an effect that is dominant over induction by glucocorticoids, retinoic acid, and glucagon.[23,24] We were interested in determining whether PEPCK is a target gene for the action of PPAR-α or -γ agonists in liver, potentially influencing hepatic glucose output. Recently, Davies et al[25] have reported that troglitazone can inhibit expression of PEPCK mRNA in primary hepatocytes cultured from normal or Zucker diabetic fatty (ZDF) rats.

Some of the effects of insulin on liver are mediated by the mitogen-activated phosphokinase (MAP kinase) signal-transducing pathway.[26] Insulin also induces expression of a negative regulator of MAP kinase activity, MAP kinase phosphatase 1 (MKP-1).[27,28] In studies using microarray technology, we identified MKP-1 as a gene whose mRNA is reduced in the liver of animals treated with a rexinoid (S. Dana and D. Crombie, unpublished observation, April 1998). MKP-1 is a member of a family of dual-specificity phosphatases that recognize the motif TXY in mitogen- and stress-activated protein kinases.

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These enzymes dephosphorylate at both threonine and tyrosine residues to inactivate the kinase.[29] Thus, MKP-1 serves as a negative feedback regulator of MAP kinase activity, and down-regulation might potentiate insulin signaling through this pathway.[27]

Clearly both fibrates and TZDs have multiple activities that address risk factors associated with cardiovascular disease and type 2 diabetes. Compounds have been identified that activate both PPAR-α and PPAR-γ,[30-32] and these might be more effective agents for the treatment of metabolic disorders by ameliorating both dyslipidemia and hyperglycemia. In particular, the trisubstituted ureidofibrate derivative GW2331 activates both PPAR-α and PPAR-γ in cotransfections using a GAL4 reporter and a GAL4 ligand-binding domain chimera generated from the mouse, human, or Xenopus receptor.[30] We treated ZDF rats with agonists of either PPAR-α (fenofibrate) or PPAR-γ (rosiglitazone), as well as with the PPAR-α/γ coagonist GW2331. We report the effects of these compounds on plasma glucose and triglyceride levels and on expression of the genes encoding AOX, HD, apoC-III, PEPCK, and MKP-1 in the livers of treated animals. We find that treatment with fenofibrate or GW2331 induces AOX and HD mRNAs and reduces the expression of apoC-III, PEPCK, and MKP-1. Treatment with rosiglitazone, on the other hand, modestly increases expression of MKP-1 and apoC-III and does not affect expression of the other 3 genes. However, rosiglitazone increases expression of glucose transporter 4 (GLUT4) and PEPCK in skeletal muscle and white adipose tissue. Our results suggest that the mechanisms by which a PPARγ agonist decreases glucose and triglyceride concentrations in the ZDF rat are distinct from those of a PPAR-α–specific agonist. Thus, combining PPAR-α and PPAR-γ activities in a single compound can be anticipated to provide significant advantages over current monotherapies for type 2 diabetes and cardiovascular disease.

MATERIALS AND METHODS

Animals and Treatments

Male ZDF rats were purchased from Genetic Models, Inc (Indianapolis, IN) and housed on a 12-hour light-dark lighting regimen (lights on at 6 AM). Animals had free access to food (Purina 5008, St Louis, MO) and water throughout the study. After 2 weeks of acclimation, 8-week-old animals were weighed, bled via the tail vein in the nonfasted state, and sorted into treatment groups based on glucose levels and initial body weights. From the next day, animals received single daily oral treatment (gavage) with vehicle (9.95% polyethylene glycol, 1% carboxymethylcellulose, 0.05% Tween 80), fenofibrate (50 to 500 mg/kg/d), rosiglitazone (0.1 to 10 mg/kg/d), or GW2331 (0.3 to 30 mg/kg/d). Fenofibrate was purchased from Sigma (St Louis, MO). Rosiglitazone and GW2331 were synthesized at Ligand Pharmaceuticals; molecular structure and purity were confirmed by nuclear magnetic resonance before use in vivo. After the 15th dose, food was withheld, and 3 hours later animals were anesthetized with isoflurane, blood was sampled by cardiac puncture, and animals were killed by CO_2 asphyxiation. Tissues were harvested, rinsed in phosphate-buffered saline (PBS), weighed, rapidly frozen in liquid nitrogen, and stored at −80°C. All protocols were approved by the institutional animal care and use committee.

Plasma glucose and triglyceride levels were determined colorimetrically (Sigma).

Preparation of RNA and Complementary DNA From Liver, Skeletal Muscle, and White Adipose Tissue

Frozen tissue (liver, quadriceps, or epididymal fat pad) was homogenized in TRI Reagent (Sigma), and total RNA was isolated according to the manufacturer's protocol. RNA concentration was determined by absorbance at 260 nm. Each RNA sample was reverse-transcribed using a superscript preamplification system for first-strand complementary DNA (cDNA) synthesis (Gibco BRL, Gaithersburg, MD) and oligo(dT) primers according to the manufacturer's protocol. RNA was stored at −80°C for Northern blot analysis, and cDNAs were stored at −20°C for real-time polymerase chain reaction (PCR) determinations of individual mRNA levels.

Real-Time Fluorogenic PCR Assays

Relative mRNA levels of 5 target genes and an invariant transcript, 36B4, were determined by real-time PCR[33] using a single cDNA preparation from the liver of each animal. For each transcript sequence to be quantified, a TaqMan probe and flanking primers (listed in Table 1) were chosen by Primer Express software (Perkin-Elmer, Foster City, CA). No amplicons were positioned further than 2 kb from the polyadenylation sequence. Individual PCR reactions contained cDNA prepared from 1 ng total RNA and a master mix such that final concentrations were 100 nmol/L probe, 100 to 900 nmol/L primers optimized for each target, and 1× TaqMan Universal PCR Master Mix (Perkin-Elmer, proprietary). PCR was performed and monitored by the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) for 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute. One cDNA from each experiment was designated as a standard, and serial dilutions were prepared in nuclease-free H_20. Each 96-well reaction plate contained duplicate no-template controls, duplicate standards of at least 7 concentrations covering a 2 log or more range, triplicate determinations of each unknown, and a single no–reverse transcription control for each unknown (to monitor DNA contamination of RNA preparations). Quantification of unknowns was performed by Sequence Detection Systems 1.6 software (Perkin-Elmer) and was compared with the arbitrary standard of similar composition. The same standard dilution series was used for all target gene assays within a given in vivo experiment. Slopes of Ct (threshold cycle, 10 SD over background) versus (logN) input cDNA concentration (standard curve) varied from −3.3 to −4.0, and the correlation coefficient (r^2) of the linear regression was greater than 0.95 for each assay reported.

Statistics

Data are presented as means ± SEM and were analyzed by unpaired Student *t* test. Correlations were sought by Pearson regression analysis. A P value of $<.05$ was considered statistically significant.

RESULTS

Metabolic Response to Treatment

ZDF rats were treated daily for 15 days with varying doses of fenofibrate, rosiglitazone, or the PPAR-α/γ coagonist GW2331. The resulting plasma glucose and triglyceride levels at termination are shown in Fig 1. Glucose was normalized by treatment with 1 and 10 mg/kg/d rosiglitazone or 30 mg/kg/d GW2331. Fenofibrate decreased glucose significantly at 250 and 500 mg/kg/d. Triglyceride levels were decreased by fenofibrate, rosiglitazone, and GW2331. GW2331 decreased triglyceride levels to 45% of control at 3 mg/kg/d and to 18% of control at 30 mg/kg/d. Triglyceride lowering was significant at

Table 1. Primer and Probe Sequences for Real-Time PCR Assays

Target	Encoded Protein	Probe*	Primers†
36B4	60s acidic ribosomal protein PO	j-GCTGTGGTGCTGATGGGCAAGAAC-t	*f* AGATGCAGCAGATCCGCAT
			r GGATGGCCTTGCGCA
ApoC-III	Apolipoprotein C-III	f-AGCCCAGCAGCAAGGATCCCTCTC-t	*f* CTCTGCCCGAGCTGATGAG
			r TTGTTCCATGTAGCCCTGCAT
AOX	Acyl-CoA oxidase	f-TGCCCGCCGCAGGCCATT-t	*f* TGCTGGCATCGAAGAATGTC
			r AATCCCACTGCTGTGAGAATAGC
HD	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	f-CAAGGGCGGGCCCATGTTCTATG-t	*f* GCTGGAGCCCAGTGACTACCT
			r TGCCAAGCTTTGCCATTCC
GLUT4	Glucose transporter 4	f-CTGCTTCCTTCTATTTGCCGTCCTCCTG-t	*f* CAGTATGTTGCGGATGCTATGG
			r AAATGTCCG GCCTCTGGTTT
LPL	Lipoprotein lipase	f-CACTTTCAGCCACTGTGCCATACAGAGA-t	*f* TGTCTAACTGCCACTTCAACA
			r CATACATTCCTGTCACCGTCC
MKP-1	MAP kinase phosphatase 1	f-AGCCACCATCTGCCTTGCTTACCT-t	*f* GCCAGGCCGGCATCTC
			r GCCTCTGCTTCACGAACTCAA
PEPCK	Phosphoenolpyruvate carboxykinase	f-TCATGCACGACCCCTTCGCTATGC-t	*f* GCAGAGCATAAGGGCAAGGT
			r GCCGAAGTTGTAGCCAAAGAAG

NOTE. Sequences are listed 5′ to 3′.

* Fluorescent labels in probes are j, JOE (6-carboxy-4, 7, 2′, 7′-tetrachlorofluorescein); f, FAM (6-carboxyfluorescein); t, TAMRA (6-carboxy-N, N, N′, N′-tetramethylrhodamine).

† Forward primers are designated by *f* and reverse primers by *r*.

rosiglitazone doses of 1 and 10 mg/kg/d (to 61% and 30% of control, respectively). Fenofibrate treatment significantly decreased triglyceride levels to 68% of control at the 500-mg/kg/d dose. Note that triglyceride lowering appears to be more sensitive to GW2331 than does glucose lowering. Although all animals gained weight throughout the study, animals treated with rosiglitazone gained more weight at all doses than animals treated with vehicle, and both fenofibrate and GW2331 restrained weight gain compared with vehicle (data not shown).

Hepatic Gene Expression Profiles in ZDF Rats Treated With PPAR Agonists

The liver is a target organ for compounds that activate PPAR-α.[34] In vitro DNA binding and transactivation assays indicate that PPAR-α and -γ recognize the same DR-1 response element.[34] In preliminary experiments, we compared the effects of fenofibrate and rosiglitazone on expression in liver of HD, a marker of PPAR-α activity, and MKP-1, a novel target gene identified by a differential expression technique. As determined by Northern blotting, fenofibrate dramatically induced HD expression and reduced MKP-1 expression in the liver (data not shown). Rosiglitazone, on the other hand, slightly increased MKP-1 expression and had no apparent effect on HD mRNA levels. To better compare changes in gene expression with the dose responses of plasma glucose and triglyceride levels, more quantitative assays were developed for several mRNAs of interest. Because the 2 compounds had opposing effects on MKP-1 gene expression and the 2 receptors generally recognize the same response elements, the in vivo effects of a combined PPAR-α/γ ligand, GW2331, were studied to see if a single pattern of response dominates or if they cancel each other.

Real-time fluorogenic PCR assays provide a quantitative and sensitive means of determining levels of multiple transcripts in small samples, which is impractical by Northern or nuclease protection assays. Development of the real-time PCR assays for AOX, HD, apoC-III, PEPCK, MKP-1, GLUT4, and LPL is described in Materials and Methods.

PPAR Target Genes in Lipid Metabolism Are Differentially Regulated in Liver in a Subtype-Specific Manner

Fibrates induce AOX and HD mRNAs and reduce the expression of apoC-III mRNA in rodent liver.[7,10] In the present study, AOX and HD mRNAs were induced by fenofibrate, consistent with the ability of fibrates to induce peroxisomal proliferation and β-oxidation of fatty acids (Fig 2). At 50 mg/kg/d, this effect was maximal for AOX (5-fold) and near maximal for HD (23-fold), although this dose was insufficient to cause a significant decrease in triglyceride levels. In contrast, rosiglitazone did not alter expression of either of these transcripts at doses at which it was efficacious at lowering triglycerides and glucose. As expected, apoC-III mRNA decreased in a dose-dependent manner with fenofibrate treatment. This change was significant at all doses tested, with a reduction to 24% at 500 mg/kg/d ($P < .00001$). ApoC-III gene expression increased 1.6-fold with rosiglitazone treatment at 10 mg/kg/d ($P < 0.01$).

The PPAR-α/γ coagonist GW2331 induced the β-oxidation genes, AOX and HD, similarly to the pure PPAR-α agonist fenofibrate. Treatment with GW2331 dramatically reduced apoC-III mRNA, the level decreasing to 13% ($P < .001$) at 3 mg/kg/d and to 5.5% ($P < .0005$) at 30 mg/kg/d compared with vehicle controls. Therefore, this reduction is dominant over any induction via PPAR-γ.

ApoC-III mRNA Levels in Liver Correlate With Plasma Triglyceride Levels for Animals Treated With Fenofibrate or GW2331

In humans and rodents, plasma triglyceride levels correlate with circulating apoC-III protein levels.[9] In this study, triglycerides correlated positively with apoC-III mRNA levels in

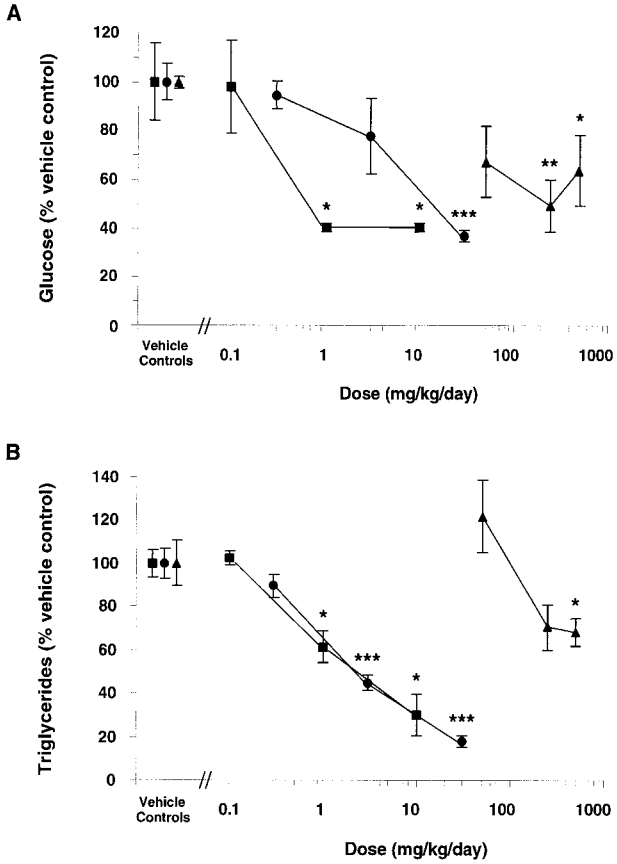

Fig 1. Dose responses of (A) plasma glucose and (B) triglyceride levels in ZDF rats after 15 days of treatment with vehicle, fenofibrate (n = 5), rosiglitazone (n = 4), or GW2331 (n = 4). Values are expressed as means ± SEM. (▲), fenofibrate (vehicle controls 505 ± 12 mg/dL glucose and 653 ± 69 mg/dL triglycerides); (■), rosiglitazone (vehicle controls 411 ± 70 mg/dL glucose and 819 ± 51 mg/dL triglycerides); (●), GW2331 (vehicle controls 476 ± 36 mg/dL glucose and 817 ± 57 mg/dL triglycerides). *$P < .05$; **$P < .01$; ***$P < .001$ *v* vehicle-treated controls.

fenofibrate- and GW2331-treated rats (Fig 3). This was most significant for GW2331-treated animals ($r = .89$, $P < .0001$). The correlation was significant for fenofibrate-treated animals as well ($r = .46$, $P < .05$). Animals treated with rosiglitazone, on the other hand, showed a weak negative correlation between triglycerides and apoC-III mRNA ($r = .49$, NS, data not shown). LPL mRNA was induced in a dose-dependent manner, to 3.2-fold the level of lean controls at 10 mg/kg/d rosiglitazone ($P < .01$) in white adipose tissue from these animals (data not shown). Increased LPL activity may be at least partly responsible for the 70% decrease in triglycerides compared with vehicle-treated control animals (Fig 1).

PEPCK and MKP-1 Genes Are Regulated by PPAR-α in Liver

Hepatic levels of PEPCK and MKP-1 mRNAs were monitored as indicators of glucose metabolism and insulin responsiveness. PEPCK mRNA is induced by fibrates and TZDs in adipocytes[21,22] and has recently been reported to be reduced in isolated rat hepatocytes after treatment with another TZD, troglitazone.[25] Regulation of MKP-1 by PPARs has not been documented. The expression of both PEPCK and MKP-1 in liver was reduced by the PPAR-α agonist fenofibrate, but not by the PPAR-γ agonist rosiglitazone (Fig 4). We found that PEPCK mRNA levels varied considerably among animals. This variance may be a consequence of regulation of this gene by multiple circulating hormones such as insulin, glucocorticoids, glucagon, and retinoids.[23,24] Despite this, PEPCK mRNA was significantly decreased compared with vehicle at all doses of fenofibrate, to 12% and 13% of vehicle-treated control values at 250 and 500 mg/kg/d, respectively. In contrast, rosiglitazone did not significantly alter PEPCK mRNA. MKP-1 mRNA was also significantly reduced by fenofibrate at all doses, to 11% of control at the maximal dose ($P < .00001$). Although there was not a clear dose response, MKP-1 mRNA increased with rosiglitazone treatment. This increase was significant at 10 mg/kg/d, with a 2.9-fold increase over vehicle-treated control values ($P < .01$).

As with the lipid metabolism targets, PEPCK and MKP-1 mRNAs responded similarly to GW2331 and fenofibrate. Reduction of both transcripts was less sensitive to GW2331, but nevertheless the response was significant for PEPCK at 30

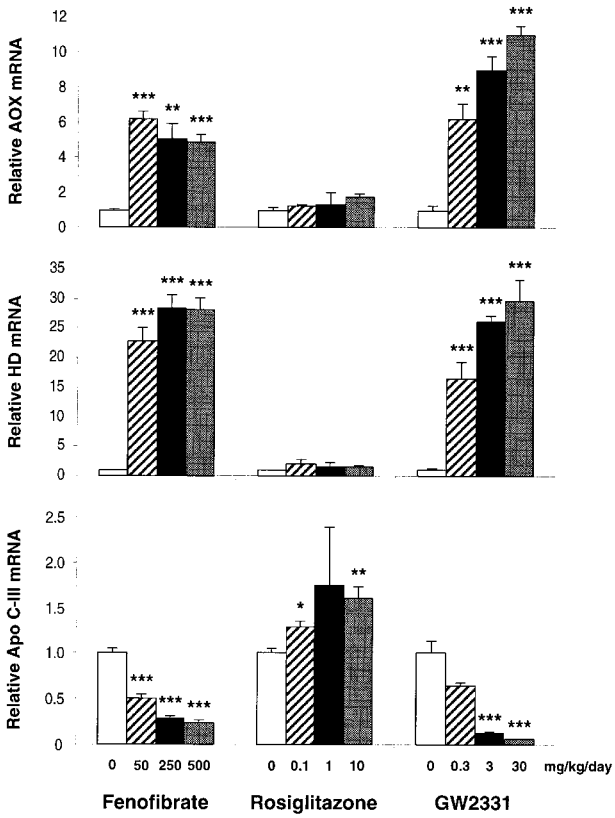

Fig 2. Dose-responsive gene expression profiling in the liver. Values are normalized to 36B4 mRNA in the same sample and relative to vehicle-treated controls and are presented as means ± SEM. n = 5 animals for fenofibrate, 4 animals for rosiglitazone, and 4 animals for GW2331. Real-time PCR determinations were done in triplicate. *$P < .05$; **$P < .01$; ***$P < .001$ *v* vehicle-treated controls.

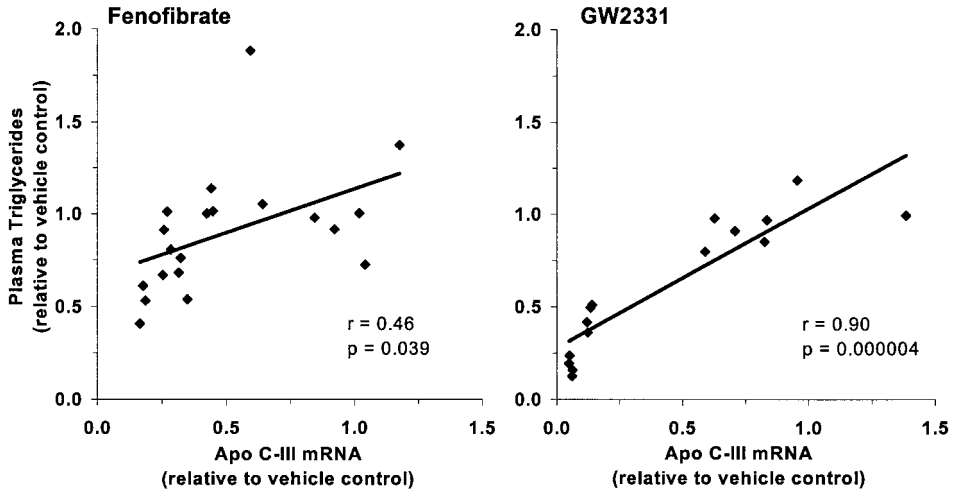

Fig 3. Correlation between individual apoC-III mRNA levels in liver and triglyceride levels in plasma. All values are relative to vehicle-treated controls.

mg/kg/d (19% of vehicle-treated control values, $P < .05$) and for MKP-1 at 3 mg/kg/d (26%, $P < .01$) and 30 mg/kg/d (29%, $P < .01$).

Evidence of PPAR-γ Activation in Peripheral Tissues

Rosiglitazone was more potent than either fenofibrate or GW2331 at glucose lowering and as potent and efficacious as GW2331 at triglyceride lowering but did not effect similar changes in gene expression in the liver. Because TZD treatment is known to stimulate glucose disposal in muscle and fat, it was necessary to document gene expression changes in these tissues to verify that the physiologic changes were associated with PPAR-γ–mediated activity. PEPCK and GLUT4 mRNAs were measured in both skeletal muscle and white adipose tissue from the same animals analyzed for liver gene expression. PEPCK and GLUT4 mRNAs were increased compared with vehicle-treated controls in both muscle and fat after 15 days of treatment with rosiglitazone (Fig 5). These messages were also measured in tissues from animals treated with GW2331. GLUT4 mRNA was increased 2.7-fold in white adipose tissue at 30 mg/kg/d GW2331 ($P < 10^{-5}$) but not significantly in skeletal muscle. Surprisingly, PEPCK mRNA was not induced in white adipose tissue after GW2331 treatment. PEPCK is induced by fenofibrate or TZDs in adipocytes.[21,22] White adi-

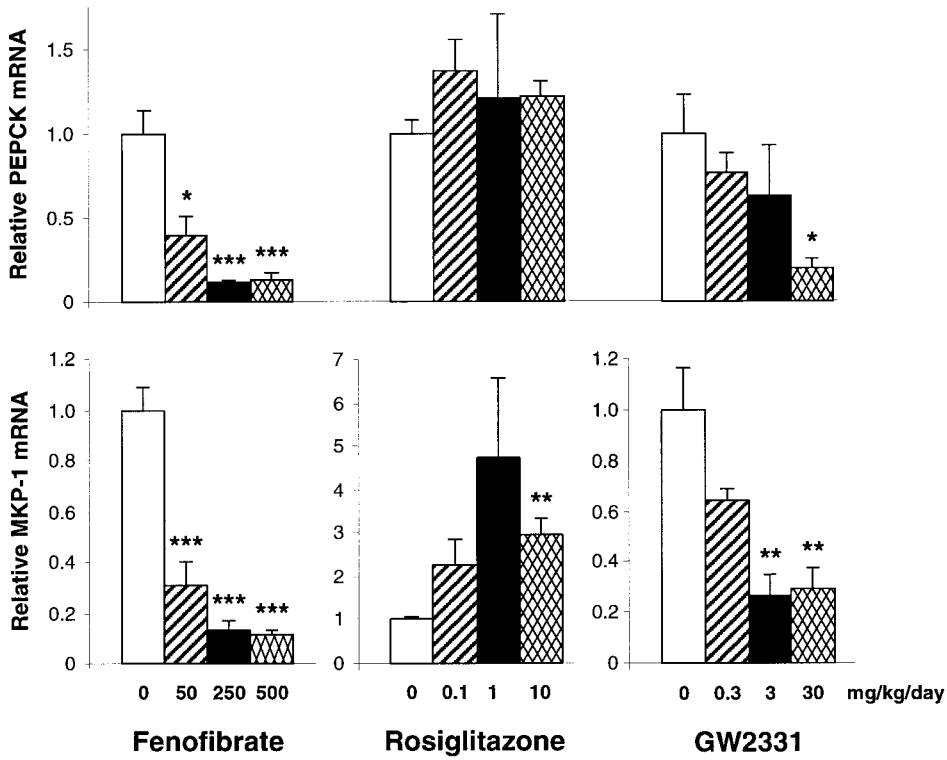

Fig 4. Dose response of PEPCK and MKP-1 gene expression in liver. Values determined by TaqMan real-time PCR assays as in Fig 2. Note the differences in *y*-axis scales for MKP-1 mRNA. **$P < .01$; ***$P < .001$ *v* vehicle-treated controls.

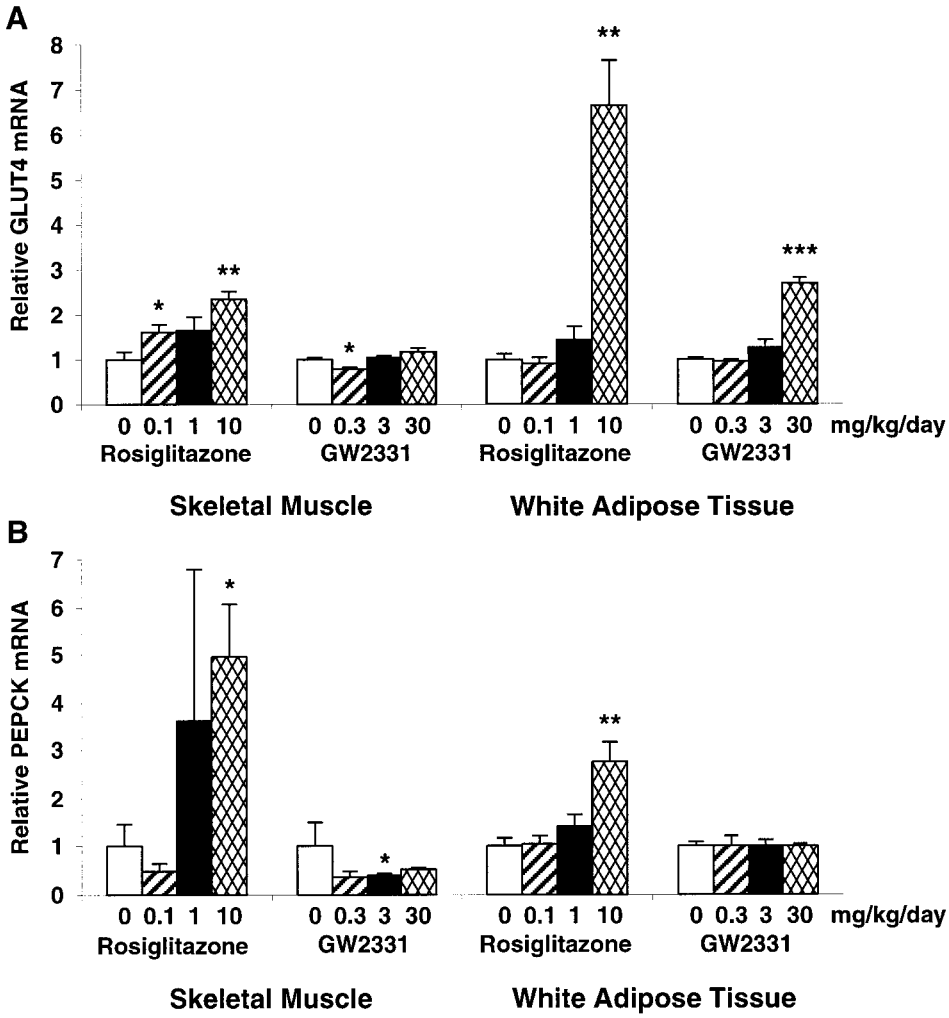

Fig 5. Rosiglitazone influence on (A) GLUT4 and (B) PEPCK gene expression in skeletal muscle and white adipose tissue. mRNA determinations were by real-time PCR as in Fig 2. *$P < .05$; **$P < .01$; ***$P < .001$ *v* vehicle-treated controls.

pose and muscle tissues were not harvested from the fenofibrate treatment groups, so we have no data on GLUT4 or PEPCK in response to fenofibrate. It can be concluded that rosiglitazone treatment is effecting changes in gene expression in skeletal muscle and white adipose tissue and that GW2331 is a weak PPAR-γ agonist in adipose tissue in this rat model.

DISCUSSION

We have examined the differences in gene expression profiles in livers of ZDF rats in response to a PPAR-α–specific agonist, fenofibrate, a PPAR-γ–specific agonist, rosiglitazone, and a PPAR-α/γ coagonist, GW2331. Both fenofibrate and GW2331 induced AOX and HD genes and reduced expression of apoC-III, PEPCK, and MKP-1 mRNAs. Furthermore, in these treatment groups, apoC-III mRNA levels correlated with plasma triglyceride levels. Rosiglitazone had no effect on hepatic expression of AOX, HD, and PEPCK genes and increased apoC-III and MKP-1 mRNAs. In contrast, it was more potent at lowering plasma glucose levels than fenofibrate or GW2331 and more efficacious and potent than fenofibrate at lowering triglyceride levels. Rosiglitazone activates reporter genes driven by PPAR response elements derived from AOX and HD promoter sequences when cotransfected with a human PPAR-γ expression plasmid in HepG2 and CV-1 cells (Mukherjee et al[3] and Patricia Hoener, unpublished results, January 1997). Rosiglitazone induces PEPCK gene expression in 3T3-L1 adipocytes, but probably through an upstream enhancer not involved in liver-specific expression.[21,22] Furthermore, rosiglitazone has been shown to induce LPL mRNA and activity in adipose tissue, which is thought to be a mechanism by which it decreases triglyceride levels.[15,35] We confirmed rosiglitazone-induced regulation of GLUT4, PEPCK, and LPL transcripts in adipose tissue and also demonstrated induction of GLUT4 and PEPCK in skeletal muscle. Given the low level of PPAR-γ expression in skeletal muscle, it is unclear whether changes in muscle are direct or secondary, for example, to signaling via adipose tissue. Nevertheless, the observed changes in expression of these genes in both tissues may contribute to the ability of rosiglitazone to decrease glucose and triglyceride levels. Alternatively, other target genes may be involved in mediating these effects.

The patterns of expression observed in response to these 3 classes of PPAR modulators are consistent with PPAR-α–predominant regulation in the liver in this animal model. Both

apoC-III and MKP-1 mRNAs were modestly induced in the livers of rats treated with rosiglitazone. Whether the mechanism involves direct transactivation by PPAR-γ in liver remains to be determined. In animals treated with the coagonist GW2331, the dominant response is down-regulation of these 2 genes consistent with activation of PPAR-α. In cotransfection experiments with an AOX PPRE reporter, GW2331 strongly activates both PPAR-α and PPAR-γ, verifying its activity as a coagonist in vitro (Keith Marschke, personal communication, August 2000), although the binding affinity is higher for PPAR-α than for PPAR-γ.[30] Induction of GLUT4 in adipose tissue after GW2331 treatment may be via PPAR-γ. It will be important to follow up with PPAR-α/γ coagonists with better PPAR-γ activity in the ZDF rat. In this experiment, GW2331 was equivalent to rosiglitazone in triglyceride lowering and equally efficacious but somewhat less potent at glucose lowering. The results suggest a model in which PPAR-α agonists improve both carbohydrate and lipid metabolism in the liver and PPAR-γ agonists improve glucose use in the periphery. A combination of both PPAR-α and -γ activities in a single therapy should improve glycemic control and serum lipid profiles, thereby minimizing the microvascular as well as macrovascular complications of type 2 diabetes. Two other novel insulin sensitizers with the ability to activate both PPAR-α and -γ have been reported to improve lipid metabolism in rats.[31,32] It would be interesting to compare more balanced α/γ coagonists to fenofibrate and rosiglitazone after short-term dosing to identify primary transcriptional effects in the 3 tissues by differential expression technology.

ApoC-III protein levels correlate with triglyceride levels in plasma, and fibrates are reported to improve hypertriglyceridemia in part by decreasing expression of the apoC-III gene in liver.[10-12] We have confirmed negative regulation of the apoC-III gene via PPAR-α in ZDF rats. Furthermore, we have identified 2 additional genes, PEPCK and MKP-1, whose expression in the liver is reduced in response to PPAR-α ligands. Gervois et al[36] have demonstrated that fibrates induce Rev-erba mRNA in liver cells and propose that Rev-erba may function as a mediator of negative gene regulation by PPAR-α.[36] There are AP-1 binding sites in human PEPCK and MKP-1 promoters, suggesting negative interference of AP-1–dependent transcription as another possible mechanism for repression by these compounds.[37,38]

This report is the first demonstration of negative regulation of the PEPCK gene in liver by a fibrate. Although it is paralleled by a decrease in circulating glucose, further investigation is needed to determine the extent to which PPAR-α activation is directly responsible for the observed reduction in PEPCK mRNA and whether it fully explains the glucose lowering. Glucose production in rat hepatoma cells (H4IIE) is reduced by fenofibrate and GW2331, but not by rosiglitazone, supporting this idea; and this may be a useful model for determining the mechanism (J. Bilakovics, unpublished observations, April 1999). This may differ for the various TZDs because troglitazone inhibits expression of PEPCK mRNA in primary rat hepatocytes.[25]

We identified a novel potential target gene for PPAR-α in liver, MKP-1. Two protein tyrosine phosphatases (PTPases) are already implicated in insulin signaling and the pathogenesis of type 2 diabetes. PTPase 1B and PTPase LAR (leukocyte common antigen-related) interfere with insulin signaling in skeletal muscle by dephosphorylating tyrosine residues on the insulin receptor and/or its proximal substrates such as IRS-1 and shc.[39-42] Consistent with this, a PTPase-1B null mouse has improved insulin sensitivity and is resistant to diet-induced obesity.[43] MKP-1 is a distal negative effector of insulin action through the MAPK signaling pathway. Overexpression of MKP-1 in a rat fibroblast cell line expressing the human insulin receptor decreased insulin-induced MAPK activity and interfered with activation of a *c-fos* SRE reporter.[28] Hyperosmotic stress induces MKP-1 mRNA in H4IIE cells,[44] favors glycogen breakdown,[45] and induces PEPCK mRNA.[46] All of these observations are consistent with a link between MKP-1 expression and glucose homeostasis in liver. An MKP-1–deficient mouse has been created that develops normally and has no obvious phenotype.[47] It would be important to measure fasting glucose levels and insulin responses in liver of these animals, especially following dietary challenges.

Whether chronic reduced expression of MKP-1 in response to PPAR-α agonists in diabetic rats is a mechanism for improved insulin responsiveness in the liver remains to be established. Reducing hepatic MKP-1 activity would increase MAP kinase activity and could amplify insulin responses. Liver responses to insulin include regulation of glycogen synthesis, glycogenolysis, and gluconeogenesis. The MAP kinase pathway does not appear to be crucial to the immediate effects of insulin on enzymatic activities but is involved in mediating changes in gene expression induced by insulin, presumably by altering the phosphorylation state of transcription factors.[26,48] We have also measured MKP-1 mRNA in skeletal muscle and white adipose tissue from the same animals and see no significant difference between control and treated levels with rosiglitazone or GW2331 (Patricia Hoener, unpublished results). Hyperinsulinemic euglycemic clamp studies would be helpful in determining the relative importance of hepatic glucose production and peripheral glucose disposal in glycemic control by PPAR modulators.

Because we monitored changes in gene expression after 15 days of treatment, it is not clear whether any are primary transcriptional responses. In *db/db* mice, changes in AOX, HD, and MKP-1 mRNAs in liver are seen within 6 hours of a naive dose of fenofibrate. ApoC-III and PEPCK mRNA levels do not change significantly by that time but are reduced after 3 and 14 days of oral dosing (J. Bilakovics, S. Dana, P. Hoener, unpublished observations, June 1999 and August 2000). In addition, fenofibrate produced similar changes in AOX, HD, apoC-III, and PEPCK mRNAs in nondiabetic animals (h apo AI transgenic mice),[49] arguing that the changes in gene expression are not secondary to glycemic control (R. Kauffman, S. Dana, unpublished observations, September 1999).

In summary, we have developed sensitive assays for the quantification of gene expression changes to profile tissue-specific effects of modulators of the intracellular receptors PPAR-α and PPAR-γ in a rat model of type 2 diabetes. In the liver, PPAR-α agonists cause significant changes in levels of transcripts encoding proteins mediating lipid catabolism, glucose production, and intracellular signaling, with examples of both up-regulated and down-regulated genes. In contrast,

PPAR-γ agonists have minimal effects on expression of these genes and may act primarily in the periphery in this model. Regulation of LPL, GLUT4, and PEPCK and mRNAs in adipose and muscle tissues substantiate this. Reduction of PEPCK mRNA by PPAR-α agonists is a novel observation and is consistent with the reduced plasma glucose in fenofibrate-treated animals. We propose that reduced expression of MKP-1 potentiates insulin signaling through MAP kinase pathways in the liver. By modifying hepatic lipid and carbohydrate metabolism through PPAR-α and improving insulin sensitivity in skeletal muscle and adipose tissue through PPAR-γ, PPAR-α/γ coagonists should prove more effective than current insulin sensitizers that work through PPAR-γ alone. Appropriate PPAR-α/γ coagonists can be expected to afford glycemic control while minimizing microvascular and macrovascular complications, offering improved therapy for type 2 diabetes.

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