

PPAR α and PPAR γ Regulation of Liver and Adipose Proteins in Obese and Dyslipidemic Rodents

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Zucker fatty rats and *ob/ob* mice are both frequently used hyperlipidemic and insulin-resistant spontaneous genetic models of obesity. We used them to study the effect of PPAR agonists on the protein-expression level in liver and white adipose tissue. PPAR α -agonist treatments of the rats resulted in that 27% of the quantified hepatic proteins were altered; implicating pronounced peroxisome proliferation and increase in capacity for β -oxidation of fatty acids although no correction of plasma triglycerides were obtained. On treatment with PPAR γ agonists, adipose proteins were regulated to a much larger extent in the rats compared to mice, 18% and 2%, respectively.

Keywords: proteomic • hyperlipidemic • *ob/ob* mice • Zucker rats • peroxisome proliferator-activated receptor • insulin resistance • rosiglitazone • darglitazone • WY14,643

Introduction

Treatment of type-2 diabetes patients with nuclear receptor PPAR γ agonists improve insulin sensitivity while treatment with PPAR α agonists (fibrates) improve the dyslipidemia associated with insulin resistance.^{1–4} Although dyslipidemic patients have been treated with PPAR α agonists for several decades, the mechanism of action of these substances are not fully understood. A similar situation exists for the more recently developed PPAR γ activators. One reason is that transcription-factor agonists have impact on the expression of many genes and proteins, either directly or indirectly, and in a tissues-specific manner (for reviews, see refs 2,3,5).

PPARs are members of a superfamily of nuclear receptors which heterodimerize with RXR and bind to specific PPAR response elements (PPREs) in the promoter region of target genes. The PPREs were originally identified as direct repeats (DR1 or DR2) of AGGTCA (reviewed by ref 6), but it is now acknowledged that the flanks or the PPRE are of importance for further specificity with respect to PPAR isoform, tissue, and recruitment of co-activators and co-repressors.⁷ PPAR α is mainly expressed in tissues where FA oxidation occurs and it regulates genes involved in TG and FA metabolism in mitochondria and peroxisomes (in rodents).⁸ Therefore, it is expected that treatment of animals with PPAR α activators will mainly influence genes and proteins in liver, especially in rodents where the expression of PPAR α in the liver is high.

PPAR γ , on the other hand, is highly expressed in tissues that store fat and is involved in synthesis and storage of fat (review).³

Gene- and protein-expression analyses are used to evaluate the effect of PPAR agonists. The strength of both techniques are that they allow analyses of a large number of proteins or genes simultaneously. Gene-expression analysis is faster and less expensive but lack the possibility to inform about changes in amounts of enzymes and proteins, which would be more relevant to follow than the amount of mRNA. The proteomic technology, when performed by 2D-gel electrophoresis and MS, allows studies of PTMs of proteins, a capability utilized in the present study. Studies of PTMs would be of extreme importance since the activity of many enzymes is regulated by their state of modification rather than on their total concentration in cells and tissues. The recent introduction of fluorescent staining of proteins in PAGE gels facilitates reliable quantification of proteins present in low amounts and with a linear response over several decades.⁹ This in combination with improvements in the sensitivity of MS analysis and an almost complete genetic coverage of the rat and mice genomes has increased the potential of the proteomic techniques. Because the PPARs directly and indirectly regulate a large number of cellular processes as discussed above unbiased protein expression techniques should be suitable for studies of their actions.

Some literature is available on gene expression analysis of liver and adipose tissue from rodents treated with PPAR activators.^{10–15} However, there are few reports about protein expression in rodents treated with PPAR-activators in doses relevant for studies of metabolism. In addition to the studies of liver from *ob/ob* mice treated with WY14,643 or rosiglitazone from our laboratory,^{16–18} a study encompassing several tissues of the same rodent model treated with rosiglitazone has been published.¹⁹ The latter, however, only reports the identity of a

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Table 1. Plasma Factors in *ob/ob* Mice Treated for 7 d with Either PPAR α or PPAR γ Activators^a

plasma levels of:	vehicle	WY14,643 (180 μ mol/kg/d)	rosiglitazone (2.5 μ mol/kg/d)
TG (mM)	4.27 \pm 1.06	1.32 \pm 0.32 ^b	1.67 \pm 0.72 ^b
glucose (mM)	32.9 \pm 6.2	17.5 \pm 5.7 ^b	16.0 \pm 7.9 ^b
insulin (nM)	6.46 \pm 2.19	5.44 \pm 1.48	6.08 \pm 3.05
body-weight gain (g)	3.4 \pm 0.64	3.4 \pm 0.87	4.68 \pm 0.61 ^b

^a Results expressed as mean \pm SE ($n = 5$). ^bSignificantly different from the vehicle-treated mice ($p < 0.05$)

Table 2. Plasma Factors and Body and Liver Weight for Zucker *Fa/Fa* Rats Treated with Either a PPAR α or a PPAR γ Activator for 3 Weeks

	vehicle	WY14,643 (30 μ mol/kg/d)	darglitazone (1 μ mol/kg/d)
plasma TG (mM)	5.9 \pm 1.2	7.3 \pm 1.4	1.0 \pm 0.1*
plasma glucose (mM)	7.5 \pm 0.5	10.5 \pm 1.2*	6.3 \pm 0.1
plasma insulin (nM)	4.8 \pm 1.4	5.1 \pm 0.7	0.6 \pm 0.2*
body weight (g)	599 \pm 13.6	585 \pm 20.9	681 \pm 19.9
body-weight gain (g)	163 \pm 11.2	122 \pm 4.6	238 \pm 8.6*
epididymal fat pad (g)	8.8 ($n = 3$)	8.6 ($n = 2$)	14.3 ($n = 3$)
liver (g)	25.3 \pm 2.6	37.4 \pm 3.1*	17.9 \pm 0.4

very small set of regulated hepatic or WAT proteins. Proteomic analyses after PPAR γ activation have been reported for mouse WAT²⁰ or mouse adipocytes.^{21,22} However, to our knowledge, there are no proteomic studies of livers from obese rats treated with any PPAR agonist although gene-expression analyses have been reported for different rat strains.^{14,15,23–25}

In the present study, we have used a proteomic approach to follow how PPAR α and γ agonists affect metabolic proteins in liver and WAT of two rodent models of obesity, insulin resistance and dyslipidemia caused by faulty leptin pathway. In earlier studies from this laboratory, protein-expression analyses were performed on *ob/ob* mice treated with the PPAR α -activator WY14,643 or the PPAR γ -activator rosiglitazone.^{16–18} Both treatments resulted in reduction of plasma TG and glucose down to levels seen in lean sex- and age-matched mice (*OB/?*). Treatment with either of the two PPAR activators lead to a substantial number of regulated liver proteins (2-D gel analysis, pH 3–10, M_w approximately 100–10 kDa). The epididymal fat pads of the very same animals were analyzed in the present study. In addition, we have performed proteomic analyses of liver and WAT from Zucker rats subjected to treatment with PPAR α and PPAR γ agonists. We thereby reveal a lack of correlation between apparent increased capacity of rat liver to oxidize FA and plasma TG level. Clarifying this paradox in the rat model may provide a deeper understanding of the connection between liver enzyme levels and regulation of plasma lipid profiles.

Materials and methods

Animals. Seven weeks old obese male *ob/ob* mice (Umeå strain) were purchased from Bomholtgård Breeding and Research Centre, Denmark and 8 weeks old male Zucker *fa/fa* rats from Charles River Wiga GmbH, Suffield, Germany. All experimental procedures were approved by the Local Ethics Committee on Animal Experiments (Göteborg region, Sweden). For further details concerning housing and dosing see.^{16,26} Drugs were given daily by gavage, Tables 1 and 2. Unless noted 5–6 animals were used and analyzed separately. Plasma lipids, glucose and insulin were measured as described in ref 26 and

are presented for the *ob/ob* mice in Table 1 and for the Zucker rats in Table 2.

Microscopy. Liver specimens from Zucker rats treated with either WY14,643 for 3 weeks (30 μ mol/kg/d) or with rosiglitazone (10 μ mol/kg/d) were fixed in 3% glutaraldehyd. They were rinsed in 0.1 M phosphate buffer, treated with 4.5 mM di-amino benzidine in 0.1 M glycine buffer and post fixed in 2% OsO₄ in 0.1 M phosphate buffer. The specimens were dehydrated in increasing concentrations of ethanol and propylenoxide and then embedded in epoxy resin, TAAB 812 (Analytical Standards AB, Sweden) and polymerized at 60 °C. The semi-thin sections (1 μ m) were stained with 0.05% toluidine blue. Ultrathin sections were contrasted with 4% uranyl acetate and lead citrate (Reynolds). The electron-microscopy examinations were made with a Philips transmission electron microscope CM10. Images were taken with a Megaview II CCD-camera and captured by the ANALYSIS computer program (both from Soft Imaging System, www.soft-imaging.com).

Two-Dimensional Electrophoresis. Approximately 100 mg of liver tissue and 0.3–1 g of the epididymal fat pad (WAT) were homogenized in 8 vol. (liver) or 2 vol. (adipose tissue) of 2 M thiourea, 7 M urea, 30 mM dithiothreitol (DTT), 0.5% IPG-buffer. This buffer, complemented with 4% (w/v) CHAPS and protease inhibitors (liver tissue) (Complete, Roche Inc), was used in the first dimension for the isoelectric focusing in Immobiline DryStrips, pH 4–7 L. The presence of thiourea in the homogenization buffer inhibits protease activity.²⁷ For the second dimension SDS-PAGE, continuous 14% T, 2.1% C gels in the format of 230 \times 200 \times 1 mm were used in most separations. In some cases, pre-cast gels were purchased from Genomic Solutions (Cat. No 0070–3716, 8–18%, www.genomicsolutions.com). Protein staining was performed with the fluorescent Ru complex, SYPRO Ruby. For further details and sources of chemicals and instruments see.²⁸ The NEPHGE analysis was performed basically as described in.²⁹

Most 2D-gel analyses performed in the present study used dithiothreitol (DTT) as reductant during the IEF. However, as recently pointed out DTT has a pK of 8.7 leading to migration of charged DTT out of the focusing gel during the IEF.³⁰ This problem was solved 2002 when Amersham Biosciences launched the reductant hydroxyethylidisulfide.³¹ Some 2D-gel analyses were performed using this reagent that significantly improved the separation of basic proteins.

Quantification and matching of gel spots were made using the software PDQuest 6.1.0 or 7.1.0 (Bio-Rad Lab. Hercules, CA). In cases where a protein was found in more than one spot, the statistical analyses were performed on the separate gel spots. The intensities of all matched spots from the groups of animals treated with PPAR activators were compared pairwise with the control group (log values, Student's t-test, $p < 0.05$, assuming equal distributions).

For the analysis of protein regulations in WAT of *ob/ob* mice, a special procedure was used because of the quantitative dominance in the gels of a few proteins and the low level of many proteins; the dominating protein spots originating from serum albumin, hemoglobin, and aFABP were excluded in the normalization. Two separate gel separations and quantitative analyses of the same adipose samples were performed and only those protein spots that were significantly changed in both analyses are reported as regulated.

Mass Spectrometry. Protein spots of interest were excised from the 2-D gels, subjected to tryptic digestion and the peptides were analyzed by MS and database mining.²⁸ Two

Table 3. Primers and Probes Used for Room Temperature PCR Analyses

gene	primers/probes	detection method
r PPAR γ	Rn00440945-m1 (Applied Biosystems)	taqman Assays
r PPAR γ 2	forward: 5' GGT GGA ACT CTG GGA GAT CC 3' reverse: 5' TGA GGG AGT TTG AAG ACT CTT C 3'	SYBR Green
r PPAR α	forward: 5' CGG GAT GTC ACA CAA TGC A 3' reverse: 5' GCCTTC AGT TTT GCT TTC TCA GA 3'	SYBR Green
r 36B4	forward: 5'-TTC CCA CTG GCT GAA AAG GT-3' reverse: 5'-CGC AGC CGC AAA TGC-3'	SYBR Green

microliters of the peptide extracts and 1 μ L of the matrix were loaded onto a Teflon masked MALDI-TOF target. The matrix solution was comprised of α -cyano-4-hydroxycinnamic acid (Agilent, Böblingen, Germany) that was mixed 1:1 in 50% acetonitrile with 0.1% trifluoroacetic acid. Internal mass calibration was achieved using tryptic autodigestion products (842.51 and 2211.11 Da). MALDI-TOF MS analysis was performed on a Perseptive Biosystems STR mass spectrometer (Framingham, MA) in reflector mode.

Peptide-mass fingerprinting was used for protein identification. Peak detection for all MALDI-TOF mass spectra was made manually using the PepEx annotation tool (BioBridge AB, Lund, Sweden). The extracted peak tables (minimum signal-to-noise was set to 10) were subsequently used for database searching with Mascot Daemon Peptide Mass Fingerprinting software (Matrix Science, UK) in Swiss-Prot and TrEMBL databases. To accept an identification, the following criteria must be fulfilled: (i) $p < 0.05$ according to the Mascot scoring algorithm, using DM < 35 ppm, one missed cleavage allowed and maximum 15 masses submitted, and (ii) minimum of 4 masses and 50% of submitted masses matched.

To analyze potential PTMs of annexin I, gel plugs from the 2-D gels were subjected to trypsinolysis. The extracted peptides were separated by capillary reversed-phase chromatography (300 μ m i.d. \times 100 mm, Zorbax C18, Agilent Technologies). The LC effluent, 4 μ L/min, was directly fractionated onto the MALDI-target plate using an in-house developed MALDI spotter (T Miliotis, unpublished). The LC fractions were allowed to dry and 0.5 μ L of matrix solution was applied.

Analysis of mRNA. RNA was isolated with TrizolR (Invitrogen, www.invitrogen.com) from approximately 50 mg of rat liver according to manufacturer's instructions. The quality of the total RNA was checked with RNA 6000 Nano Assay reagent Kit, and Agilent 2100 Bioanalyzer (www.agilent.com/chem/labonachip) and the quantity was determined spectrophotometrically. DNA contamination was removed from RNA samples with DNA-free TM (Ambion, www.ambion.com) and cDNA first strand synthesis was performed using Superscript First-Strand Synthesis for RT-PCR (Invitrogen). A 15-ng portion of cDNA was used for the RT-PCR as listed in Table 3 using TaqmanR Universal PCR master mix or SYBR Green PCR master mix (Applied Biosystems, www.appliedbiosystems.com). The endogenous control used was acidic ribosomal phosphoprotein P0 (r36B4, Swiss-Prot. P19945).

Results

Proteomic Analysis of *ob/ob* Mice Treated with a PPAR α or a PPAR γ Agonist. Treatment of *ob/ob* mice with 180 μ mol/kg/d of WY14,643 or 2.5 μ mol/kg/d rosiglitazone for 1 week resulted in significant reduction of plasma concentrations of triglycerides (TG) and glucose, Table 1. Two-D gel analyses of liver proteins from these mice earlier performed in our laboratory^{16–18} showed large changes of protein profiles for both

treatment groups. It was therefore of interest to study epididymal fat-pad proteins of the same animals using the same proteomic technique.

We earlier showed that thiourea in the buffer used for homogenization and isoelectric focusing of WAT proteins substantially improved the quality of the 2-D gels, a procedure that later was shown also to inhibit proteolytic activity.²⁷ Approximately 10% of the 500 quantified spots were significantly changed ($p \geq 0.05$) but either the fold-change was small or the intensity of the regulated spots was very low. To filter out false regulations the protein extracts were reanalyzed using the same type of 2-D gels and by selecting only those protein spots that were significantly changed in the duplicate analyses; one protein was found to be changed by PPAR α treatment and 11 by the PPAR γ -treatment (Supporting Information, Table 1). Of the latter, heat shock cognate protein 71 kDa was down-regulated. Four other proteins, transthyretin, vimentin, 3-keto acyl CoA thiolase (mitochondrial), and semiclotin + seminal vesicle secretory protein IV, were up-regulated. In addition, four minor albumin spots (one representing the full-length sequence and the other three degradation products) were changed. An alkaline form of annexin 1 was down-regulated and an acidic form up-regulated on treatment with both PPAR activators, Figure 1. Transthyretin and albumin are produced in the liver and therefore the protein in the WAT probably originate from blood in the tissue. Transthyretin hepatic mRNA level but not plasma protein level has earlier been shown to be regulated by treatment of mice with a PPAR α agonist.³² To summarize; of the regulated proteins in WAT of *ob/ob* mice, only one is an enzyme involved in energy metabolism. This enzyme, 3-ke-toacyl-CoA thiolase that participates in the β -oxidation of fatty acids, was up-regulated in response to PPAR γ but not PPAR α . It is present in both mitochondria and peroxisomes and the peroxisomal enzyme has been shown to have a functional PPRE.³³

In addition to the treatment-regulated protein spots more than 100 nonregulated proteins were identified by MS.²⁸ The identified proteins that participate in metabolism are listed in Supporting Information, Table 1, grouped according to their roles in the cell. Of these metabolic proteins, 34, participate in the fatty-acid metabolism but are not changed in amount on treatment of the mice with either the PPAR α - or the PPAR γ -activator. It should be pointed out that neither the amount of aFABP nor acyl-CoA binding protein were changed although they are known to have functional PPRE's,^{34,35} reviewed by.³⁶ A preliminary Northern blot analysis of the aFABP mRNA, however, showed a two times higher level in the rosiglitazone-treated mice vs the vehicle treated (unpublished).

Treatment of Obese Zucker (*fa/fa*) Rats with a PPAR α or a PPAR γ Agonist. Four doses of WY14,643 (3, 10, 30, and 300 μ mol/kg/d) were given for 3 weeks to Zucker rats. Despite the enlargement of the liver seen at all doses, significant decrease in plasma TG was only obtained at the highest dose (from 5.8

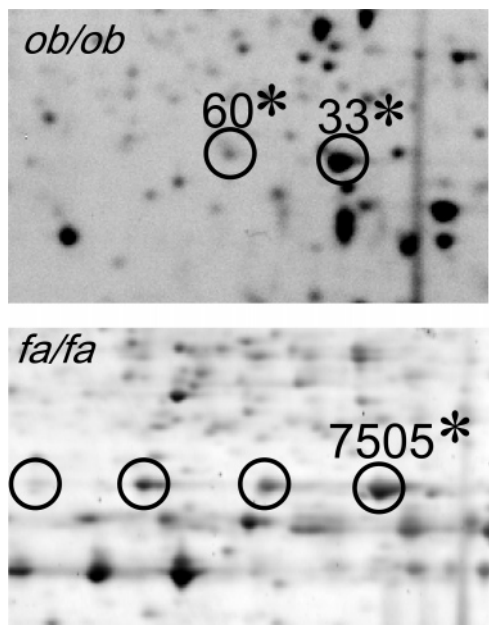


Figure 1. 2D-gel separation of different molecular forms of annexin 1 in the adipose tissue of *ob/ob* mice (top) and *fa/fa* rats (bottom). The spots identified as annexin 1 by MS are encircled. Spots that differ significantly between PPAR-activator treated animals and vehicle-treated ones are marked with * ($p \leq 5\%$). Figures on gels refer to unique spot numbers as shown in²⁸ and Supporting Information Figure 2.

to 2.7 mM, $p < 0.036$, $n = 4$). As the body-weight gain of the highest dose was unexpectedly small (50% of the control group, $p < 0.026$) due to a reduced food intake, the highest dose at which there was no significant reduction in weight gain was chosen for the further studies (30 $\mu\text{mol/kg/d}$).

On the basis of our earlier studies of the effects of darglitazone on Zucker rats,²⁶ a dose of 1 $\mu\text{mol/kg/d}$ was chosen. This dose caused a significant decrease of plasma TG after 3 weeks of treatment. Plasma values of TG, glucose, and insulin, as well as body weight, liver, and epididymal fat-pad weights are summarized in Table 2. There was a large difference in the response of the Zucker rats to the treatment with WY14,643 and darglitazone, the latter normalizes the plasma TG values to those of lean animals, whereas WY14,643 does not give any effect.

Histology of the Rat Liver. Microscopy at low magnification showed that the liver of untreated rats contained a large number of fat-filled vacuoles, Figure 2. The fat droplets differed in size from 250 nm to 20 μm and were found in almost all cells. Treatment of the rats with 30 $\mu\text{mol/kg/d}$ of WY14,643 resulted in a dramatic decrease of this liver steatosis, Figure 2. It was observed that near the central veins fewer fat droplets remained after treatment than close to the portal zone (not shown). In the untreated rats, it was difficult to estimate how many mitochondria and peroxisomes were present due to the many and large fat droplets while treatment with WY14,643 resulted in appearance of large areas with high density of both mitochondria and peroxisomes (not shown). As comparison two animals were treated with the PPAR γ -activator rosiglitazone which resulted in amelioration of the steatosis concomitant with some increase in the number of peroxisomes (not shown).

Proteomic Analysis of Zucker Rat Liver. A typical 2-D gel analysis of Zucker rat hepatic proteins is shown in Supple-

mentary information, Figure 1 (pH 4–7, 12% SDS-PAGE and NEPHGE). Quantification of spot volumes of the two groups of PPAR-treated rats and comparison with the vehicle-treated one showed that 160 spots were differently expressed in livers of PPAR α -treated animals and 60 in the PPAR γ -treated ($p < 5\%$). As several very basic enzymes were expected to be regulated in liver of the rats treated by a PPAR α activator¹⁸ and the resolution in the pH range above 8 was not optimal (DTT used as reductant), we also performed a separation using NEPHGE focusing followed by SDS-PAGE electrophoresis. Although the most basic proteins did not give spots that were sharp enough to quantify using the software PDQ, a series of them were found by visual inspection to be strongly up-regulated by the PPAR α treatment. Identified proteins are grouped in Supporting Information, Table 2, according to their known biological function. After this study was completed the reductant hydroxyethylthiolate,³¹ that allows focusing of proteins with pI above 8, was introduced into the market. Combining this reductant with the use of 8–16% gradient SDS-PAGE gels resulted in extremely good separations that allowed identification of several basic proteins by MS (not shown). The main conclusion from this comparison is that reduction with hydroxyethylthiolate allows as many basic proteins to be visualized on 2-D gels as the NEPHGE focusing. However, when using NEPHGE focusing, in tube gels, more high-molecular weight proteins enter the 2-D gels compared to when the focusing has been performed with IPG-strips with an immobilized pH gradient. This explains why, e.g., peroxisomal bifunctional enzyme, 78 kDa, could be identified only from the NEPHGE gels.

Enzymes involved in pathways that generate energy from FAs were strongly up-regulated by the PPAR α treatment; mitochondrial and peroxisomal β -oxidation, the Krebs cycle and the mitochondrial respiratory chain, Figure 3 and Supplementary information, Table 2. Examples of such proteins are 3-ketoacyl-CoA thiolase B, peroxisomal bifunctional enzyme, hydroxymethylglutaryl-CoA synthase, acyl-CoA thioester hydrolase, acyl-CoA dehydrogenase and isomerases and Apo E. Mitochondrial hydroxymethylglutaryl-CoA synthase which is a key enzyme in ketogenesis was up-regulated. A protein tentatively identified as phosphoenol pyruvate carboxy kinase, PEPCK, was down-regulated 2-fold. This enzyme regulates glyconeogenesis and has a functional PPRE in adipose tissue.³⁸ On the gene level this has been reported earlier to occur after treatment with a PPAR α activator.¹⁰ Glycerol-3-phosphate dehydrogenase, which participates in both glyconeogenesis and glycolysis, is up-regulated almost 3 times, while a protein tentatively identified, as hepatic ketohexokinase is down-regulated to 0.6.

In addition to the above-mentioned regulations, enzymes participating in several other pathways were influenced; NO activation, urea cycle, creatine synthesis, homocysteine metabolism, metabolism of xenobiotics, heat-shock, and stress proteins, Supporting Information, Table 2. Some enzymes participating in amino acid metabolism were either up- or down-regulated, a phenomenon earlier noted in *ob/ob* mice¹⁸ and by gene-expression analysis of rats treated with a PPAR α agonist.³⁹ Both argininosuccinate synthase and 4-hydroxyphenylpyruvate dioxygenase were down-regulated by treatment of the Zucker rats and *ob/ob* mice¹⁸ with WY14,643.

Zucker rats treated with the PPAR γ activator darglitazone showed a much lower degree of regulation of liver proteins compared to those treated with the PPAR α agonist, Figure 3

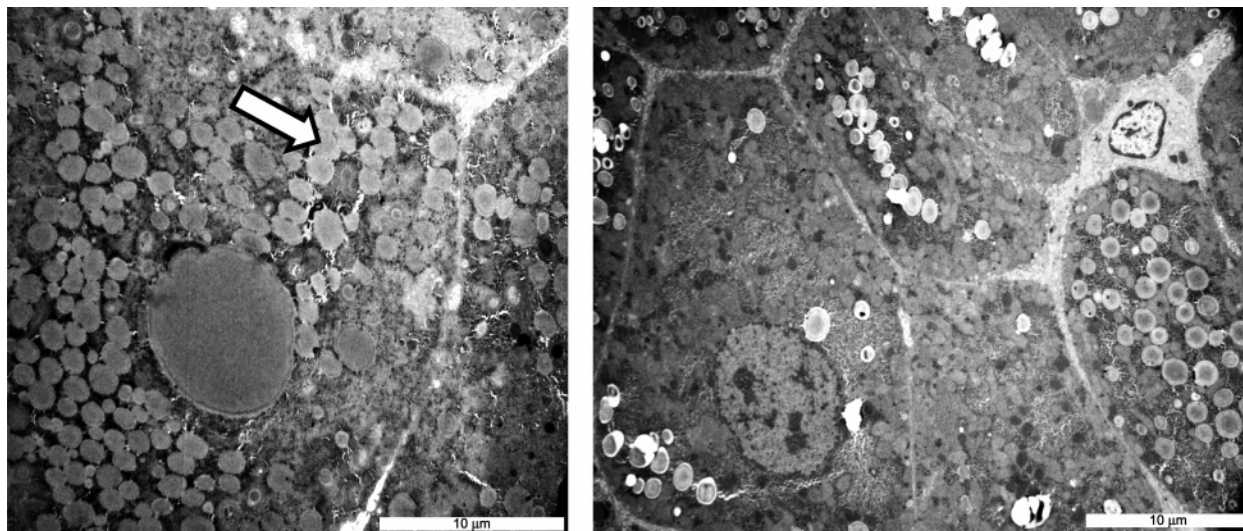


Figure 2. Images of electron microscopy of hepatocytes from obese and dyslipidemic Zucker rats before (left) and after (right) treatment for 3 weeks with WY14,643 (30 $\mu\text{mol/kg/d}$). The arrow points to a lipid droplet, bar = 10 μm .

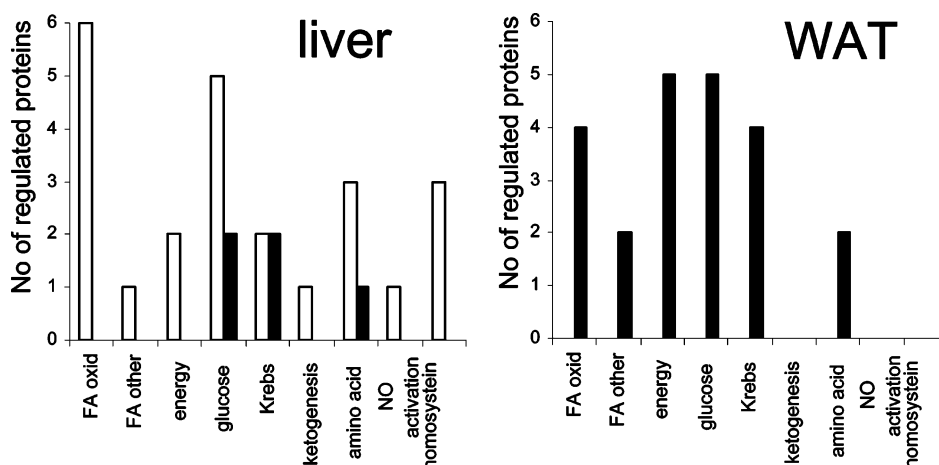


Figure 3. Summary of protein regulations in liver and adipose tissue of Zucker rats that had been treated with a PPAR α agonist (open bars) or a PPAR γ agonist (filled bars). "FA other" summarizes proteins involved in FA synthesis, binding and transport; "energy", energy transfer/redox/electron handling; "glucose", glycolysis and/or glycogenesis. For identities of regulated proteins, see Supplementary Information, Tables 2 and 3.

and Supporting Information, Table 2. Three enzymes belonging to central pathways (fatty-acid, glucose, and amino acid metabolism) were regulated in the same way as by treatment with WY14,643; pyruvate dehydrogenase, ketohexokinase, and succinyl-CoA synthase. The only enzyme belonging to either of the above-mentioned pathways, 4-hydroxyphenylpyruvate dioxygenase, was regulated in opposite direction to that by WY14,643. In addition, stress proteins and prohibitin was regulated by PPAR γ .

Proteomic Analysis of Zucker Rat Adipose Tissue. Two-D gel analysis of proteins from the epididymal fat pads of the Zucker rats treated with WY14,643 or darglitazone were performed using DTT- or hydroxyethylthiol-disulfide-reduction.³¹ The latter resulted in superior resolution of basic proteins and therefore such a gel is shown in Supporting Information, Figure 3. For the PPAR α -treated animals only 3 very weak protein spots were regulated while for the PPAR γ -treated rats approximately 80 of the 450 quantified spots were statistically changed ($p \leq 0.05$).

In the PPAR γ -treated animals, enzymes participating in the handling or β -oxidation of FAs were up-regulated as were some

enzymes involved in glycolysis, Figure 3. Several proteins with roles in stress reactions (heat-shock proteins), cell structure (vimentin, actin), and protein modifications (protein disulfide isomerases) were influenced. Two proteins known to be present in different molecular forms were of special interest. One protein spot identified as adiponectin was decreased on treatment with darglitazone, whereas another form was unchanged in amount. Adiponectin, which is produced in the adipose tissue,⁴⁰ can have different mobility on 2-D gels as it is glycosylated before it is secreted into the plasma.⁴¹ Three proteins appearing in the plasma but having hepatic origin were found to be up-regulated in the proteomic analysis of WAT. These were tentatively identified as fibrinogen, alanine aminotransferase, and anti-thrombin III.

In the proteomic analysis of mice, two spots were identified as annexin 1, Figure 1 and Supporting Information, Tables 1 and 3. The more basic form was down-regulated and the acidic form up-regulated on treatment of the mice with either WY14,643 or rosiglitazone. For the Zucker rats the same phenomenon was observed for annexin 1, the basic spot 7505 decreased in volume when the rats had been treated with

Table 4. Number of Regulated Protein Spots Obtained by Treatment of Mice and Rats with PPAR α or PPAR γ Agonists

species	drug	tissue	no. of quantified spots	no. of spots with $p < 0.05$ (% of quantified)
zucker	WY14,643	liver	600	169 (27%)
zucker	darglitazone	liver	600	58 (10%)
<i>ob/ob</i> mice	WY14,643	WAT	500	1 (0.2%)
<i>ob/ob</i> mice	rosiglitazone	WAT	500	11 (2%)
zucker	WY14,643	WAT	380	3 (3%)
zucker	darglitazone	WAT	450	79 (18%)

Table 5. Analyses of mRNA for PPAR's in Liver of *fa/fa* Rats Treated with PPAR Agonists

treatment/strain	response		
	PPAR γ /36B4 (E4)	PPAR γ 2/36B4 (E5)	PPAR α /36B4 (E3)
vehicle/lean, <i>FAI</i> ?	5.0 \pm 2.5	2.5 \pm 1.3	3.3 \pm 0.7
vehicle/obese, <i>fa/fa</i>	6.9 \pm 3.0	10 \pm 8.3	4.8 \pm 1.1
darglitazone/obese, <i>fa/fa</i>	5.8 \pm 3.3	6.4 \pm 1.6	4.6 \pm 1.0
WY14,643/obese, <i>fa/fa</i>	13 \pm 8.6	27 \pm 5.4	9.0 \pm 4.4 ^a

^a $p < 0.05$ vs obese vehicle.

darglitazone, Figure 1. Several PTMs of annexin 1 has been published, phosphorylation,⁴² glycosylation,^{43,44} and N-terminal acetylation.⁴⁵ We attempted to search for these modifications in the different spots by LC-MS but were not successful.

mRNA of PPAR α and PPAR γ in the Liver of Zucker Rats. PPAR α , PPAR γ 1 and PPAR γ 2 are examples of proteins not appearing on 2D-gels and this is the reason mRNA analysis was used to estimate their hepatic expression. The expression of PPAR γ 2 was approximately 1/10 of that of total PPAR γ (γ 1 + γ 2). The differences in mRNA expression of total PPAR γ or PPAR γ 2 observed in obese vs lean rats were not statistically significant, Table 5. Neither did the increased hepatic expression of mRNA for PPAR γ observed by WY14,643 treatment reach significance. It should be noted that the level of expression of PPAR γ was extremely low. For the quantification, 15 ng of cDNA was used, and the Ct value was >32 , which corresponds to a few copies of PPAR γ mRNA per cell. Treatment with the PPAR α agonist WY14,643, however, increased the levels of mRNA for PPAR α 2-fold ($p < 0.05$).

Discussion

We compared two commonly used animal models with genetic defects leading to obesity; *ob/ob* mice⁴⁶ and *fa/fa*, Zucker,⁴⁷ rats. The former do not produce leptin, whereas the latter lack the leptin receptor and both therefore do not regulate their food intake, but overeat and become obese, dyslipidemic and insulin resistant on a chow diet. In the present study, protein expression analysis was performed on the two tissues being the main targets for PPAR α and PPAR γ activators, the liver and white adipose tissue, respectively. Literature data on proteomics for rodents treated with the two types of PPAR agonists are scarce. The reports from *ob/ob* mice treated with rosiglitazone^{19,20} utilize higher doses than used in our study thus making the results not comparable. Probably, the composition of food, duration, and doses of the treatments are of importance for plasma parameters and level of regulation of proteins. For these reasons, comparisons of different drugs and tissues should preferentially be performed with animals treated and fed in parallel and of the same origin.

It is now generally accepted that treatment with PPAR α agonists can lead to reduction of body-weight gain in rodents¹⁰ and body weight in primates.⁴⁸ For the Zucker rats, we did not obtain a reduction of plasma TG without giving WY14,643 in doses so high that the food intake was decreased, a situation that should be avoided. We used the highest dose at which no significant reduction in weight gain was seen for WY14,643 (30 μ mol/kg/d). The dose of WY14,643 given to the *ob/ob* mice (180 μ mol/kg/d) did not reduce the weight gain although lowering of plasma TG was obtained, Table 1. Unfortunately, very few publications report influences on body weight for the experimental animals why conclusions in the literature may not always be possible to compare.

Our proteomic analysis of mouse WAT showed that very few proteins were regulated by the treatments with PPAR agonists, while in the rat PPAR α greatly influenced liver proteins and PPAR γ WAT proteins, Figure 3.

One advantage with 2-D gel separations is that differences in PTMs can be detected. In our study, as in many other proteomic studies (see, e.g., refs 18 and 49), some proteins are identified in more than one gel spot. When proteins appear at different apparent molecular weights the redundancy could be caused by either PTMs or by cleavage by endogenous proteases.⁴⁹ However, when more than one spot is obtained from a protein at the same molecular weight but with different *pI*, the reason probably is differences in PTM. Examples of this is annexin 1 in the adipose tissue of both the mice and rats and adiponectin detected in two different spots in the adipose tissue of the rats. Adiponectin, which is produced in adipose tissue, has been detected as several differently glycosylated forms.⁴¹ In a study were protein-expression analysis was performed on a murine cell line in different stages of the adipogenesis, two different adiponectin spots were identified by MS, one of them up-regulated on adipogenesis.²² Such a difference in modification may reflect differences in maturation of the protein and thereby possibly the rate of secretion.

Several isoforms of annexin 1 were found in the adipose tissue of the mice and rats. Treatment with a PPAR γ agonists affected the relative distribution of some of the spots identified as annexin 1. Annexin 1 (also called lipocortin 1, recently reviewed refs 50 and 51) has been shown to be a substrate, and thus a potential competitive inhibitor, of the insulin receptor kinase in rat liver *in vivo*,⁴² and of human placental insulin receptors *in vitro*.⁵² Further, treatment of mature 3T3-L1 adipocytes with the PPAR γ -agonist troglitazone suppressed the expression of annexin 1,⁵³ a finding which was thought to be one mechanism by which troglitazone might improve insulin-signaling in insulin-resistant tissues. Our findings are in line with these three observations with the apparent shift in *pI* that might reflect increased phosphorylation by the insulin receptor kinase in tissues from PPAR γ -treated animals thus indicating insulin-sensitization *in vivo*. Further MS analyses were done to detect differentially phosphorylated peptides by MS, but no such differences were found. Thus, we cannot exclude the possibility that the pattern of annexin 1 spots could have been caused by other PTMs, such as enzymatic or nonenzymatic glycosylation. An annexin 1 knock-out mouse has recently been created,⁵⁴ and it would be interesting to compare insulin-signaling in that model with that of wild-type mice.

There could be several reasons for the detection of protein regulations in animals treated with PPAR α or PPAR γ agonists; the gene coding for a protein could have a response element

for PPAR, PPRE, and therefore be directly regulated by the activation of either of the PPARs. Examples of this are peroxisomal bifunctional enzyme,⁵⁵ 3-ketoacyl-CoA thiolase,³³ acyl-CoA thioester hydrolase,³⁷ Apo E,⁵⁶ and hydroxymethylglutaryl-CoA synthase.^{57,58} Supplementary information, Table 2. In some cases, it has been shown that a PPRE may be functional in one tissue but not in another, e.g., Apo E, which was up-regulated in rat WAT by the PPAR γ agonist but down-regulated in rat liver by the PPAR α agonist. Apo E has been shown to have a PPAR γ responsive PPRE in human macrophages.⁵⁶ It is worth noting that Galetto et al.⁵⁶ report that the Apo E PPRE is not responsive to PPAR α although our data showed a clear down-regulation in the liver.

Several regulated proteins were identified for which no functional PPRE have been identified. Examples are; ATP synthase β -chain, cytochrome b5 and most proteins listed as "other" in Supplementary Information, Tables 1-3. In such cases, the expression of proteins may be secondary to the treatment with the PPARs. The treatment period was 1 week for the mice and 3 for the rats which gave changes in the concentration of many metabolic products and intermediates.⁵⁹ Several of them could influence the expression of genes, i.e., cause indirect regulation of proteins. Our study cannot discriminate between direct and indirect regulations.

There was a large overlap in identities of liver proteins regulated by WY14,643 in the *ob/ob* mice¹⁸ and Zucker rats; a clear increase in proteins that handle fatty acids, oxidize them in the mitochondria or peroxisomes or are important for the production of ATP, Supporting Information, Tables 2 and 3. Our proteomic study show that these effects earlier demonstrated at the mRNA level¹⁰⁻¹⁵ are translated to the protein level. Another important regulation that was consistent in the two rodents treated with WY14,643 was hydroxymethylglutaryl-CoA synthase. This enzyme, that catalyzes the synthesis of ketone bodies, used as fuel by, e.g., the brain and skeletal muscle, was up-regulated in both the mice and rats. In addition enzymes participating in amino acid metabolism, and heat-shock proteins were influenced in both species.

On the gene level, it has also been shown that enzymes in the glycerol pathway (glycerol kinase and glycerol-3 phosphate dehydrogenase)⁶⁰ are up-regulated in the liver by PPAR α agonists, a response also shown at the protein-expression level by the Zucker rats in our study.

There was a difference in the hepatic response to PPAR γ agonists in mice and Zucker rats. In the rats, fewer protein spots were significantly regulated and more importantly none of the changes implied strong modifications of the metabolism of FAs, Figure 3, while in the mice many metabolic enzymes were regulated.¹⁸ The reason for the high level of regulation of metabolic enzymes in the mice has been attributed to the presence of PPAR γ ^{216,61} in the liver of obese animals. In the liver of the Zucker rats, we found that mRNA for PPAR γ (γ 1 + γ 2) was very low and not significantly different from that of the lean rats. The mRNA for PPAR γ 2 was approximately 3 times higher in the obese rats than the lean ones (not statistically significant). These changes were smaller than those seen for *ob/ob* mice⁶² which may be the reason for the discrepancy in protein regulation between these two rodent models. In addition, in the liver of the Zucker rats the level of PPAR γ 2 did not seem to increase on treatment with a PPAR γ agonist as was reported for rat hepatocytes.⁶³ Further, the level of mRNA for PPAR γ 1 and PPAR γ 2 was extremely low in the rat livers. For the house-keeping gene used, r36b4, the Ct value was ap-

proximately 20, whereas it was >32 for PPAR γ 2, corresponding to a few copies of mRNA/cell. The level of mRNA for PPAR α , however, was significantly up-regulated when the Zucker rats were treated with WY14,643. Such an up-regulation has earlier been demonstrated by immuno-histochemistry on rats treated with clofibrate, a PPAR α agonist.⁶⁴

Neither the mice nor the rats showed significant changes in WAT proteins after treatment with the PPAR α agonist, WY14,643, 0.2% and 3% or quantified spots, respectively. This result was expected since there are no reports indicating substantial expression of PPAR α in this tissue.

Our proteomic study of the adipose tissue of Zucker rats treated for 3 weeks with the PPAR γ agonist darglitazone show that several enzymes which participate in energy metabolism were up-regulated, Figure 3. Proteins like acyl-CoA dehydrogenase, pyruvate dehydrogenase, malate dehydrogenase, ATP synthase are all up-regulated. This indicates increased mitochondrial activity or number of mitochondria. The latter has been suggested to be an effect of a transformation from white to brown adipocytes.⁶⁵ However, as pointed out by Wilson-Fritch et al.²¹ mitochondriogenesis is coupled to adipogenesis per se. It is also known that PPAR γ activators increase the adipose-tissue mass^{26,66} both by increased sequestering of fat and biosynthesis. When rats are treated with the PPAR γ agonist troglitazone, the number of small adipocytes increase.^{66,67} Small adipocytes, a wanted effect of the treatment, are considered to be more insulin sensitive.⁶⁸

The analysis of mouse WAT, showed that only one protein involved in metabolism of fat was regulated by the PPAR γ agonist; 3-ketoacyl-CoA thiolase, Supporting Information, Table 1, which contrasts significantly to the result of Wilson-Fritch et al.²⁰ However, these authors gave their mice 5 times higher doses of rosiglitazone and the treatment period was twice as long as that used by us. The animals used in the study of Wilson-Fritch et al.²⁰ also were older and thereby twice as heavy as ours (approximately 70 g vs 35-45 g).

While the phenomenon of decreased adipocyte cell size is well documented for rats treated with PPAR γ agonists^{66,67} and recently also in mice treated with rosiglitazone,⁶⁹ we have not been able to demonstrate such a decrease for the *ob/ob* mice treated with rosiglitazone (unpublished). Our results are in line with what was observed for *ob/ob* mice treated with rosiglitazone by Wilson-Fritch et al.²⁰ The difference in adipose size obtained could be caused by the different doses of the PPAR γ given.

Among the mouse adipose proteins that were detected and quantified but did not change in amount on treatment with the PPAR γ agonist was aFABP. Our preliminary Northern analyses of the mRNA of this gene, however, showed a doubling of the amount, which is in analogy with what has been shown several times (review ref 36). This discrepancy could be related to a difference in ca. dian rhythm of the mRNA and protein as recently demonstrated for UCP3 and pyruvate dehydrogenase kinase.⁷⁰ In the adipose tissue of the rats, the protein hFABP was strongly up-regulated (7 times) while aFABP was not changed. It has earlier been shown that it is the isoprotein hFABP that is regulated in rat liver¹⁵ by PPAR γ agonists, while aFABP is regulated in mice.⁷¹

White adipose tissue of rats treated with the PPAR γ agonist showed three regulated proteins that are of hepatic origin; fibrinogen, alanine aminotransferase, and anti-thrombin III. Since the whole epididymal fat pad was used for the analyses

also blood proteins appeared on the gels. The increased level of ALAT has earlier been reported after treatment with PPAR α agonist.⁷²

Both *ob/ob* mice and Zucker rats have been useful in development of PPAR agonists for use in the clinic⁷³ as changes in physiological parameters in the rodents have been predictive to data obtained in man.⁷⁴ In addition, these two rodent models of obesity and dyslipidemia are frequently used throughout the scientific community to study effects of modulating a range of nuclear receptors. Using the proteomic technique we found that the PPAR γ agonist rosiglitazone in *ob/ob* mice did not regulate adipose proteins to the extent expected although such a response was seen in the rat adipose. The second unexpected finding was that despite that the PPAR α agonist WY14,643 strongly up-regulated several β -oxidation enzymes in Zucker liver this did not lead to decreased plasma TG. The mice, on the other hand, obtained normalized levels of TG after just 7 days of treatment. Our data show that the action of PPAR agonists differ more between the two rodent species than earlier demonstrated, a finding that that deserves further investigations to fully explain.

Abbreviations. FA, fatty acid; TG, triglyceride; WAT, white-adipose tissue; PPAR, peroxisomal proliferator-activator receptor; PPRE, peroxisomal proliferator-respons element; NEPHGE, nonequilibrium pH gel-electrophoresis; PTM, post-translational modification

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Supporting Information Available: Supporting Information, Tables 1–3 and Supporting Information Figures 1–3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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