JPET Fast Forward. Published on October 31, 2005 as DOI: 10.1124/jpet.105.093831

JPET# 93831

Blood glucose lowering nuclear receptor agonists only partially normalize hepatic gene expression in *db/db* mice

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JPET Fast Forward. Published on October 31, 2005 as DOI: 10.1124/jpet.105.093831
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Running Title

Blood Glucose Lowering Drugs and Hepatic Gene Expression

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Number of

Text pages	23	
Tables	2	
Figures	6	+ 3 Supplementary Figures
References	40	

Number of words

Abstract	220
Introduction	333
Discussion	402

ABBREVIATIONS PPAR, peroxisome proliferator-activated receptor; QPCR, quantitative real-time reverse-transcriptase PCR; SREBP, sterol regulatory element-binding protein; UPR, unfolded protein response.

Section

Endocrine and Diabetes

ABSTRACT

Agonists of the nuclear receptors peroxisome proliferator-activated receptor (PPAR) γ, PPARα, and liver X receptors (LXR) reduce blood glucose in type 2 diabetic patients and comparable mouse models. Since the capacity of these drugs to normalize hepatic gene expression is not known we compared groups of obese diabetic *db/db* mice treated with agonists for PPARγ (rosiglitazone [Rosi]; 10 mg/kg/day), PPARα (Wy 14643 [Wy]; 30 mg/kg/day), and LXR (T0901317 [T09]; 40 mg/kg/day) and from untreated non-diabetic littermates (*db/+*) by oligonucleotide microarrays and quantitative reverse transcriptase polymerase chain reaction. The 10-day treatment period of *db/db* mice with Rosi, Wy, and T09 altered expression of 300, 620, and 735 genes including agonist-specific target genes, respectively. However, from the 337 genes differentially regulated in untreated *db/+* vs *db/db* animals, only 34 (10%), 51 (15%) and 82 (24%) were regulated in the direction of the *db/+* group by Rosi, Wy and T09, respectively. Gene expression normalization by drug treatment involved glucose homeostasis, lipid homeostasis and local glucocorticoid activation. In addition, our data pointed to hitherto unknown interference of these nuclear receptors with growth hormone receptor gene expression and endoplasmic reticulum stress. However, many diabetes-associated gene alterations remained unaffected or were even aggravated by nuclear receptor agonist treatment. These results suggest that diabetes-induced gene expression is minimally reversed by potent blood glucose lowering nuclear receptor agonists.

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INTRODUCTION

Modern anti-diabetic agents targeting nuclear receptors are designed to modulate blood glucose levels and gene expression on a molecular level. Transcriptional regulation by agonists of peroxisome proliferator-activated receptor (PPAR)γ, PPARα and LXR comprise genes involved in gluconeogenesis, fatty acid metabolism and ketogenesis (Willson et al., 2000; Venkateswaran et al., 2000). PPARγ is a critical transcription factor involved in energy balance and activated by well-established anti-diabetic drugs, the thiazolidinediones (Lehmann et al., 1995). Nuclear receptor agonist or fatty acid dependent activation of PPARα promotes peroxisomal proliferation, hepatic fatty acid oxidation and the generation of ketone bodies thereby providing substrates for energy metabolism in peripheral tissues (Issemann and Green, 1990). LXRα, the predominant LXR paralogue in liver (Repa et al., 2000) regulates intracellular cholesterol and bile acid metabolism as well as expression of sterol regulatory binding protein (SREBP)-1c, the major lipogenic transcription factor (Schultz et al., 2000; Repa et al., 2000). Activation of LXR is associated with down-regulation of key genes involved in hepatic gluconeogenesis (Stulnig et al., 2002a; Cao et al., 2003; Laffitte et al., 2003). Moreover, nuclear receptors can modulate each other's gene expression as shown for PPARγ and LXRα (Ide et al., 2003; Seo et al., 2004) pointing to a close relation of their transcriptional regulations and metabolic function. Agonists of PPARγ, PPARα and LXR all decrease blood glucose concentrations in type 2 diabetes patients and/or comparable animal models (Lehmann et al., 1995; Guerre-Millo et al., 2000; Laffitte et al., 2003) by regulating gene expression. In order to address the question whether normalization of blood glucose levels by these nuclear receptor agonists is accompanied by normalized gene expression, we analyzed genome-wide hepatic gene expression profiles of diabetic *db/db* mice treated with nuclear receptor agonists and their untreated non-diabetic littermates and compared them with

those of untreated *db/db* mice. By providing a comprehensive overview of drug-induced changes in gene expression in obese diabetic mice our data revealed that reversal of diabetes-associated alterations in hepatic gene expression occurs only to a very limited extent.

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MATERIAL AND METHODS

Animals. Male C57BL/KsJ-lepr^{db}/lepr^{db} diabetic (*db/db*) mice and their nondiabetic littermates (*db/+*) were purchased from Charles River Laboratories Inc. (Germany) at seven weeks of age and maintained under standard light (12h light/dark) and temperature conditions (23°C). During one week of acclimatization mice were provided with a low-fat standard rodent diet (<3% fat; N1324, Altromin, Germany) and water *ad libitum*.

Treatment. For the experiment, the low-fat standard diet was either mixed with vehicle alone (ethanol; untreated) or supplemented with 0.005% (w/w) PPARγ agonist Rosiglitazone (Rosi; Avandia, Smithkline Beecham, PLC, Middlesex, U.K.; corresponding to approximately 10 mg/kg/day; Hori et al., 2002), 0.02% PPARα agonist Wy-14.643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid; Wy; Sigma-Aldrich, St Louis, MO, U.S.A.; corresponding to approximately 30 mg/kg/day; Edvardsson et al., 1999) or 0.025% of the synthetic LXR agonist T0901317 (N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1(trifluoromethyl)-ethyl]phenyl]-benzenesulfonamide; T09; generously provided by Amgen Inc., formerly Tularik Inc.; corresponding to approximately 40 mg/kg/day; Stulnig et al. , 2002a) followed by extensive evaporation of ethanol. Four groups of *db/db* (untreated, Rosi, Wy, T09; n = 5) and one group of *db/+* mice (untreated; n = 8) were treated for 10 days.

Tissue and blood analyses. Mice were anesthetized with Isoflurane and sacrificed by neck dislocation after cardiac puncture. The liver was immediately cut into small homogenous regions, snap frozen in liquid nitrogen and kept at -80°C until isolation of total RNA. Blood samples were drawn from the tail vein before starting the experimental diets. EDTA plasma separated from cardiac blood was stored in aliquots at -20°C until further analyses. Blood glucose was measured by an automated analyzer (ALCYON 300i, Abbott Laboratories,

Illinois, U.S.A.) at the beginning and the end of the experiment. Plasma cholesterol and triglycerides were determined by the Alcyon 300i analyzer (Abbott Laboratories). Serum concentrations of non-esterified fatty acids were measured with the Wako FFA-kit (Wako chemicals, Richmond, VA, U.S.A.), insulin by ELISA (Linco Research, St. Charles, MO, U.S.A.). Liver triglycerides were determined following lipid extraction as described (Haemmerle, 2002) but by using a commercially available enzymatic reagent (Rolf Greiner Biochemica, Flacht, Germany). The study protocol was approved by the local ethics committee for animal experiments and the Guidelines for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes were followed.

RNA preparation. Total RNA was prepared by disrupting equivalent regions of approximately 50 mg of liver tissue from each animal per group in TRIzol reagent (Invitrogen) with a tissue homogenizer followed by RNA isolation according the manufacturer's instructions. Total RNA samples were checked for integrity by agarose gel electrophoresis and the 2100 bioanalyzer (Agilent, Palo Alto, CA). For microarray cDNA synthesis total RNA samples were repurified with RNeasy MinElute kit (Qiagen).

Microarray hybridization and data analysis. Ten µg of total RNA from each animal (n=3 per group) was transcribed into first strand cDNA by Superscript II (Invitrogen, Carlsbad, CA, U.S.A.) using T7-Oligo(dT) primers followed by second strand synthesis and repurification according to the manufacturer's protocols (all Affymetrix, Santa Clara, CA, U.S.A.). Following in vitro transcription, 15µg of labeled and fragmented cRNA from each individual sample were hybridized to U74Av2 GeneChips (12k) which were scanned using the GeneArray scanner and further analyzed with Microarray Suite version 5.0 software

according to the manufacturer's protocols (all Affymetrix, Santa Clara, CA, U.S.A.). Array quality criteria for all chips included control of expression report files for background, background noise, scale factor <2, internal control gene 3' to 5' ratio, and hybridization control ratios, respectively. Normalization was performed by global scaling to an average intensity of 100 arbitrary units. Gene abundances were estimated by robust multi-array analysis (Irizarry, 2003) using the probe-level modeling (affyPLM) package from Bioconductor (www.bioconductor.org). This algorithm provides for calculation of means and standard errors of logarithmically transformed estimates, reflecting the inconsistency among the different probes for the same gene. We used a strategy similar to analysis of variance and computed a consensus estimate for the variability among all groups as a basis for t-test statistics. The multiple comparisons problem was addressed by estimating the false discovery rate (FDR) in a simple manner as the ratio of the expected number of false positives (resulting from permutation analyses with samples being randomly assigned to different groups) at a given *p*-value threshold to the number of positives actually found (Irizarry, 2003; Storey and Tibshirani, 2003). Using this statistical approach comparisons of gene expression with absolute fold changes of at least 1.5-fold (increase or decrease) were selected at $p<0.01$ as a default and $p<0.001$ where indicated. Abbreviation, annotation and analyses of genes meeting the selection criteria were done by combining available information from Affymetrix, Applied Biosystems (PANTHER), Jax, Genelynx, Kegg, Ensembl, Swiss-Prot and PubMed in a Filemaker Pro database.

Hierarchical clustering analysis. Genes altered by treatment with all nuclear receptor agonists by at least 1.5-fold (in either direction) and at $p < 0.01$ between mean gene expression and untreated *db/db* animals were selected for a cluster analysis. The genes were clustered using a distance measure defined as 1 minus the correlation between gene pair

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measures, and using complete-linkage hierarchical clustering. The results are shown in Supplementary Figure 2.

Quantitative Real-Time Polymerase Chain Reaction. One µg of total RNA from each animal (n=5 per group) was treated with DNase I and reverse transcribed into cDNA by Superscript II using random hexamer priming (all Invitrogen, Carlsbad, CA, U.S.A.). Quantitative real-time polymerase chain reaction (QPCR) was performed for all samples per group using gene-specific FAM-TAMRA-labeled commercial Assays-on-Demand (Assay ID: Mm00839363_m1, G6pc; Mm00484574_m1, G6pt1; Mm00440636_m1, Pck1; Mm00662319_m1, Fasn; Mm00772290_m1, Scd1; Mm00833328_m1, Gpam; Mm00483985_m1, Crat; Mm00451571_m1, Slc25a20; Mm00443579_m1, Acox1; Mm00470091_s1, Ehhadh; Mm00478137_m1, Pex11a; Mm00550050_m1, Hmgcs2; Mm00476182_m1, Hsd11b1; Mm00439093_m1, Ghr; Mm00439561_m1, Igf1; Applied Biosystems, Foster City, CA, U.S.A.) or self-designed primer/probe combinations (SREBP-1c; ref. Stulnig et al., 2002b) normalized to 18S VIC-TAMRA labeled endogenous control (Applied Biosystems). Expression of specific mRNAs was quantitated in duplicates with a tolerated variance of ≤10% in an ABI PRISM 7000 Cycler (Applied Biosystems).

Statistics and calculations. Data are given in means ± S.E.M. unless indicated otherwise. Study groups were compared to untreated *db/db* mice by univariate ANOVA using Dunnett's t-test for post-hoc analysis except for evaluation of gene expression profiles (see above). Data from quantitative PCR and microarrays as well as other data exhibiting unequality of variances between groups according to Levene's test were log-transformed before ANOVA. The effect of diabetes was evaluated by comparing *db/+* to *db/db* mice resulting in its reciprocal ($diabetes^{-1}$) in order to facilitate direct comparisons with the normalizing effect of the compounds.

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RESULTS

Metabolic data. This study was performed to elucidate to which extent blood glucose-lowering nuclear receptor agonists alter hepatic gene expression profiles of obese diabetic mice in convergence to non-diabetic animals. Gene expression profiles of *db/db* mice treated with agonists of PPARγ (Rosi), PPARα (Wy) and LXR (T09) and untreated *db/+* mice were compared to untreated diabetic mice. Treatment with each of the compounds resulted in reductions in blood glucose concentrations near to those of non-diabetic mice (Table 1). Plasma insulin concentrations were significantly increased only in the T09-treated group indicating that stimulation of insulin secretion as shown for cultured pancreatic islets and β-cells (Efanov, 2004) could also occur *in vivo*. Body weight remained constant during the treatment except a borderline ($p = 0.088$) increase in the Rosi group. The Wy and particularly T09-treated animals showed significant hepatomegaly with increased liver to body weight ratio by 1.8 and 2.5-fold, respectively, due to the known development of hepatic steatosis by T09 as revealed by highly elevated hepatic triglyceride contents in T09-treated *db/db* mice (Table 1).

Gene expression profiling. Gene expression profiles were evaluated using 12k oligonucleotide microarrays for 3 individual mice of each group and robust multiarray analysis. A *p*-value of < 0.01 was predefined together with a fold change of ≥ 1.5 / ≤ -1.5-fold as selection criteria for profile comparisons including detection of functional groups. 337 genes were altered in untreated diabetic vs. non-diabetic mice (Table 2). Drug treatment of *db/db* mice elicited significant changes in the expression of a comparable number of genes, namely 300, 620, and 735 genes for Rosi, Wy, and T09, respectively. Estimated false discovery rates (FDR) were between 6-11% for $p < 0.01$ but about 80% of genes were altered at a $p < 0.001$ with FDR of 1-3%, respectively, indicating excellent confidence of data. Moreover, standardized logarithmic expression estimates from microarrays highly correlated

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with those from quantitative PCR (adjusted $r^2 = 0.854$, $p < 0.0005$) indicating valid relative quantitation by microarray hybridization (data not shown).

We used the reciprocal form of the diabetes effect (diabetes^{-1}), i.e. untreated non-diabetic vs. diabetic mice, in order to facilitate direct comparison with treatment effects. Treatment-induced normalization of gene expression was defined as genes that were significantly changed by a compound in the same direction as diabetes^{-1}, i.e. in convergence to *db/+* animals, without testing whether the gene actually reached the level of non-diabetic mice. From the genes (microarray probe sets) altered in *db/db* vs *db/+* mice, only 34 (10% of all genes altered in diabetes^{-1}), 51 (15%) and 82 (24%) were normalized by Rosi, Wy and T09, respectively (Fig. 1A). In total, only a set of 19 genes (6%) were normalized by all blood glucose lowering drugs (listed in Supplementary Figure 1A) indicating that these genes could be particularly important in mediating the glucose lowering effects. These genes included some with clear implication in glucose metabolism and diabetes such as those involved in gluconeogenesis (e.g., glucose-6-phosphatase, fructose-1,6-bisphosphatase) and the glucocorticoid-activating enzyme 11β-hydroxysteroid dehydrogenase type 1, whereas the functional implication of others for glucose lowering have to be assessed in detail. However, 56 (17%), 85 (25%) and 59 (18%) genes altered in *db/db* vs *db/+* mice were regulated by Rosi, Wy and T09, respectively, in the direction opposite to diabetes^{-1} (Fig. 1B). The 30 genes (probe sets) regulated by all compounds in the direction opposite to diabetes^{-1} (listed in Supplementary Figure 1B) thus possibly giving insight into augmented adaptive processes, e.g., comprised genes involved in mitochondrial and peroxisomal β-oxidation (Acaa1, Dci, Ehhadh), including the important peroxisomal biogenesis factor (Pex11a) as discussed in detail below. Treatment with nuclear receptor agonists regulated many genes not altered by diabetes^{-1} itself and resulted in considerably overlaps in gene expression profiles in diabetic mice (Fig. 1C) similar to that shown for PPARα, LXR, and retinoid X receptor in non-diabetic mice (Anderson et al., 2004). The overlap of PPARγ agonist treatment with other

compounds is noteworthy, since PPARγ is only weakly expressed in liver and thus direct crosstalk between nuclear receptors cannot be accounted for. Supplementary Figure 2 provides a tree obtained by hierarchical clustering of genes altered in parallel by all nuclear receptors agonists to highlight the correlation of their regulation. A complete list of all genes altered in *db/db* vs *db/+* mice (shown as diabetes^{-1}) or by treatment with any compound is given in Supplementary Figure 3.

Gluconeogenesis. Hepatic gluconeogenesis and glucose output is significantly enhanced in type 2 diabetes patients and comparable mouse models and contributes to high blood glucose levels. Treatment with each of the blood glucose lowering nuclear receptor agonists resulted in normalization of gluconeogenetic key enzyme gene expression as shown for phosphoenol pyruvate carboxykinase 1 (PEPCK; gene Pck1; 1.2 fold; $p < 0.05$), fructose-1,6-bisphosphatase (Fbp1) and the catalytic and transport units of glucose-6-phosphatase (G-6-Pase; G6pc and G6pt1) (Fig. 2). Interestingly, the PPARα agonist treatment led to an increased expression of fructose-2,6-bisphosphatase (Fbp2) which could imply an additional regulation of Fbp1 through competitive inhibition by fructose-2,6-bisphosphate. Expression of aldolase B (Aldo2) that catalyzes cleavage of fructose-1,6-bisphosphate and functions as a feed forward activator of pyruvate kinase was normalized by both Wy (-1.8 fold; $p < 0.002$) and Rosi (-1.4 fold; $p = 0.042$). Glucokinase (Gck) whose expression was increased by 1.4 fold in diabetes^{-1}, was normalized in the Rosi group (1.4 fold; $p = 0.003$) and T09 group (1.8 fold; $p < 0.001$). Additional transfer of glucose-6-phosphate through the pentose phosphate shunt in the LXR-treated group is indicated by elevated expression of Gck, X-linked glucose-6-phosphate dehydrogenase (G6pdx), glucose-6-phosphate dehydrogenase 2 (G6pd2), ribose 5-phosphate isomerase A (Ripa) and transketolase (Tkt; Supplementary Figure 3). In general these data indicate that treatment of *db/db* mice with different blood glucose lowering nuclear receptor agonist resulted in normalized expression of key genes involved in glucose homeostasis even though some genes were regulated in an agonist-specific manner.

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Lipogenesis. Hepatic lipogenesis and fatty acid desaturation has been implicated in various pathological conditions including obesity and diabetes (Ntambi et al., 2002). Hepatic gene expression of lipogenic enzymes, e.g., stearoyl-Coenzyme A desaturase 1 (Scd1), was generally increased in *db/db* vs *db/+* animals (Fig. 3A). Treatment of *db/db* mice with any blood glucose lowering compound led to a further strong increase of Scd1 gene expression (Fig. 3A). Scd1 expression is regulated by sterol regulatory element-binding protein-(SREBP)-1c-dependent and independent mechanisms (Miyazaki et al., 2004). Since induction of Scd1 by drugs correlated with elevated SREBP-1c expression levels only in the T09 group (Fig. 3A), Scd1 upregulation by PPAR agonists appears to occur predominantly by SREPB-1c-independent mechanisms. Parallel to Scd1 expression, fatty acid synthase (Fasn) and glycerol-3-phosphate acyltransferase (Gpam) was strongly induced by treatment with any agonist whereas expression of hepatic lipase was decreased (Fig. 3A). Thereby, the nuclear receptor agonists aggravated diabetes-associated alterations in lipogenesis in parallel with their glucose lowering effect (Way et al., 2001). Such regulation opposite to diabetes^{-1} could on the one hand indicate enhancement of adaptive processes that were already induced by diabetes itself but also worsening of detrimental diabetes-associated alterations. Recent data suggest that upregulation of lipogenesis provides an effective means to inhibit diabetes development by shifting the lipogenic burden from adipose tissue to the liver (Nadler and Attie, 2001). Altogether alterations in hepatic lipid homeostasis provoked hepatic triglyceride accumulation particularly in the T09-treated and – to a lesser extent – in Rosi-treated animals. Moreover, the LXR agonist increased blood plasma triglyceride concentrations, whereas Rosi and Wy lowered plasma triglyceride concentrations (Table 1; ref. Chisholm et al., 2003; Oakes et al., 1994). In contrast to lipogenic enzymes, genes involved in cholesterogenesis such as 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (Hmgr) and synthase 1 (Hmgcs1), isopentenyl-diphosphate delta isomerase (Idi1), farnesyl diphosphate farnesyl transferase 1 (Fdft1) and NAD(P)-dependent steroid dehydrogenase-like (Nsdhl) were not

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altered by diabetes and were differently changed by nuclear receptor agonists (Supplementary Figure 3. and data not shown).

Mitochondrial and peroxisomal β-oxidation. β-oxidation of free fatty acids provides energy and substrates for ketogenesis. Expression of genes implicated in mitochondrial fatty acid import including the carnitine acyltransferase Crat and the translocase Slc25a20 (Ramsay et al., 2001; Sekoguchi et al., 2003). Expression of both genes was significantly increased in diabetic vs. non-diabetic mice and further elevated by treatment with blood glucose-lowering nuclear receptor agonists indicating augmented mitochondrial fatty acid import (Fig. 3B). Expression of the long chain-specific carnitine acyltransferases (Cpt1a, Cpt2) was increased by diabetes and further elevated in response to Wy treatment. Expression of enzymes involved in mitochondrial fatty acyl oxidation such as dodecenoyl-Coenzyme A delta isomerase (Dci) and t acetyl-CoA dehydrogenases (Acads, Acadm, Acadl) was somewhat increased in diabetic animals and further elevated by nuclear receptor agonists (Fig. 3C and Supplementary Figure 3). Peroxisomes oxidize fatty acids by different pathways with preference for long chain and very long chain fatty acids (Van Veldhoven and Mannaerts, 1999). Expression of enzymes of the classical pathway, namely acyl-Coenzyme A oxidase 1 (Acox1), enoyl-Coenzyme A hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (Ehhadh) and acetyl-Coenzyme A acyltransferase 1 (Acaa1) was more pronounced in *db/db* vs. *db/+* animals (Lan et al., 2003) and further increased by nuclear receptor agonist treatment, particularly Wy (Fig. 3C) in parallel with peroxisomal biogenesis factor 11a (Pex11a; Fig. 3C). Notably, hepatic triglyceride accumulation particularly in T09-treated animals revealed that increased β-oxidation did not sufficiantly counteract elevated lipogenesis to prevent hepatic steatosis. Increased ketogenesis in diabetes was indicated by elevated expression of key genes 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (Hmgcs2) and 3-hydroxy-3-methylglutaryl-Coenzyme A lyase (Hmgcl; Fig. 3D). Particularly PPAR agonists further increased Hmgcl and Wy also increased Hmgcs2 expression suggesting increased

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ketogenesis. In conclusion, treatment with different blood glucose-lowering nuclear receptor agonists resulted in increased expression of most genes contributing to mitochondrial and peroxisomal fatty acid oxidation and ketogenesis thereby aggravating the diabetes-associated shift to fatty acid metabolism.

Glucocorticoid activation. Type 2 diabetes has been shown to be associated with alterations in local activation of inactive glucocorticoid precursors (cortisone, 11-dehydro corticosterone) to active glucocorticoids (corticosterone, cortisol) by 11β-hydroxysteroid dehydrogenase type 1 (Hsd11b1). Hsd11b1 is particularly expressed in human and rodent liver and adipose tissue (Bujalska et al., 2002) and increased expression has been linked to development of diabetes and the metabolic syndrome (Masuzaki et al., 2001; Aoki et al., 2001). Treatment with agonists of the PPAR and LXR family has been shown recently to affect expression of Hsd11b1 in adipose tissue and liver (Stulnig et al., 2002b; Cao et al., 2003). Treatment of either nuclear receptor agonists diminished Hsd11b1 expression even below the level of non-diabetic mice (Fig. 4). Since oxo-reductase activity of Hsd11b1 depends on NADPH, mutations in hexose-6-phosphate dehydrogenase (H6pd) synergize with weak Hsd11b1 mutations in glucocorticoid activation (Draper et al., 2003). However, H6pd gene expression was not changed in this study (Fig. 4). Notably, Hsd11b1 was one of the only 19 transcripts that were normalized by all blood glucose lowering nuclear receptor agonists (Supplementary Fig. 1A). These data emphasize the importance of 11β-hydroxysteroid dehydrogenase 1 for the development of insulin resistance and suggest the use of specific inhibitors of this enzyme in diabetes treatment.

Growth hormone signaling. Growth hormone (GH) antagonizes insulin signaling and elicits insulin resistance resulting in increased hepatic glucose production. In contrast to unchanged expression in diabetic vs. non-diabetic mice, treatment with blood nuclear receptor agonists downregulated growth hormone receptor gene (Ghr) expression (Fig. 5), whose abundance is related to GH tissue sensitivity (Dominici and Turyn, 2002; Iida et al., 2004), as

well as expression of insulin-like growth factor-1 (Igf-1), a prototype GH-responsive gene in the liver. These data indicate interference with GH action by nuclear receptor agonist treatment. Since inhibition of GH action improves insulin sensitivity (Yakar et al., 2004), this could be an additional mechanism how PPAR and LXR agonists ameliorate glucose homeostasis in diabetes.

Endoplasmic reticulum stress. Endoplasmic reticulum stress is a major contributor for the development of obesity and insulin resistance as shown very recently (Özcan et al., 2004). Obesity triggers an unfolded protein response (UPR) in liver that is controlled by X-box-binding protein-1 (XBP-1) (Yoshida et al., 2001). Notably, XBP-1$^{+/-}$ mice are prone to insulin resistance due to impaired insulin signal transduction during endoplasmic reticulum stress (Özcan et al., 2004). Glucose-regulated/binding immunoglobulin protein Grp78 (Hspa5), an endoplasmic reticulum chaperone, and the protein kinase inhibitor p58^{ipk} (Dnajc3) are upregulated during UPR and the latter has been shown to be a XBP-1 target gene as is thioredoxin domain containing protein-7 (Txndc7) (Lee et al., 2003). XBP-1 expression was significantly lowered by all blood glucose-lowering nuclear receptor agonists (Fig. 6) in parallel with Grp78, DNajc3 and Txndc7 pointing to reduced XBP-1 activity and amelioration of endoplasmic reticulum stress. Reduced expression of Grp78 in untreated *db/db* animals compared to lean mice could be secondary to the nearly twofold reduced expression of XBP-1 in untreated *db/db* vs. *db/+* mice. It is intriguing to speculate that the reduced expression of XBP-1 in *db/db* animals indicates a reduced capacity to deal with endoplasmic reticulum stress similar to that seen in XBP-1$^{+/-}$ mice whereas further reduction of the expression of UPR genes by nuclear receptor agonist treatment of diabetic mice reflects a decline of endoplasmic reticulum stress. Thus, reduction of endoplasmic reticulum stress could be a novel mechanism how PPAR and LXR agonists lower blood glucose concentrations in diabetic mice prone to UPR by lowered XBP-1 expression.

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DISCUSSION

Insulin-sensitizing compounds such as agonists for PPARγ and PPARα are widely used in clinical practice to ameliorate diabetes-induced alterations in glucose and lipid metabolism, respectively. In this study we show that although blood glucose lowering agonists for the nuclear receptors PPARγ, PPARα and LXR regulate expression of a large number of genes, they improve only some diabetes-associated alterations mainly by normalization of gluconeogenetic gene expression. A large number of diabetes-associated alterations in gene expression were not reversed towards levels found in non-diabetic mice but even deteriorated by drug treatment including genes implicated in lipogenesis, peroxisomal and mitochondrial function. Some of these regulations, e.g., the elevated expression of genes involved in lipogenesis, indicate that nuclear receptor agonists enhanced adaptive processes protecting obese mice from further metabolic derangements, e.g. by shifting the lipogenic burden to the liver. A gene expression study by itself cannot discriminate between causal and adaptive alterations. However, irrespective of whether diabetes-associated alterations by nuclear receptor agonist treatment were primarily of causal or adaptive nature, these changes indicate that a cure in a molecular sense, i.e., correction of causal diabetes-associated molecular alterations, has been achieved by the compounds only to a very limited extent. In addition, our study disclosed hints on possible novel modes of action how nuclear receptor agonists ameliorate blood glucose concentrations. Particularly interference with growth hormone signaling and reduction of endoplasmic reticulum stress warrant investigations in future focused studies. Moreover, the significantly increased insulin plasma concentration by T09 treatment revealed that LXR agonism or selective LXR modulation could provide a novel pharmacological approach to stimulate insulin secretion. However, issues of preventing hepatic steatosis by these compounds and possible β-cell lipotoxicity by increased SREBP-1c

expression (Efanov, 2004) have to be addressed first. Notably, the model for type 2 diabetes used here cannot discriminate between alterations induced by diabetes or obesity alone, respectively. Hence changes provoked by obesity but not by metabolic derangements cannot be overcome by the action of the compounds that did not induce weight loss. However, alterations induced by obesity and metabolic derangements are usually combined also in type 2 diabetes patients.

In conclusion, this study revealed that apart from pointing to novel modes of action, currently available blood glucose-lowering nuclear receptor agonists by far do not normalize diabetes-associated molecular alterations. Despite our advances during recent years, there is still a need for developing novel drugs for effective treatment of type 2 diabetes at the molecular level.

Acknowledgments. We thank Sylvia Molzer for technical assistance and Jelena Todoric for liver triglyceride determination.

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FOOTNOTES

Title footnote: This work was supported by CeMM – Center of Molecular Medicine, a basic research institute within the companies of the Austrian Academy of Sciences (to T.M.S. and W.W.), and the Joseph Skoda Award of the Austrian Society for Internal Medicine (to T.M.S.).

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FIGURE LEGENDS

Fig. 1. Overlap of diabetes and drug effects. Out of the 337 genes (microarray probe sets) altered by diabetes figures in overlapping wheels of panels (**A**) and (**B**) indicate the number of genes that were also regulated by PPARγ, PPARα and LXR agonists, respectively, in direction to normalize (**A**) or deteriorate (**B**) diabetes-associated changes (see Supplementary Figure 1 and 2). Panel (**C**) gives total numbers of genes regulated by the compounds illustrating considerable overlap of drug effects. Numbers with connecting lines to the wheels indicate the total number of genes regulated by the respective nuclear receptor agonist. Note that only a minority of diabetes-associated gene regulations were normalized by the treatments, and that many genes not associated with diabetes-associated alterations were regulated by the compounds.

Fig. 2. Expression of genes involved in gluconeogenesis. Diabetic *db/db* mice were treated for 10 days with Rosi, Wy, or T09, respectively, or left untreated as were non-diabetic *db/+* mice. Gene expression was evaluated by QPCR (default; n=5) or by microarrays (indicated by black dot; n=3) and is given in percent of that in untreated *db/db* mice. G6pc, glucose-6-phosphatase, catalytic subunit; G6pt, glucose-6-phosphatase, transport subunit; PEPCK (Pck1), phosphoenolpyruvate carboxykinase 1, cytosolic; Fbp1, fructose bisphosphatase 1; Aldo2, aldolase 2, B isoform; ut, untreated. Significant differences compared to untreated *db/db* are indicated as follows: *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$.

Fig. 3. Lipid homeostasis and ketogenesis. **A**) Expression of genes involved in lipogenesis. Gene expression from mice is given as detailed in legend to Fig. 2. Gene expression was evaluated by QPCR (default; n=5) or by microarrays (indicated by black dot; n=3) and is given in percent of that in untreated *db/db* mice. Fasn, fatty acid synthase; Scd1, stearoyl-

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Coenzyme A desaturase 1; Gpam, glycerol-3-phosphate acyltransferase, mitochondrial; Dgat1, diacylglycerol O-acyltransferase 1; Lipc, hepatic lipase; Srebp-1c, sterol regulatory element binding factor 1; ut, untreated; geometric mean is given for columns exceeding scale of Y-axis. Significant differences compared to untreated *db/db* are indicated as follows: *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$. **B**) Expression of genes involved in mitochondrial fatty acid import. Crat, carnitine acetyltransferase; Slc25a20, solute carrier family 25 (mitochondrial carnitine/acylcarnitine translocase), member 20; Cpt1a, carnitine palmitoyl- transferase 1, liver; Cpt2, carnitine palmitoyl- transferase 2. **C**) Expression of genes involved in mitochondrial and peroxisomal β-oxidation. Acox1, acyl-Coenzyme A oxidase 1, palmitoyl; Dci, dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenyme A isomerase); Ehhadh, enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase; Acaa1, 3-ketoacyl-CoA thiolase B, acetyl-Coenzyme A acyltransferase 1; Pex11a, peroxisomal biogenesis factor 11a. **D**) Expression of genes involved in ketogenesis. Hmgcs2, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2; Hmgcl, 3-hydroxy-3-methylglutaryl-Coenzyme A lyase.

Fig. 4. Glucocorticoid metabolism. Gene expression from mice is given as detailed in legend to Fig. 2. Gene expression was evaluated by QPCR (default; n=5) or by microarrays (indicated by black dot; n=3) and is given in percent of that in untreated *db/db* mice. Hsd11b1, hydroxysteroid 11-beta dehydrogenase 1; H6pd, hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase); ut, untreated. Significant differences compared to untreated *db/db* are indicated as follows: ‡, $p < 0.001$.

Fig. 5. Growth hormone signaling. Gene expression from mice is given as detailed in legend to Fig. 2. Gene expression was evaluated by QPCR (default; n=5) or by microarrays (indicated by black dot; n=3) and is given in percent of that in untreated *db/db* mice. Ghr,

growth hormone receptor; Igf-1, insulin-like growth factor 1; ut, untreated. Significant differences compared to untreated *db/db* are indicated as follows: †, $p < 0.01$; ‡, $p < 0.001$.

Fig. 6. Expression of genes involved in endoplasmic reticulum stress. Gene expression from mice is given as detailed in legend to Fig. 2. Gene expression was evaluated by QPCR (default; n=5) or by microarrays (indicated by black dot; n=3) and is given in percent of that in untreated *db/db* mice. Xbp1, X-box binding protein 1; Grp78 (Hspa5), heat shock 70kD protein 5 (glucose-regulated protein); Dnajc3, DnaJ (Hsp40) homolog, subfamily C, member 3; Txndc7, thioredoxin domain containing protein-7; ut, untreated; *, $p < 0.05$; †, $p < 0.01$; ‡, $p < 0.001$.

Supplementary Figure 1. Genes regulated by all nuclear receptor agonists in the direction to normalize (A) or aggravate (B) diabetes-induced alterations. Gene Ontology (GO) identifiers were added as a functional annotation. Microarray probe sets representing identical genes show up repeatedly.

Supplementary Figure 2. Clustering of genes regulated by all nuclear receptor agonists. The profiles cluster according to the pattern across all conditions including alterations between untreated diabetic and non-diabetic animals. Genes downregulated by the compounds are represented in the upper part, upregulated genes in the lower part of the figure. Several different patterns of response are observed; for example the upmost group of the lower half corresponds to genes that are up-regulated in all conditions including the diabetes^{-1} effect (i.e., *db/+* vs. *db/db* animals). On the other hand, the downmost genes of the upper half correspond to genes with increased expression in *db/+* vs. *db/db* animals but downregulated by all compounds.

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Supplementary Figure 3. Hepatic gene expression profiles of drug-treated *db/db* and untreated *db/+* compared to untreated *db/db*. Diabetic *db/db* mice were treated for 10 days with Rosi (R), Wy (W), or T09 (T), respectively, or left untreated as were non-diabetic *db/+* mice. Probe sets numbered with affy ID# represent genes identified by genbank entry numbers (genbank ID#), gene symbols and names. The fold change difference to untreated *db/db* mice is given as a color code and in numbers. The mean estimate of expression in untreated *db/db* mice is related to the mean of all genes on each microarray that was set to 100.

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TABLE 1. Animal characteristics and blood analyses

Parameter (units)	*db/db*				*db/+*
	untreated	Rosi	Wy	T09	untreated
Body weight (g)					
before treatment	42.7±1.8[a]	42.6±1.6	42.0±1.7	42.5±1.3	27.3±1.4***
end of treatment	43.7±1.7	46.7±1.9	42.3±2.7	43.4±2.4	27.9±1.4***
Liver weight (g)	2.30±0.30	2.68±0.35	3.83±0.36***	5.67±0.29***	1.43±0.13***
Liver/body weight (%)	5.2±0.5	5.7±0.6	9.1±0.9***	13.1±0.2***	5.1±0.3
Blood glucose[b] (mmol/l)					
before treatment	23.4±3.2	19.5±5.1	24.5±3.7	25.0±4.8	8.1±0.9***
end of treatment	28.7±2.0	10.4±1.7***	14.7±4.5***	11.9±4.0***	7.9±0.7***
Insulin[c] (nmol/l)	0.52±0.26	0.53±0.26	0.41±0.22	1.75±0.58***	0.37±0.19
Triglycerides[c] (mmol/l)	2.3±0.5	0.7±0.3***	1.1±0.8**	4.4±1.7	1.7±0.5
Cholesterol[c] (mmol/l)	4.2±0.9	3.5±0.6	6.7±1.0***	14.8±2.2***	3.4±0.4
NEFA[c] (mmol/l)	0.25±0.04	0.14±0.02**	0.18±0.01	0.29±0.05	0.21±0.07
Liver triglycerides (nmol/mg liver)	36.9±9.3	63.1±11.1**	33.8±5.1	174.6±40.1**	5.3±2.2**

[a] Data are given in means ± S.D.

[b] Blood parameters were analyzed from plasma unless stated otherwise.

[c] log-transformed for ANOVA.

[d] All groups were compared to untreated db/db; * $p < 0.05$; ** $p < 0,01$; *** $p < 0.001$.

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TABLE 2. Overall statistics of changes in gene expression profiles

Effect	Comparison vs untreated db/db	Number of genes[a]		
		Increased	Decreased	Total
Diabetes^{-1}[b]	db/+	155	182	337
PPARγ	Rosi	158	142	300
PPARα	Wy	362	258	620
LXR	T09	402	333	735

[a] Number of genes changed at least ±1.5-fold and a $p<0.01$.

[b] The diabetes effect was given as reciprocal (*db/+* vs *db/db*; diabetes^{-1}) in order to facilitate direct comparisons with normalizing effects of drugs.

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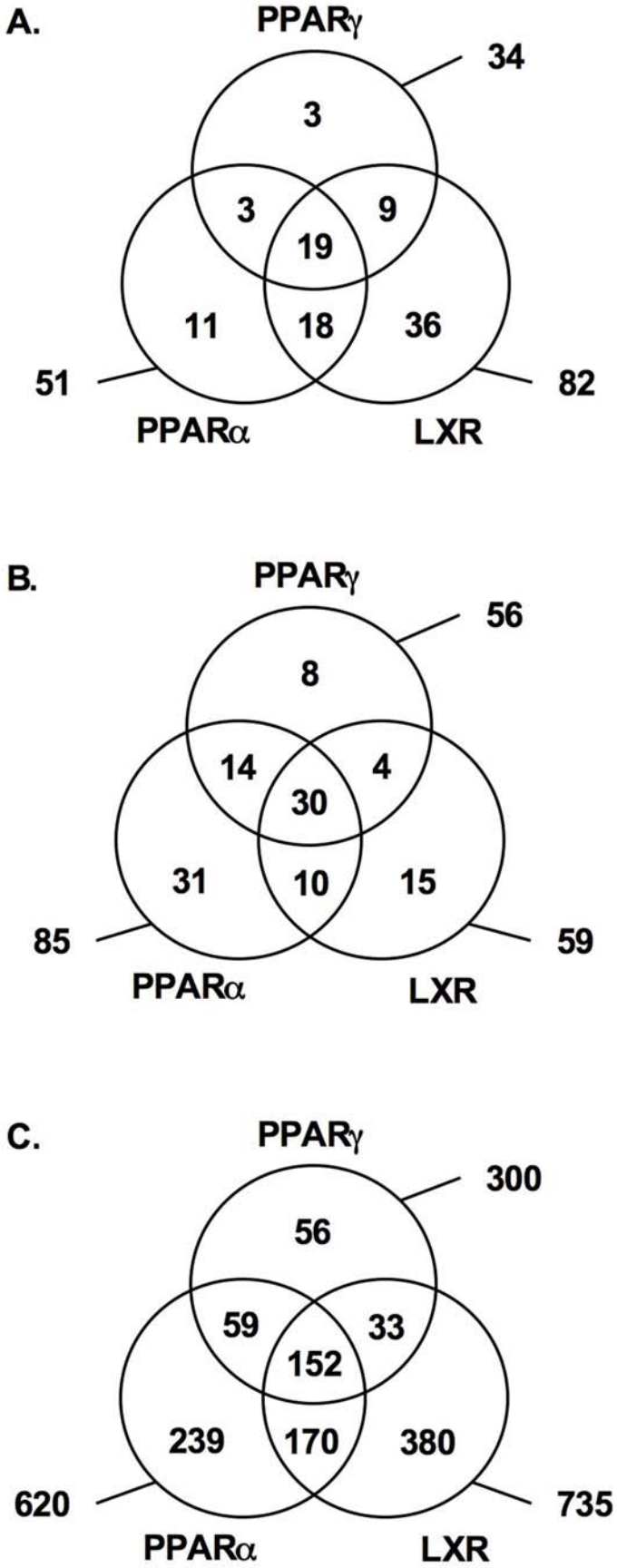

FIG. 1

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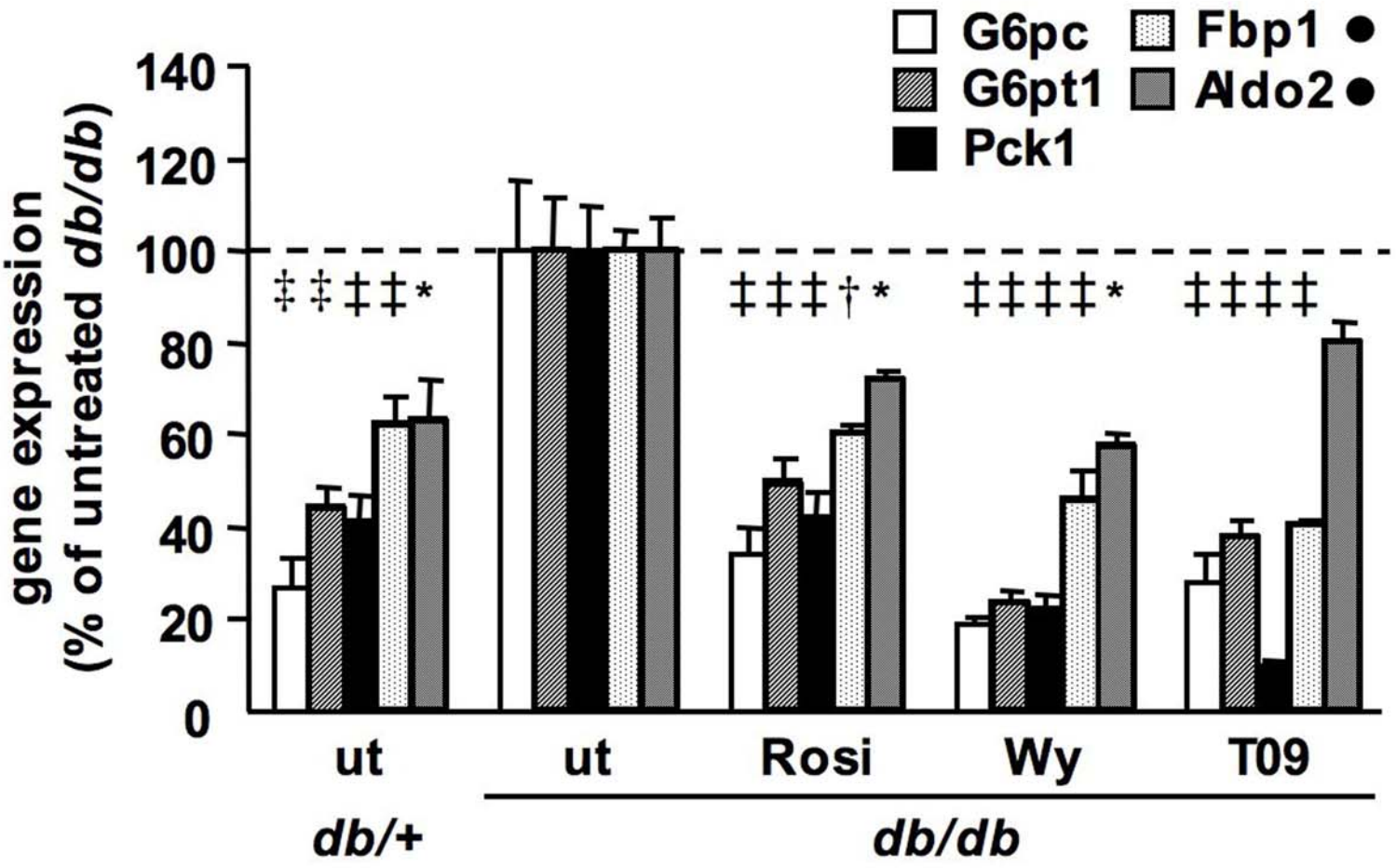

FIG. 2

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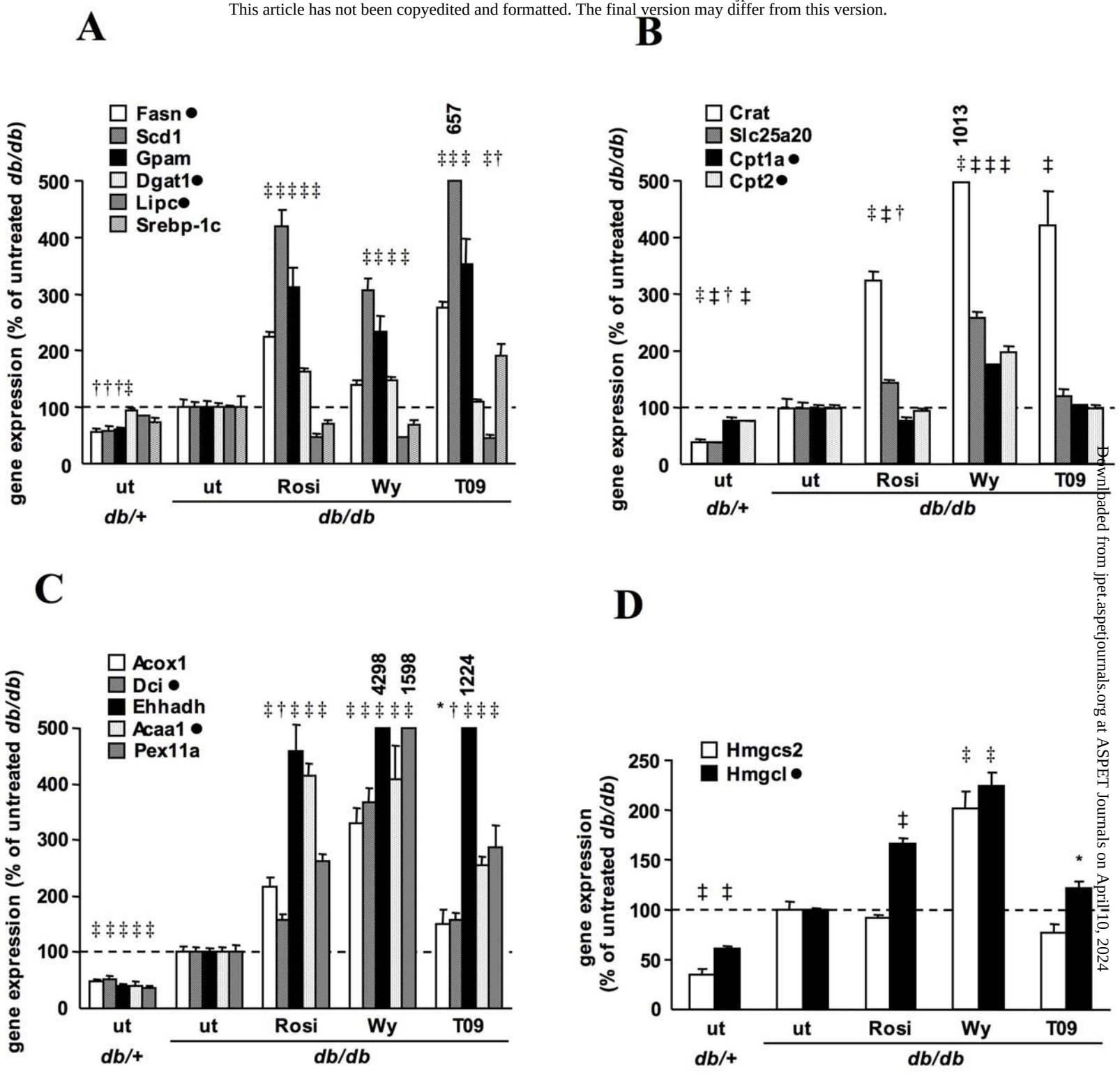

FIG. 3

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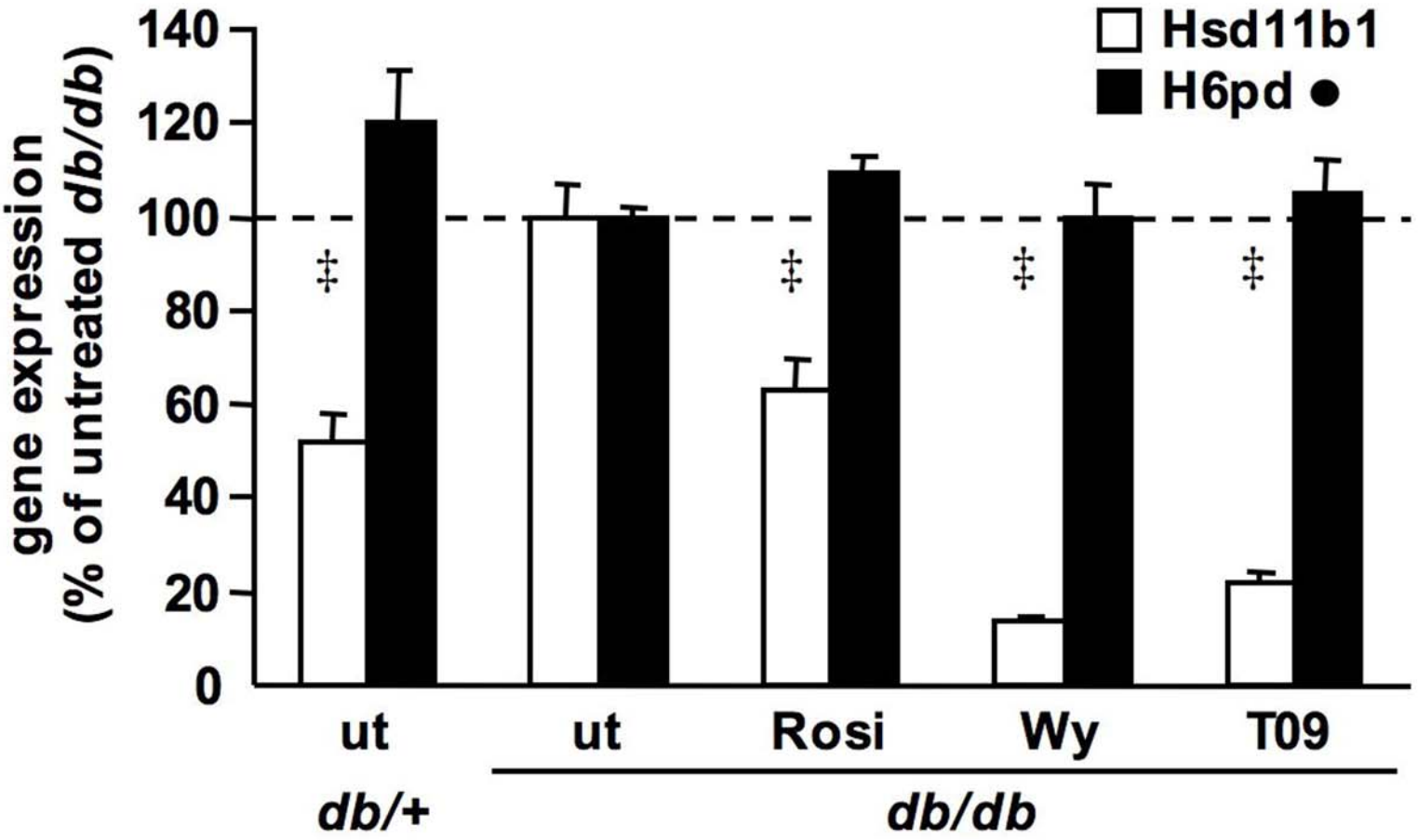

FIG. 4

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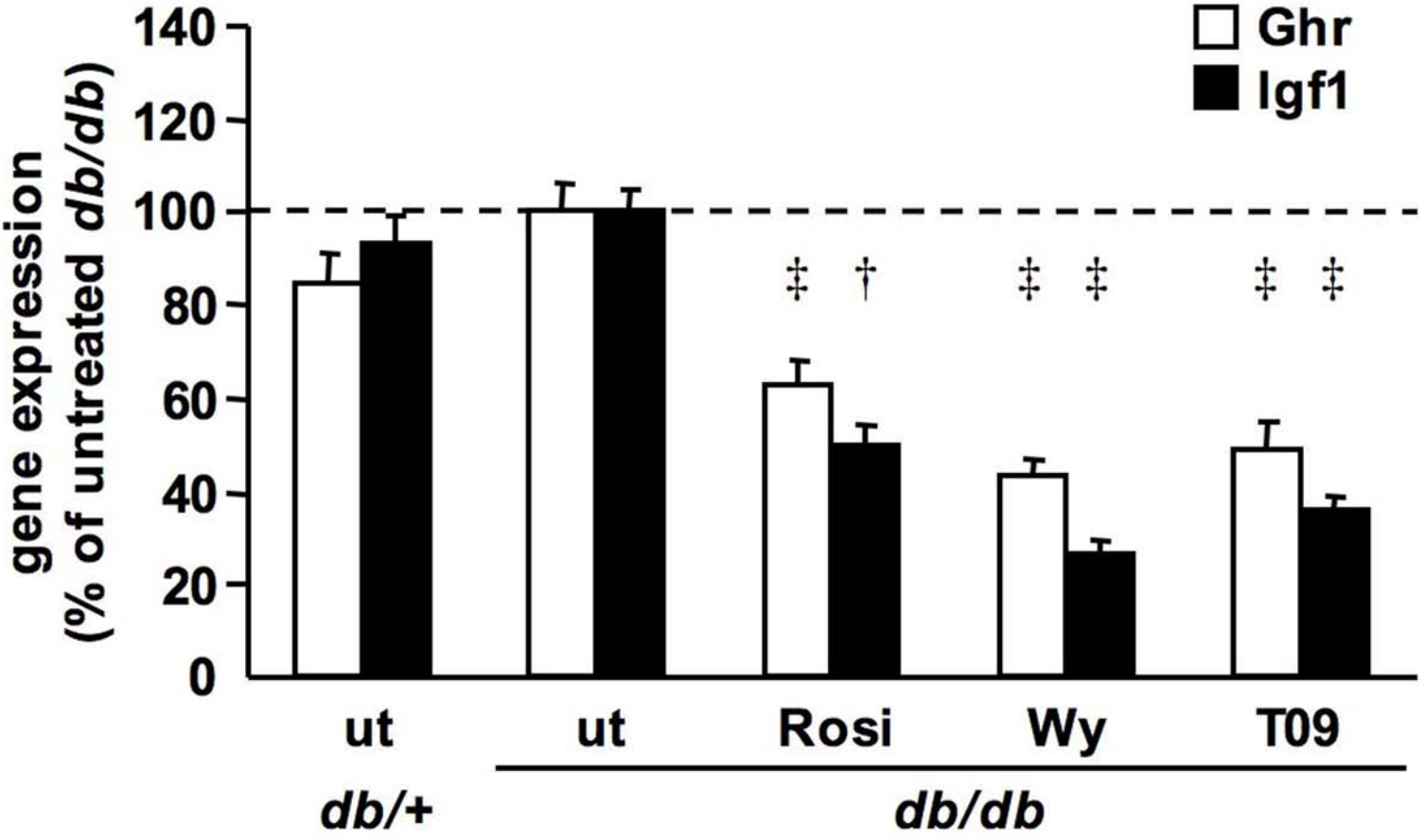

FIG. 5

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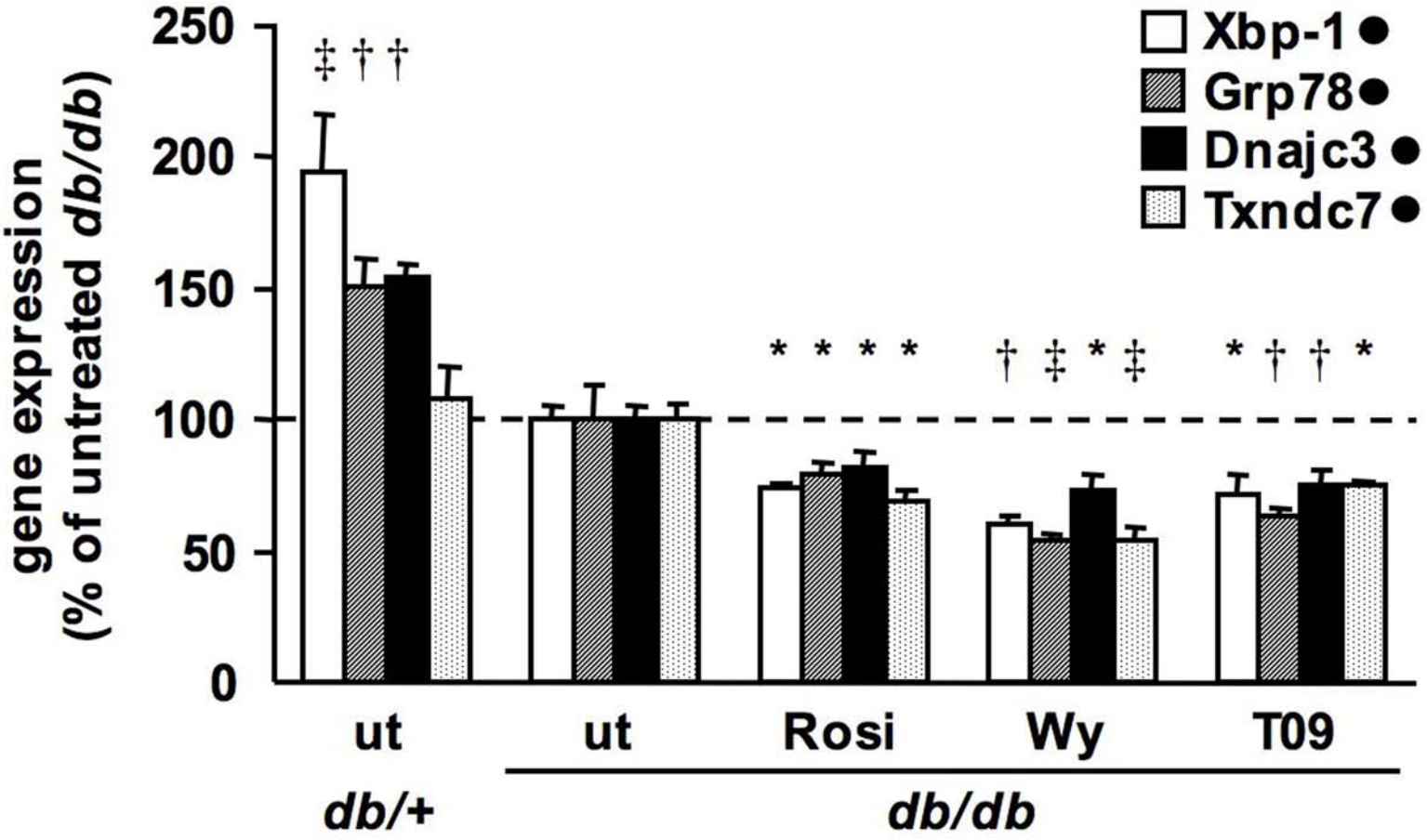

FIG. 6