The UDP-glucuronosyltransferase 1A9 enzyme is a Peroxisome Proliferator-Activated Receptor alpha and gamma target gene.

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SUMMARY

Peroxisome proliferator-activated receptor (PPAR) alpha and gamma are ligand-activated transcription factors belonging to the nuclear receptor family. PPARα mediates the hypolipidemic action of the fibrates, whereas PPARγ is a receptor for the anti-diabetic glitazones. In the present study, the UDP-glucuronosyltransferase (UGT) 1A9 enzyme is identified as a PPARα and PPARγ target gene. UGTs catalyze the glucuronidation reaction, which is a major pathway in the catabolism and elimination of numerous endo- and xenobiotics. Among the UGT1A family enzymes, UGT1A9 metabolizes endogenous compounds, including catecholestrogens, and xenobiotics, such as fibrates and to a lesser extent troglitazone. Treatment of human hepatocytes and macrophages and murine adipocytes with activators of PPARα or PPARγ resulted in an enhanced UGT1A9 expression and activity. In addition, disruption of the PPARα gene in mice completely abolished the PPARα agonist-induced UGT1A9 mRNA and activity levels. A PPAR response element (PPRE) was identified in the promoter of UGT1A9 at position −719 to −706 bp by transient transfection and electromobility shift assays. Considering the role of UGT1A9 in catecholestrogen metabolism, PPARα and PPARγ activation may contribute to the protection against genotoxic catecholestrogens by stimulating their inactivation in glucuronide derivatives. Furthermore, since UGT1A9 is involved in the catabolism of fibrates, these results suggest that PPARα and PPARγ may control the intracellular level of active fibrates.
INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) belong to the family of nuclear receptors that are ligand-activated transcriptional regulators of gene expression. Upon ligand-activation, PPARα and PPARγ regulate gene transcription by dimerizing with the Retinoid X Receptor (RXR) and binding to PPAR response elements (PPREs) within the regulatory regions of target genes (1). These PPREs usually consist of a direct repeat of the hexanucleotide AGGTCA sequence separated by one or two nucleotides (DR1 or DR2) (1). Furthermore, both PPARα and PPARγ negatively interfere with pro-inflammatory transcription factors pathways by a mechanism termed transrepression (2). PPARα is highly expressed in tissues such as liver, muscle, kidney and heart, where it stimulates the β-oxidative degradation of fatty acids (3). PPARγ is predominantly expressed in adipose tissue, where it promotes adipocyte differentiation and lipid storage (4). Both PPARα and PPARγ are expressed in cells of the immune system, where they regulate the inflammatory response, influence cell proliferation and apoptosis and modulate lipid homeostasis (5,6).

Fatty acids (FAs) and derivatives are natural ligands for PPARα and PPARγ. Natural eicosanoids derived from arachidonic acid via the lipoxygenase pathway, such as 8-hydroxytetraenoic acid (8-HETE), 15-HETE and leukotriene B4 (LTB4), as well as oxidized phospholipids activate PPARα (7-9). PPARγ is a receptor for eicosanoid metabolites formed via the cyclooxygenase (15d-PGJ2) and lipoxygenase pathways (15-HETE, 9-hydroxyoctadecadienoic acide [9-HODE] and 13-HODE) (10). Synthetic agonists of PPARs are used in the treatment of metabolic diseases, such as dyslipidemia and type 2 diabetes. The antidiabetic glitazones (e.g. troglitazone, rosiglitazone and pioglitazone), which are insulin sensitizers, are high-affinity ligands for PPARγ (7). The hypolipidemic fibrates (gemfibrozil, bezafibrate, cipofibrate and fenofibrate) are PPARα ligands (7). Both natural and synthetic ligands of PPARs share similar metabolic pathways, since eicosanoids, fibrates and troglitazone are excreted as glucuronide conjugates in humans (11-14).

Glucuronides are naturally occurring conjugation metabolites of xenobiotics and endobiotics (15). Glucuronide conjugation (or glucuronidation), the process underlying their formation, is considered detoxifying, because glucuronides usually possess less intrinsic
biological or chemical activity than their parent aglycones and are rapidly excreted. UDP-glucuronosyltransferase (UGT) enzymes catalyze the glucuronidation reaction, which consists in the addition of the glycosyl group from a nucleotide sugar (UDP-glucuronic acid, UDPGA) to a wide variety of endogenous and exogenous compounds (15). Endogenous glucuronidated substances are for instance androgens, estrogens, progestins, bile acids, fatty acids, retinoids and bilirubin (16). Numerous exogenous compounds are glucuronidated by UGTs, including environmental compounds, plant steroids and important pharmacological agents (such as non-steroidal anti-inflammatory drugs, opioids or fibrates) (11,16). More than 60 mammalian UGT isoforms have been separated into 2 families and 4 sub-families based on their primary amino acid sequence identity (17). In humans, members of the UGT1A sub-family are encoded by a complex gene, which contains at least 17 exons spanning over 200 kb (18). Located on chromosome 2q37, the UGT1A gene complex leads to the production of 13 different mRNAs by alternative splicing: while exons 14 to 17 are shared between all UGT1A isoforms, the 13 first exons encode the amino terminal part of each protein, and share between 37 and 90% amino acid sequence identity (16). The common carboxyl part of UGT1A proteins contains the cofactor UDPGA binding site, whereas the substrate specificity of each enzyme is determined by the amino terminal part of the protein (17).

Belonging to the UGT1A sub-family, UGT1A9 catalyzes the conjugation of various bulky phenols, N-hydroxy-arylamines, steroids and several drugs (16,19). UGT1A9 glucuronidates a variety of endogenous molecules, such as retinoic acid or thyroid hormones (T3 and T4) and is mainly involved in the metabolism of catecholestrogens (4-hydroxyestradiol [4OH-E1] and 4-hydroxyestrone [4OH-E2]) (20,21). Recent studies revealed that UGT1A9 also catalyzes glucuronidation of the synthetic PPAR activators, gemfibrozil, fenofibrate and troglitazone (22-25). Furthermore, preliminary data indicate that UGT1A9 may conjugate natural PPARα and PPARγ agonists, such as 15-HETE and 13-HODE (Bélanger et al., personal communication). Human UGT1A9 is expressed in various tissues, including liver, intestine, kidney, mammary gland and ovary (21).

Several studies in both humans and animals reported that treatment with PPARα activators results in enhanced glucuronidation activity and UGT expression (26-29). In the Helsinki Heart Study population, gemfibrozil treatment resulted in a significant elevation of
plasma androstane-3α, 17β-diol glucuronide levels (26). In rats, but not in mice, fenofibrate increases bilirubin glucuronidation in liver microsomes (27,28). In the same line, clofibrate induces the bilirubin-conjugating UGT1A1 protein in microsomes from rat liver (29). In rodents, clofibrate induces the glucuronidation of an antithrombotic thioxyloside (LF 4.0212), which is catalyzed by UGT1A9 in humans (30). Based on these observations, we investigated in the present study whether UGT1A9 expression is regulated by PPARα and PPARγ agonists. Our results demonstrate that UGT1A9 is a positively regulated PPARα and PPARγ target gene. This increased expression is observed in human hepatocytes, macrophages, as well as in mouse 3T3-L1 adipocytes. Induction of UGT1A9 gene expression is accompanied by an increased glucuronidation activity of catecholestrogens and fibrates. This positive regulation of UGT1A9 expression occurs at the transcriptional level by binding of PPARα and PPARγ to a DR1 response element located at –719 to –706 bp in the promoter region of the UGT1A9 gene. Furthermore, the role of PPARα in the fibrate-dependent induction of UGT1A9 is established by the absence of fibrate response in PPARα-null mice.
MATERIAL AND METHODS

Animal studies. Animal studies were performed in compliance with European Union specifications regarding the use of laboratory animals. Details of experimental conditions have been described previously (31). Briefly, male Sv/129 homozygous wild type (+/+) (n=6) and PPARα null (-/-) (n=6) mice (32) (kind gift of Dr. F. Gonzalez, National Cancer Institute, National Institutes of Health, Bethesda, USA) were fed for 17 days with standard mouse chow diet containing 0.2 % (wt/wt) fenofibrate or not. At the end of the treatment period, the animals were fasted for 4 h, sacrificed and livers were removed immediately, weighed, rinsed in 0.9% (wt/vol) NaCl, frozen in liquid nitrogen, and stored at -80°C until RNA or microsome preparation.

Cell culture. Mononuclear cells were isolated from blood of healthy donors by Ficoll gradient centrifugation and cultured as described (5,33). Mature monocyte-derived macrophages were used for experiments after 10 days of culture. For treatment with PPARα and PPARγ agonists, medium was changed to medium without serum but supplemented with 1% nutridoma HU (Roche, Mannheim, Germany). Human monocytic THP-1 cells (ATCC, Rockville, Maryland) were maintained in RPMI 1640 medium (Life-Technologies, Cergy-Pontoise, France) containing 10% fetal calf serum (FCS) and differentiated for 72h with 100 nM PMA. Differentiated cells were incubated for 24 h in the presence or absence of the indicated PPAR activators. Human primary hepatocytes were isolated as described previously (34) and incubated for the indicated times in dexamethasone-free William’s E medium containing fenofibric acid (250 µM). Mouse 3T3-L1 preadipocytes cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL) containing 10% FCS and differentiated in adipocytes according to the method of Bernlohr et al. (35). Two days post-confluent cells were switched to differentiation medium (DMEM, 10% FCS, 10 µg/ml insulin, 1 µM dexamethasone and 0.5 mM 3-methyl-1-isobutylxanthine, IBMX) for 10 days. Cells were then cultured in post-differentiation medium (DMEM, 10% FCS and insulin) with or without rosiglitazone (1 µM). Isolation and stable expression of the human UGT1A9 cDNA in human embryonic kidney (HEK) 293 cells has been previously described (21).

RNA purification and Reverse-Transcription (RT). Total RNA from cells was isolated using Trizol as specified by the supplier (Life Technologies). RNA was isolated from mouse livers
by the acid guanidium thiocyanate/chloroform method (36). Two micrograms of total RNA from cultured cells and mouse livers were reverse transcribed using random hexamer primers and 200 units of MMLV reverse transcriptase (Life Technologies).

**Real-time PCR.** Reverse transcribed UGT1A9 and 28S cDNAs were quantified by real-time PCR on a MX 4000 apparatus (Stratagene) using the previously described specific primers for UGT1A9 and 28S (37,38). Oligonucleotides for murine UGT1A9 were mUGT1A9F (sense) 5’-GAAGAACATGCATTTTGCTCCT-3’ and mUGT1A9R (antisense) 5’-CTGGGCTAAAGAGTTGCTGTCATAGTC-3’. PCR amplifications were performed in a volume of 25 µl containing 100 nM of each primer, 4 mM MgCl₂, the Brilliant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene) and SYBR Green 0.33X (Sigma-Aldrich). The conditions were 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. UGT1A9 mRNA levels were subsequently normalized to 28S mRNA. A non-parametric Mann-Whitney test was used to analyze for significant difference between the experimental groups of wild type and PPARα null mice.

**Plasmid cloning and site-directed mutagenesis.** A 1.1 kb genomic fragment containing a portion of the UGT1A9 promoter (from nucleotides +53 to –1115) was amplified by PCR, using forward 5’-CTAGCAGAAGCTTCAGTAGGTGGGAGAAATACCAGCAC-3’ and reverse 5’-CTAGCAGCTCGAGGAAGACCGTCTCTTACTGGCAAG-3’ primers into which Xho I and Hind III sites were respectively introduced. The PCR amplification was performed in a final reaction volume of 50 µl under the following conditions: denaturation at 96 °C for 3 min, 35 cycles of 15 sec at 94 °C, 40 sec at 60 °C and 15 sec at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR product was cloned into the Xho I and Hind III restriction sites of the pGL3 vector to generate the A9prom construct. Mutations were introduced in the DR1 site using the Quick Change Site Directed Mutagenesis Kit (Stratagene) and the A9promDR1_m (5’-TGACATCACCTCTGA_TCAAGGAGTGCTCAG -3’) oligonucleotide (nucleotides in bold are the mutated bases). The A9DR1-TKpGL3 and A9DR1mt-TKpGL3 plasmids were obtained by cloning three copies of the corresponding annealed oligonucleotides in the thymidine kinase promoter-driven luciferase reporter (TKpGL3) vector.
Transient transfection assays. For all assays, 40x10^3 COS cells were transfected with 100 ng of the indicated luciferase reporter plasmids, 50 ng of the pCMV-βgalactosidase expression vector, and with or without 20 ng of the pSG5-hPPARα or pSG5-hPPARγ expression vectors, as indicated. All samples were complemented with pBS-SK+ plasmid (Stratagene) to an identical amount of 500 ng/well. COS cells were transfected using the ExGen reagent (Euromedex) for 3 h at 37°C, and subsequently incubated overnight with DMEM 0.2% FCS, and then treated for 24 h with either DMSO (vehicle), Wy14643 (50µM) or rosiglitazone (1µM), as indicated.

Electrophoretic Mobility Shift Assays (EMSA). Human PPARα and PPARγ and mouse RXRα proteins were synthesized in vitro using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). Double-stranded oligonucleotides were end labeled with [γ-^32P]-ATP using T4-polynucleotide kinase. For EMSA using in vitro produced proteins, PPARα, PPARγ and/or RXR proteins were incubated for 15 min at room temperature in a total volume of 20 µl containing 2.5 µg poly(dI-dC) and 1 µg herring sperm DNA in binding buffer as previously described (39). The radiolabeled probes (A9DR1wt: 5’-GACATCACCTCTGACCTCAAGGAG-3’; A9DR1mt5’: 5’-GACATAAAAATCTGACCTCAAGGAG-3’ and A9DR1mt3’: 5’-GACATCACCTGTAAAAATCAAGGAG-3’; where underlined nucleotides represent response element half-sites and bases in bold are mutated) were added and the binding reaction was incubated for a further 15 minutes at room temperature. The protein complexes were resolved by 4% non-denaturing polyacrylamide gel electrophoresis in 0.25X Tris-Borate-EDTA (TBE) at room temperature. For supershift experiments, anti-PPARα or anti-PPARγ antibodies (0.2 µg) (Santa-Cruz) were pre-incubated for 20 minutes in the binding buffer before the addition of in vitro produced proteins as described above. For competition experiments, the indicated excess quantities of unlabeled oligonucleotides were added to the binding reaction just before the labeled probes.

Adenovirus generation. The recombinant adenovirus (Ad-GFP) and (Ad-PPARα) were obtained by homologous recombination in Escherichia Coli (40) after insertion of the cDNAs into the pAdCMV2 vector (Q.BIOgene, Illkirch, France). Viral stocks were then created as previously described (41). Viral titers were determined by plaque assay on HEK 293 cells and defined as pfu/ml. 0.8x10^6 HepG2 cells were infected at an input multiplicity (MOI) of 100 virus particles per cell, by adding virus stocks directly to the culture medium. After 3 h of
infection, cells were subsequently incubated for 24 h with either DMSO (Vehicle) or Wy14643 (50 µM).

**Microsome purification and glucuronidation assays.** The human liver microsome preparation, purchased from the Human Cell Culture Center, Inc (Laurel, MD) was from a pool of female subjects. Microsomal proteins were purified from HEK 293 cells stably expressing UGT1A9, THP1 cells treated or not with PPAR activators or from wild type or PPARα-null mouse livers as previously described (21). Enzyme assays were performed as reported (21). Briefly, microsomes were incubated with 25 µM [14C]-UDP-glucuronic acid, 2 mM unlabeled UDPGA and 200 µM of substrates in a final volume of 100 µl of glucuronidation assay buffer (21). Assays were performed at 37°C for 30 min for fibrates, 1 h for 4OH-E1 and 4OH-E2 or 4 h for glitazones, and were terminated by adding 100 µl of methanol. 100 µl of glucuronidation assays were applied onto a Thin Layer Chromatography (TLC) plate (Merck, Darmstadt, Germany) and migrated using a toluene:methanol:acetic acid (7:3:1) mix. The extent of substrate glucuronidation was analyzed and quantified by PhosphorImager analysis (Biorad).
RESULTS

UGT1A9 catalyzes glucuronidation of fibrates and troglitazone.

Recent studies indicated that UGT1A9 catalyzes glucuronidation of gemfibrozil, fenofibric acid and troglitazone, thus suggesting that this enzyme is involved in the metabolism of synthetic PPAR activators (22-25). To further characterize the role of UGT1A9 in fibrate and glitazone glucuronide conjugation, microsomes from HEK 293 cells stably expressing UGT1A9 or from human liver (as positive control) were incubated with gemfibrozil, fenofibric acid, clofibric acid, ciprofibrate, troglitazone, pioglitazone and rosiglitazone, in presence of radiolabeled cofactor UDPGA (Table 1). Whereas all fibrates were glucuronidated by microsomes from UGT1A9-HEK 293 cells and human liver, only a low level of troglitazone glucuronide formation was detected, and pioglitazone and rosiglitazone were not glucuronidated in either microsome preparation (Table 1). UGT1A9 glucuronidated all fibrates tested with a similar efficacy (between 259 and 343 pmol*min⁻¹*mg protein⁻¹ for gemfibrozil and ciprofibrate, respectively), whereas gemfibrozil was glucuronidated 2-fold more by human liver microsomes, compared to other fibrates (Table 1). Interestingly, troglitazone was glucuronidated with a 10-fold lower activity than fibrates by both UGT1A9 and human liver microsomes, while pioglitazone and rosiglitazone are not glucuronidated in human liver. These results demonstrate that UGT1A9 is able to convert fibrates and, to a lesser extent, troglitazone into their glucuronide derivatives.

PPARα and PPARγ activators induce UGT1A9 expression.

Since UGT1A9 is expressed in various tissues (21,42,43), the regulation of its gene expression by ligand-activated PPARα and PPARγ was studied in different cell types. Primary human hepatocytes, that express PPARα, were treated with fenofibric acid (250 µM) for 6, 12 and 24 h, and the level of UGT1A9 mRNA expression was determined by real time RT-PCR. An increase in the concentration of UGT1A9 mRNA was observed in fenofibrate-compared to vehicle-treated cells (Figure 1a). This effect occurred in a time-dependent manner, with a 4.2-fold increase of UGT1A9 transcript levels after 24 h of treatment.

To investigate whether PPARα induces UGT1A9 expression, human hepatoma HepG2 cells were infected with an adenovirus coding for PPARα (Figure 1b). Wy14643 treatment induced a 2-fold higher UGT1A9 expression in non-infected as well as in control Ad-GFP-infected cells. Infection with the PPARα-adenovirus produced a more than 3-fold
increase in UGT1A9 expression when compared to non-infected or Ad-GFP-infected cells, and the addition of Wy14643 resulted in 4-fold higher UGT1A9 mRNA levels (Figure 1b). These results demonstrate that PPARα over-expression positively regulates the expression of UGT1A9.

In differentiated murine adipocyte 3T3-L1 cells, that express PPARγ at high levels (44,45), rosiglitazone treatment resulted in 3-fold higher expression of UGT1A9 comparatively to vehicle-treated cells (Figure 1c).

Monocyte-derived macrophages, that express both PPARα and PPARγ (5), were treated with increasing concentrations of the PPARα agonist Wy14643 or the PPARγ activator rosiglitazone (Figure 2a). Both treatments resulted in an increased UGT1A9 expression. In monocyte-derived macrophage THP-1 cells that also express both nuclear receptors (5), treatment with Wy14643 resulted in a 2-fold induction of UGT1A9 mRNA, whereas rosiglitazone provoked a 2.4-fold accumulation of this transcript (Figure 2b). Taken together, these results demonstrate that activation of PPARα or PPARγ induces the UGT1A9 gene expression in different human and mouse cell types.

**PPARα and PPARγ activators induce UGT1A9 activity in human THP-1 cells.**

To determine whether the induction of UGT1A9 expression is accompanied by changes in its activity, THP-1 cells, in which UGT1A9 is induced both by PPARα and PPARγ activators, were treated with Wy14643 (50 µM) or rosiglitazone (1 µM) for 36 h and analyzed for their glucuronidation efficiency using 4OH-E₁, 4OH-E₂, gemfibrozil, fenofibric acid or rosiglitazone as substrates (Figure 3). Glucuronidation of rosiglitazone by microsomes purified from THP-1 cells was detected, but too low to be quantifiable (data not shown). However, treatment with Wy14643 or rosiglitazone provoked an increased glucuronidation of all other substrates tested. As such, Wy14643 induced 4OH-E₁, 4OH-E₂, gemfibrozil and fenofibric acid glucuronidation 2-, 1.7- 2.2- and 1.9-fold, respectively; whereas rosiglitazone-induced glucuronidation of these substrates by respectively 2.1-, 1.9-, 2.2- and 1.6-fold (Figure 3). These results clearly demonstrate that both PPARα and PPARγ agonists induce the glucuronidation activity of UGT1A9 in THP-1 cells.

**PPARα gene disruption abolishes fibrate-induction of murine UGT1A9.**

The effect of PPARα gene disruption on the induction of UGT1A9 mRNA and activity levels by fenofibrate was investigated in mice (Figures 4). In wild-type mice,
fenofibrate treatment provoked a 2-fold increase in UGT1A9 mRNA levels as compared to control animals (Figure 4a). By contrast, fenofibrate failed to induce UGT1A9 gene expression in PPARα-null mice. Moreover, glucuronidation assays using liver microsomes revealed that fenofibrate treatment increases the levels of 4OH-E₂, 4OH-E₃, gemfibrozil and fenofibric acid glucuronidation activity in wild type, but not in PPARα-null mice (Figure 4b-e). These results demonstrate that PPARα is required for the induction of UGT1A9 expression and activity by fenofibrate.

**PPARα and PPARγ activate the UGT1A9 gene promoter.**

To decipher the molecular mechanisms of UGT1A9 induction by PPAR activators, a 1.1 kb fragment of the human UGT1A9 gene promoter was cloned in front of the pGL3-luciferase reporter gene (Figure 5a). Cotransfection of this plasmid in COS cells in the presence or absence of mRXRα, hPPARα or hPPARγ and subsequent incubation with Wy14643 or rosiglitazone, resulted in an increased activity of the UGT1A9 promoter (Figure 5b&c). The UGT1A9 promoter contains a DR1 sequence at position −719 to −706. To test whether this site could mediate the induction by PPARα and PPARγ, mutations were introduced in the context of the 1.1 kb UGT1A9 promoter construct (Figure 5a). Mutation of this DR1 site abolished the induction of UGT1A9 promoter activity by ligand-activated PPARα and PPARγ (Figure 5b&c).

Next, the wild type and mutated DR1 sites were cloned in multiple copies upstream of the luciferase reporter gene driven by the heterologous thymidine kinase promoter (TKpGL3), and cotransfection experiments were performed (Figure 6). Luciferase activity was increased upon cotransfection of the plasmid containing 3 copies of the wild type DR1 with the PPARα or PPARγ plasmids. This activity was further enhanced by addition of their respective ligands (Figure 6b). In contrast, no change in activity was observed when either the empty TKpGL3 vector or the TKpGL3 vector containing 3 copies of the mutated DR1 were transfected. Taken together, these data indicate that the UGT1A9 promoter DR1 is a positive cis-acting element by which ligand-activated PPARα and PPARγ induce human UGT1A9 promoter activity.

**PPARα and PPARγ bind the PPRE within the UGT1A9 gene promoter.**

To determine whether human PPARα or PPARγ bind the PPRE in the UGT1A9 promoter, electromobility shift assays (EMSA) were performed using this response element as...
radiolabeled probe (Figure 7). As expected, in the absence of RXR, neither PPARα nor PPARγ bound the probe (Figure 7a & b, lane 2). In the presence of RXR both PPARα and PPARγ bound the DR1 site (Figure 7a & b, lane 4). Furthermore, these complexes were supershifted by anti-PPARα or anti-PPARγ antibodies (lane 5). While both PPARα/RXR and PPARγ/RXR heterodimers bound to the wild type probe, no protein-DNA complex was observed when using the mutated A9DR1mt5' and A9DR1mt3' probes (Figure 7a & b, lanes 9 & 13). For competition experiments, increasing amounts (1, 10, 50 and 100-fold excess) of unlabeled oligonucleotides encompassing either a consensus DR1 site (DR1cons.) or the A9DR1wt, A9DR1mt5' or A9DR1mt3' sites were added to binding reactions containing either PPARα or PPARγ in the presence of RXR (Figure 7c & d). Both PPARα and PPARγ binding on the A9DR1wt was strongly competed by the DR1 consensus site, and to a lower extent by the UGT1A9 DR1 itself (Figure 7c & d). By contrast, the mutated A9DR1 did not efficiently compete for PPARα or PPARγ binding to the DR1. Taken together, these data demonstrate that PPARα and PPARγ bind to the UGT1A9 PPRE site at position -719 to -706.
DISCUSSION

In the present study, we identify UGT1A9 as a novel PPARα and PPARγ target gene. The induction of UGT1A9 mRNA by PPARα or PPARγ agonists was observed in human hepatocytes and macrophages, human THP-1 and HepG2 cell lines and murine 3T3-L1 adipocytes. The identification of a PPRE in the UGT1A9 gene provides the first evidence for a direct regulation of a human UGT enzyme by PPARs.

As previously reported (22-25), UGT1A9 catalyzes the glucuronide conjugation of gemfibrozil, fenofibric acid and troglitazone; however the present study also evidences the role of this enzyme in glucuronidation of ciprofibric and clofibric acids. In contrast glucuronidation of rosiglitazone and pioglitazone was not detected with both microsomal preparations from UGT1A9 expressing HEK 293 cells and human liver. This absence of glucuronidation is consistent with previous observations indicating that only traces of pioglitazone and rosiglitazone glucuronides are detected in human urine, whereas their major metabolites correspond to sulfate conjugates (46,47). The present study, consistent with previous reports (24), indicates that gemfibrozil is glucuronidated with a 2- to 3-fold higher activity than other fibrates by human liver microsomes. This observation may explain, at least partly, the very short plasma half life of this compound when compared to other fibrates (48). However, UGT1A9 glucuronidates all fibrates with similar rates, and this difference of plasma half life may involve other UGT enzymes, such as UGT1A1, UGT2B7 or UGT2B15 which also conjugate both gemfibrozil and fenofibrate (24).

UGT1A9 catalyzes glucuronidation of many drugs and exogenous compounds. Thus UGT1A9 induction by PPARα and PPARγ agonists may stimulate the catabolism of these substances. Drug or xenobiotic metabolizing enzymes play central roles in the biotransformation, metabolism and/or detoxification of foreign compounds that are introduced in the human body. Glucuronide conjugates are among the major types of naturally occurring metabolites of xenobiotics (49). Whereas the nuclear receptors pregnane-X receptor (PXR) and constitutive androstane receptor (CAR) play essential roles in the control of drug metabolizing enzyme expression, PPARs are also important xenobiotic sensors, at least in rodents (50-52). Thus, PPARα regulates, in rats, the expression of various cytochrome P450s (CYP) such as CYP1A2, 2A1, 2B1, 2B2 and 3A4, as well as conjugating enzymes such as
glutathione S-transferase, GSTA1 and GSTM2 (50). Furthermore, in mice PPARα positively regulates the expression of mdr2 (or P-glycoprotein, Pgp, abcc2), a major transporter of glucuronide derivatives localized in the canalicular membrane of hepatocytes (53,54). These data indicate that PPARs are involved in the control of cellular metabolic processes, and the identification of UGT1A9 as a PPAR target gene demonstrate a direct role of these nuclear receptors as regulators of the metabolism of endogenous and exogenous compounds in humans.

Since UGT1A9 catalyzes the glucuronidation of fibrates and troglitazone, its induction by PPARs suggests that synthetic agonists of nuclear receptors may regulate their own metabolism through up-regulation of UGT enzymes. Interestingly, this enzyme also glucuronidates polycyclic aromatic hydrocarbons (PAHs), such as 6-hydroxychrysene or 4-methylumbelliferone (55), and PAHs induce UGT1A9 and UGT1A6 expression in Caco2 cells (55). Whereas the molecular mechanisms of this transcriptional regulation of PAH-inducible UGTs have not been clearly established, this may occur through the aryl hydrocarbon receptor (55). Very recent observations indicate that UGT1A9 also catalyzes the glucuronidation of arachidonic and linoleic acid metabolites, such as 5-HETE, 12-HETE, 15-HETE or 13-HODE (Bélanger et al. personal communication). Some of these compounds, such as 15-HETE and 13-HODE, are endogenous PPARα and PPARγ ligands (7). This suggests that not only synthetic, but also natural activators of PPARs may control the expression of enzymes involved in their inactivation pathways. Identification of UGTs as target genes of receptors activated by their substrates suggests that drug-conjugating enzymes may control intracellular levels of receptor ligands. Furthermore, metabolites of the cyclooxygenase and lipoxygenase pathways, such as 12-, 15-HETE and 13-HODE are highly synthesized in smooth muscle cells and/or endothelial cells of the atherosclerotic plaque (56-58). The release of these pro-inflammatory molecules stimulates the inflammatory response and activates monocyte adhesion and migration in the vascular sub-endothelium (56-59). PPARα and PPARγ are highly expressed in cells of the arterial wall, where they control the expression of numerous genes involved in the inflammatory response, macrophage differentiation, apoptosis and cholesterol efflux (10). Thus the PPAR-dependent induction of HETEs and 13-HODE glucuronidation catalyzed by UGT1A9 in macrophages may potentially contribute to the anti-inflammatory effect of PPARα and PPARγ ligands.
Increased UGT1A9 expression is accompanied by an enhanced catecholestrogen glucuronidation activity in PPAR agonist-treated THP-1 cells. Catecholestrogens such as 4OH-E1 and 4OH-E2 are formed from estrogen through a hydroxylation process catalyzed by CYP1A1, CYP1A2 and CYP1B1 (60), and are major endogenous substrates for UGT1A9 (21). The glucuronidation of catecholestrogens is a potentially important catabolic pathway for the elimination of these genotoxic steroid metabolites from a given tissue, and the prevention of cell damages they can induce (61,62). Indeed, the hydroperoxide-dependent oxidation of catecholestrogens to quinones and the NADPH-dependent reduction of the quinones back to hydroquinones, yield semiquinone free radical intermediates and superoxide radicals (63). The generation of free radicals has been postulated to mediate DNA damage leading to tumor development (61-63). Thus by enhancing catecholestrogen glucuronidation catalyzed by UGT1A9, PPAR activators may contribute to protect cells against the potential carcinogenic formation of free radicals.

UGT1A9 expression and glucuronidation activity are induced following PPARγ activator treatment of various cell types, including mouse adipocyte 3T3-L1 cells. Interestingly, there are accumulating evidences that PPARγ ligands inhibit estrogen biosynthesis in breast adipose tissue (64). Local estrogen biosynthesis is catalyzed by the P450 aromatase (CYP19), and recent data established that ligands for PPARγ and RXR inhibit aromatase expression in human breast adipose tissue (65). While this inhibition was shown to be independent of DNA-binding, it is believed that PPARγ agonists may have useful therapeutic benefits in the management of breast cancer by reducing local estrogen biosynthesis. The data presented here demonstrate that PPARγ not only decreases estrogen biosynthesis, but could also locally induce their catabolism to catecholestrogen glucurononides. Thus, the UGT1A9-induced expression may be part of a complex process by which PPARγ activators decrease estrogen levels in adipose tissue.

In conclusion, the present study demonstrates that PPARα and PPARγ activators enhance UGT1A9 expression and activity. However, whereas all clinically used fibrates are glucuronidated, the only glitazone conjugated by this enzyme (ie, troglitazone) is no longer used in clinic (66), and pioglitazone and rosiglitazone are not glucuronidated. These observations suggest that treatment with agonists of both receptors may affect the transcriptional activity of PPARα while PPARγ-dependent pathways may not be affected. In
addition, molecules which activate both PPAR\(\alpha\) and PPAR\(\gamma\) are actually under development and examination of their glucuronidation by UGT1A9 may be useful to understand their pharmacokinetic properties which may influence their hypolipidemic and hypoglycemic activities.
ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. PPARα induces human UGT1A9 mRNA in hepatocytes and HepG2 cells, and PPARγ stimulates murine UGT1A9 expression in 3T3-L1 adipocytes.
(a) Primary human hepatocytes were treated with DMSO (vehicle) or fenofibric acid (250 µM) for 6, 12 or 24 h.
(b) HepG2 cells were infected with Ad-PPARα or Ad-GFP for 3 h and subsequently stimulated for 24 h with vehicle or Wy14643 (50 µM).
(c) 3T3-L1 adipocytes were treated or not with rosiglitazone (1µM) for 24 h. Human and murine UGT1A9 RNA levels were measured by real-time RT-PCR. Values are expressed as means ± SD (n=3), relative to the control set as 1.

Figure 2. PPARα and PPARγ activators increase UGT1A9 mRNA in human primary and THP-1 macrophages.
(a) Primary human macrophages were treated with Wy14643 (25, 50 or 75 µM) or rosiglitazone (0.5, 1 or 2 µM) for 24 h.
(b) Differentiated THP-1 macrophages were treated for 24 h with DMSO (Vehicle), Wy14643 (50 µM) or rosiglitazone (1µM). UGT1A9 mRNA levels were measured by real-time RT-PCR, and expressed relative to control set as 1. Values are means ± SD (n=3).

Figure 3. PPARα and PPARγ activators increase glucuronidation activity in THP-1 macrophages.
Microsomes purified from PPAR agonist- or vehicle-treated THP-1 macrophages were incubated with [14C]UDPGA and 4-hydroxyestradiol, 4-hydroxyestrone, gemfibrozil or fenofibric acid (200 µM) as indicated in materials and methods. Radiolabeled glucuronide derivatives were subsequently analyzed by thin layer chromatography and quantified by PhosphorImager analysis. Values represent the means ± SD (n=3).

Figure 4. PPARα is required for the induction of UGT1A9 gene expression by fibrates.
Wild type (+/+) and PPARα null (-/-) mice were treated with 0.2% (wt/wt) fenofibrate mixed in chow diet (■) or chow diet (□) for 17 days (a). Total RNA was extracted from livers and
analyzed for mUGT1A9 expression by real-time RT-PCR. Values are expressed as means ± SD (n=6), relative to the control set as 1. Statistically significant differences between untreated- and fenofibrate-treated mice are indicated by asterisks (Mann-Whitney test: ***, p < 0.001).

(b-e) Microsomes were incubated with radiolabeled UDPGA (25 µM), unlabeled UDPGA (2 mM) and 4-hydroxyestradiol (b), 4-hydroxyestrone (c), gemfibrozil (d) or fenofibric acid (e) (200 µM) as indicated in materials and methods. Radiolabeled glucuronide derivatives were subsequently analyzed by thin layer chromatography and quantified by PhosphorImager analyses. Values represent means ± SD (n=3).

Figure 5. PPARα and PPARγ induce UGT1A9 promoter activity via a PPRE located between –719 and -706 bp.
(a) Schematic representation of the human UGT1A9 gene promoter: 1.1 kb of the UGT1A9 promoter was cloned in front of the luciferase reporter gene, the potential PPAR response element was mutated by site-directed mutagenesis. Mutated bases are underlined, whereas arrows represent response element half-sites.
(b&c) COS cells were transfected with the indicated human UGT1A9 promoter-driven luciferase (Luc) reporter plasmids (100 ng) in the absence or presence of pSG5-mRXRα, pSG5-hPPARα (b) or pSG5-hPPARγ2 (c) (20 ng) and a CMV-driven β-galactosidase expression plasmid (pCMV-β-gal, 50 ng). Cells were subsequently treated or not with Wy14643 (50 µM) or rosiglitazone (1 µM) for 24 h. Values are expressed as fold-induction of the controls (pGL3) set at 1, normalized to internal β-galactosidase activity as described in materials and methods. Values represent the means ± SD.

Figure 6. PPARα and PPARγ activate the DR1-719 response element.
(a) Three copies of the wild type or mutated PPREs were cloned upstream of the thymidine kinase (TK) minimal promoter-driven luciferase reporter (TKpGL3).
(b&c) COS cells were transfected with the indicated plasmids (100 ng), pCMV-β-gal (50 ng) and in the absence or presence of pSG5-mRXRα, pSG5-hPPARα (b) or pSG5-hPPARγ2 (c) (20 ng). Cells were subsequently treated or not with Wy14643 (50 µM) or rosiglitazone (1 µM) for 24 h.
Values are expressed as fold-induction of the controls (pGL3) set at 1, normalized to internal β-galactosidase activity as described in materials and methods. Values represent the means ± SD.

**Figure 7. PPARα and PPARγ bind to the PPRE in the UGT1A9 promoter.**

(a & b) Electrophoretic Mobility Shift Assays (EMSA) were performed with end-labeled wild type or mutated A9DR1 probes in the presence of unprogrammed reticulocyte lysate, RXR, PPARα (a), PPARγ (b) both RXR and PPARα or PPARγ as indicated. Supershift experiments were carried out using anti-PPARα or anti-PPARγ antibodies (0.2 µg).

(c & d) Competition EMSA on radiolabeled A9DR1 probe were performed by adding 1-, 10-, 50- or 100-fold molar excess of the indicated cold consensus DR1 (DR1 cons.), A9DR1wt, A9DR1mt5' or A9DR1mt3' oligonucleotides in EMSA with unprogrammed reticulocyte lysate, RXR and PPARα or PPARγ.
Table 1

*In vitro* glucuronidation of fibrates and glitazones by microsomes isolated from human liver and HEK 293 cells expressing UGT1A9. Results represent the values obtained in two independent experiments.

N.D.: Glucuronide Not Detected

<table>
<thead>
<tr>
<th></th>
<th>Glucuronide formation (pmol<em>min⁻¹</em>mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UGT1A9</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>292 - 259</td>
</tr>
<tr>
<td>Fenofibric acid</td>
<td>301 - 264</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>310 - 310</td>
</tr>
<tr>
<td>Ciprofibric acid</td>
<td>343 - 322</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>30 - 25</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pioglitazone</td>
<td>N.D.</td>
</tr>
</tbody>
</table>
Figure 1
**Figure 2**

(a) UGT1A9 mRNA (Fold Induction) with Wy14643 (µM) and Rosiglitazone (µM) concentrations: 0, 25, 50, 75, 0.5, 1, 2.

(b) UGT1A9 mRNA (Fold Induction) with Vehicle, Wy14643, and Rosiglitazone.
Figure 4

a) mUGT1A9 mRNA (Fold Induction) with significant induction (***).

b) Glucuronide formation of 4-Hydroxyestradiol (pmol min⁻¹ mg protein⁻¹).

c) Glucuronide formation of 4-Hydroxyestrone (pmol min⁻¹ mg protein⁻¹).

d) Glucuronide formation of Gemfibrozil (pmol min⁻¹ mg protein⁻¹).

e) Glucuronide formation of Fenofibric acid (pmol min⁻¹ mg protein⁻¹).
Figure 5

Relative Luciferase Activity (Fold Induction)

(b) Comparison of luciferase activity with different promoters and receptors.

(c) Further analysis showing the effect of Rosiglitazone on luciferase activity.
Figure 6

Relative Luciferase Activity (Fold Induction)

TKpGL3  A9DR1  A9DR1<sub>mt</sub>  RXR<sub>α</sub>  PPAR<sub>α</sub>  Wy14643

TKpGL3  +  +  +  +  +  +  +  +  +  +
A9DR1  -  -  -  +  +  +  +  +  +  +
A9DR1<sub>mt</sub>  -  -  -  +  +  +  +  +  +  +
RXR<sub>α</sub>  -  -  -  +  +  +  +  +  +  +
PPAR<sub>α</sub>  -  -  -  +  +  +  +  +  +  +
Wy14643  -  -  -  +  +  +  +  +  +  +

TKpGL3  +  +  +  +  +  +  +  +  +  +
A9DR1  -  -  -  +  +  +  +  +  +  +
A9DR1<sub>mt</sub>  -  -  -  +  +  +  +  +  +  +
RXR<sub>α</sub>  -  -  -  +  +  +  +  +  +  +
PPAR<sub>α</sub>  -  -  -  +  +  +  +  +  +  +
Rosiglitazone  -  -  -  -  -  -  -  -  -  -
Figure 7
The UDP-glucuronosyltransferase 1A9 enzyme is a peroxisome proliferator-activated receptor alpha and gamma target gene

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