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RXR activators molecular signalling: involvement of a PPARα-dependent pathway in the liver and kidney, evidence for an alternative pathway in the heart

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1 In this study we compared the molecular signalling elicited by rexinoids, selective retinoid X receptor (RXR)-activators, in several organs (i.e. liver, kidney, heart) and in hepatocytes of various species.

2 RXR plays the pivotal role of a hetero-dimerization partner for the members of the class II subset of nuclear receptors which regulate the transcription of numerous target genes, following chemical activation. Several of these selective activators are currently used to treat hyperlipidaemia (fibrates), type II diabetes (glitazones), or skin disorders (retinoic acid). Although these therapeutic pathways are not fully elucidated, receptor activation is considered a pre-requisite for efficacy. Therefore RXR, which accepts numerous dimeric partners, is considered a worthwhile pharmacological target.

3 We analysed a number of biochemical and molecular responses to rexinoids which were given orally to mice. Our results showed a prominent involvement of the peroxisome proliferator-activated receptor (PPARα) as a majority of the observed hepatic and renal regulations were abolished in PPARα-knockout animals. Therefore we documented the species-specificity of these rexinoid actions which were reproduced in rat primary hepatocyte cultures but not in cultures of rabbit or human origin. Conversely, we established that the regulation of the pyruvate dehydrogenase kinase (PDK4) gene in the heart, by rexinoids, is independent of PPARα expression.

4 Our results support the obligatory expression of the active, although quiescent, PPARα to sustain a subset of relevant regulations attributable to rexinoids in the liver and kidney. Their cardiac molecular signalling unveiled an alternate transduction pathway and therefore opens new prospects in the therapeutic potential of rexinoids.

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Keywords: Nuclear receptor; rexinoid; retinoid; RXR; PPAR; fibrate; liver; kidney; heart; pyruvate dehydrogenase kinase

Abbreviations: CYP4A, cytochrome P450 4A; DMSO, dimethylsulphoxide; PDK4, pyruvate dehydrogenase kinase 4; PPAR, peroxisome proliferator-activated receptor; PP, peroxisome proliferators; RAR, retinoic acid receptor; RXR, retinoic X receptor; TG, triglycerides; TR, thyroid hormone receptor

Introduction

Retinoids represent a pharmacological class of natural and synthetic Vitamin A derivatives. They are used in clinical practice to treat dermatological disorders such as acne, photodamage, psoriasis, and various skin cancers (Boehm *et al*., 1995). Reported side effects include mucocutaneous toxicity, hypertriglyceridaemia and teratogenesis (Thacher *et al*., 2000). Since no substitutes are available displaying equivalent benefits with less adverse effects, research efforts have been focused on obtaining new active compounds of the same class that present a better therapeutic index (Nagpal & Chandraratna, 2000).

Retinoids modulate numerous fundamental physiological processes such as cellular differentiation, proliferation and apoptosis. Biological effects of retinoids are mediated *via* two distinct families of nuclear receptors, which belong to the class II of the nuclear receptor family (Mangelsdorf *et al*., 1995): RAR (retinoic acid receptor) and RXR (retinoid X receptor). Both receptors display three subtypes (α, β, γ) encoded by distinct genes. RXRα abrogation in murine hepatocytes results in a phenotype of impaired metabolic pathways (cholesterol, fatty acids, bile acids, steroids, xenobiotic metabolisms) (Wan *et al*., 2000) coupled with an altered regeneration capability of the liver parenchyma (Imai *et al*., 2001).

The members of this class of nuclear receptor modulate gene expression by acting as ligand-dependent transcription factors. Upon binding and/or activation by specific activators, they bind as receptor dimers to DNA gene regulatory sequences and subsequently modulate the transcription of an array of target genes. Remarkably, RXR represents the dimerization partner for several class II receptors. Therefore, RXR plays a key role in nuclear signalling pathways involving its dimeric partners (e.g. RAR, the peroxisome proliferator-activated receptors (PPAR), the thyroid hormone receptor (TR), the Vitamin D receptor, the pregnane X receptor, the liver X receptor and the constitutive androstane receptor. RXR is consequently regarded as a master regulator

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of numerous biological pathways, especially in the liver where many of its partners are expressed and are active.

A RXR-selective agonist, commonly named rexinoid, is being used to treat cutaneous T cell lymphoma (Hurst, 2000; Talpur *et al*., 2002). Additionally, it has been reported that administration to humans of RXR selective agonists generates hypertriglyceridaemia (Rizvi *et al*., 1999). This observation in animal models remains controversial since an increase (Davies *et al*., 2001; Claudel *et al*., 2001), or a decrease in triglyceride levels (Mukherjee *et al*., 1998; Lenhard *et al*., 1999) or an absence of effect (Standeven *et al*., 1996; 1997) has been reported.

Because of the central position of RXR in nuclear signalling, this investigation was based on the assumption that xenobiotic-activation of the receptor could result in the recruitment of quiescent dimeric partner(s), in the absence of its (their) specific activators, which would lead to observable pharmacological effects. Furthermore, in light of the modulation of peroxisome proliferator-target genes by rexinoids (Mukherjee *et al*., 1998), a phenomenon referred to as the permissiveness of the dimer, special emphasis was given in this study to the characterization of a putative interaction between the signalling pathways of the rexinoids and of the peroxisome proliferators (PP). The liver represents the most relevant organ in this perspective as it is the first and main target of xenobiotics following oral administration and since PP signalling is well documented due to a marked PPARα expression in hepatic parenchyma. Diverse, structurally unrelated, PP molecules activate this receptor isoform including phthalate plasticizers and fatty acids. Of particular interest was the widely prescribed fibrate class of lipid-lowering drugs. Following long-term administration to rodents, these molecules elicit a pleiotropic response including hepatomegaly, peroxisome proliferation and hepatocarcinoma. The non-genotoxic hepatocarcinogenicity of PP was shown to be restricted to rodents, as long as they display a functional PPARα signalling pathway in the organ (Peters *et al*., 1998). Subsequently, fibrates, like the fenofibrate, have been safely and beneficially prescribed to humans for decades to normalize cholesterol and triglyceride circulating levels while no sign of hepatic adverse effects have been substantiated (Gariot *et al*., 1983; 1987).

Besides possible applications in dermatology, we wanted to document the potential of reference rexinoids administered orally. Indeed, the involvement of RXR in various signal transduction pathways identifies it as a relevant pharmacological molecular target for putative therapeutic interventions on these pathways e.g., lipid or glucose metabolism or actions of hormones, vitamins and sterols. For rexinoids, sustaining a molecular signal could be theoretically achieved by RXR *per se* (RXR active dimers) or by recruiting quiescent heterodimeric partners. We report that there was cross-talk, activated by rexinoids, between RXR and PPARα-dependent signalling pathways in the liver and kidney, *in vivo*. This signalling pathway was not exclusive since at least one biochemical change associated with rexinoid exposure was resistant to deletion of the PPARα. Remarkably, experiments conducted on the heart, a non-prototypical PP target organ, have led to the novel conclusion that, in this environment, rexinoids could regulate a prototypical PPARα downstream target gene in a PPARα-independent manner, thus showing the capability of rexinoids to recruit alternate, organ-specific, signalling pathways. While extending our knowledge regarding the potency range of rexinoids, these observations will help in re-focussing the assessment of their therapeutic potential and safety.

Methods

Animals

C57 BL/6J 8-week-old (except for Figure 6, 32-week-old) male mice were obtained from Charles River (Les Oncins, France). Age-matched PPARα-deficient males on a C57 BL/6J genetic background (Lee *et al*., 1995; Costet *et al*., 1998) were bred at the INRA transgenic animal facility according to European Union guidelines for animal care. Male Sprague-Dawley rats (weighing 200–250 g) were purchased from Charles River (Les Oncins, France). Male white New-Zealand rabbits (weighing 1.8–2 kg) were obtained from the INRA rabbit breeding facility (Station de Recherche Cunicole, Toulouse, France).

Experimental procedure

In vivo studies were conducted under the E.U. guidelines for the use and care of laboratory animals and protocols were approved by an internal ethical committee. Animals were assigned to treatment groups following randomization according to their bodyweight. Oral administration to mice (100 mg kg^{-1}) was performed by gavage once daily for eight consecutive days at 0900 h. Animals were fed *ad libitum* with free access to water during the course of the experiment. Prior to euthanasia by cervical dislocation, mice were fasted for 2 h. Tissues were dissected, weighed, snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until RNA extraction.

Lipids and lipoprotein measurements

Following anaesthesia with isoflurane (Centravet, Toulouse, France) in a rodent anaesthesia chamber (T.E.M., Angers, France), blood was collected from the retro-orbital sinus in coagulation accelerating tubes (Capiject®, CML, Nemours, France). Samples were allowed to clot for approximately 2 h, and then centrifuged at $4^{\circ}C$ to separate serum. Serum samples were assayed for triglycerides (TG) using an enzymatic endpoint assay and for apolipoprotein AI and B using immunoturbidimetric assays. Serum cholesterol and TG were measured in the Hematochemical Department of Aventis Crop Sciences, France (automated dosages).

Primary cultures of rat and rabbit hepatocytes

Rat hepatocytes were isolated by collagenase perfusion according to Seglen (1976) with slight modifications (Gomez-Lechon *et al*., 1984). Cell suspensions, containing more than 80% trypan blue-excluding cells, were seeded into 100 mm diameter collagen coated dishes (density of 9×10^6 live cells per dish in 6 ml nutrient medium). Cells were maintained in Williams' E medium supplemented with foetal calf serum (5%), insulin (5 μg ml^{-1}), dexamethasone (10 μM), and antibiotics. Six hours after seeding, the culture medium was changed to remove foetal calf serum. Twenty-four hours after plating, the culture medium was renewed in the presence

of the test compounds which were dissolved in DMSO ($n=3$ for each experimental condition). The final DMSO concentration in the medium was 0.1% (v v^{-1}). In the control condition, the medium was supplemented with DMSO alone. Cultures were stopped 24 h post-treatment and the cells were collected by scraping them in TRIzol reagent prior to RNA analysis.

Rabbit hepatocytes were isolated and cultured as described by Daujat *et al*. (1987). Except for the perfusion method, cell culture and treatments were similar to those described for rat hepatocytes.

Cultures of human hepatocytes

Fresh human liver tissue was obtained from surgical procedures from consenting donors undergoing partial hepatectomy, in general compliance with ethical committee approved protocols. Cryopreservation, cell culture and total RNA extractions from human hepatocytes were conducted at the laboratory of Pharmaco-Toxicologie Cellulaire et Moleculaire (INRA, Antibes, France). Treatments lasted for 24 h with control plates receiving DMSO alone.

RNA analysis

Total RNA was isolated from frozen tissues using TRIzol reagent according to the manufacturer's instructions and was precipitated in isopropanol. Twenty micrograms of total RNA per lane were loaded and analysed by electrophoresis on 1.2% agarose denaturing gels. Following capillary transfer on nylon membranes (Genescreen plus, NEN Life Science, Boston MA, U.S.A.) hybridization was performed with ^{32}P-radiolabelled cDNA probes. After washing, the membranes were exposed to a phosphor screen and analysed using the Storm phosphorimager (Molecular Dynamics, Bondoufle, France) and ImageQuant® analysis software. Dot blot analysis was performed using a microfiltration apparatus (BioRad, Marnes-la-Coquette, France) with five micrograms of total RNA per dot. Hybridization and image analysis were conducted as described above. Ratio values of transcript levels (gene of interest/β-actin) were used to establish histogram representations of Northern blot analyses.

Materials

Test compounds, RXR selective agonists LGD1069 and LG100268, were synthesized and characterized by the Chemistry Department at Galderma R&D. Fenofibrate was purchased from SIGMA-ALDRICH Chemicals, France. Suspensions in a 0.5% (w v^{-1}) carboxy-methyl-cellulose solution were prepared in purified water only once and used for the whole course of the study. Collagenase was from Roche, Meylan, France and TRIzol reagent from Invitrogen, Cergy Pontoise, France. Assays for triglycerides and for apolipoproteins AI and B were obtained from Sigma, Saint-Quentin-Fallvier, France (assay nos. 336, 356, 357).

Statistical analysis

Results are presented as mean ± s.e.mean. Statistical significance of differences among experimental groups were assessed by analysis of variance (ANOVA). Where there were differences among the groups, these were subjected to Tukey multiple comparison test using a proprietary statistical software package (S+, Insightfull, Paris, France). The *P*-value for statistical significance was set at 0.05.

Results

In order to compare our investigations to relevant data sources, we selected RXR-specific and PPARα-specific ligands. LGD1069 and LG100268 are known RXR(α,β,γ) selective agonists (Lansiaux *et al*., 2000) which have been extensively investigated since their first documented studies in 1994 and 1995. Fenofibrate was selected as a prototypical member of the fibrate subclass of PP. Chemical structures of these compounds are presented in Figure 1.

After treatment of wild type C57BL/6J adult male mice with LGD1069, a RXR-selective activator, we analysed two hepatic parameters reported to be specifically modulated following rodent exposure to PP. The first, of pathophysiological relevance, is that of liver enlargement associated with hepatocyte peroxisomal proliferation. The second parameter, of transcriptional relevance, is the increased abundance of liver transcripts encoding the CYP4A microsomal enzyme. As shown in Figure 2A, LGD1069 treatment resulted in an approximate 20% increase in liver weight, while a comparable dose of fenofibrate produced a 65% increase. Northern blot analysis of the CYP4A mRNA levels indicated a 6.8 and 19.5 fold induction in LGD1069 and fenofibrate-treated groups, respectively, compared to vehicle treated-mice (Figure 2B). These hepatic effects of PP are known to be reversible. Therefore, we submitted identically-treated groups to a 7-day withdrawal period. As in fibrate-treated mice, both parameters decreased to control values in LGD1069-treated mice (Figure 2C,D). Tissue samples were submitted to histological examination. No major histopathological modifications (necrosis, steatosis, fibrosis) were noted. Examination of liver sections revealed a mild, reversible, hepatocyte hyperplasia and hypertrophy in both treated groups (data not shown).

PP molecular signalling has been extensively documented and is prevalently observed in rodents (Roberts *et al*., 2000). It is consistently reproduced in liver samples and in primary hepatocyte cultures within a given species (Elcombe & Mitchell, 1986). We have compared the abundance of various

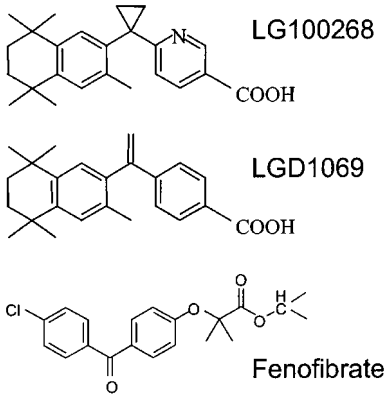

Figure 1 Chemical structures of tested compounds.

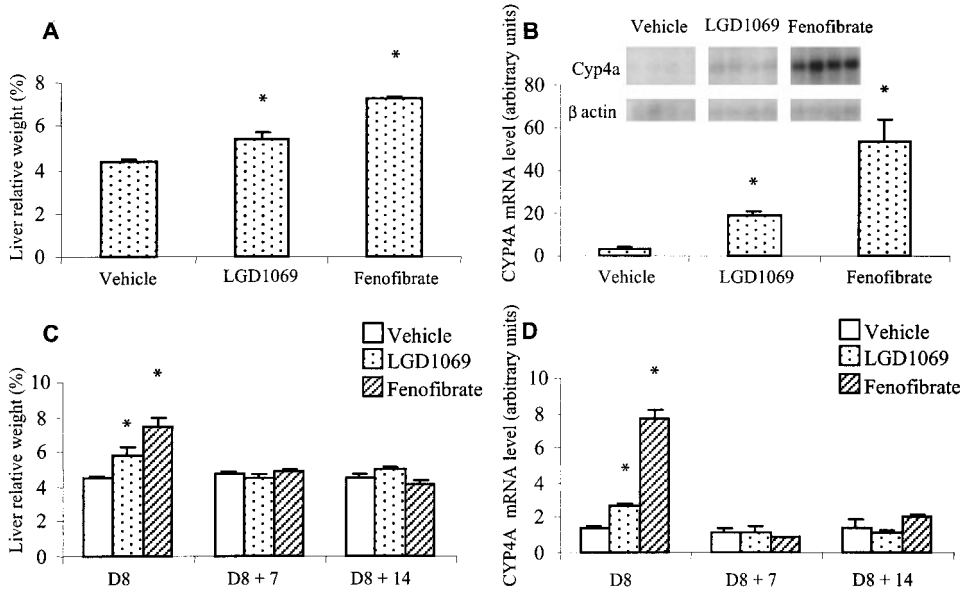

Figure 2 Hepatic effects induced by LGD1069 or fenofibrate. (A) Hepatomegaly following treatment with LGD1069 (100 mg kg^{-1} day^{-1}) or fenofibrate (100 mg kg^{-1} day^{-1}) in C57 BL/6J mice for 8 days. Values are ratios (liver weight/body weight) $\times 100, \pm$s.e.mean ($n=5$). (B) CYP4A transcript levels in the liver of control, LGD1069 and fenofibrate-treated mice. Total RNA (20 μg) was separated by electrophoresis and hybridized with a CYP4A cDNA probe. Densitometric analysis was performed and represented as mean $\pm$ s.e.mean ($n=4$ per group). Absolute values were normalized by the β actin transcript level. (C, D). Time-course of the regression of hepatic effects following 7 and 14 days of withdrawal of treatment. Mice were initially treated for 8 days with LGD1069 or fenofibrate (100 mg kg^{-1} day^{-1}). Values are mean $\pm$ s.e.mean ($n=5$). (C) Hepatomegaly reversibility. (D) Reversibility of CYP4A transcript induction. Asterisks (*) indicate that the observed value for the treated group is statistically different from the value obtained from the group receiving the vehicle alone.

relevant transcripts in hepatocyte cultures of rat, rabbit or human origin exposed to rexinoids (LGD1069, LG100268) or to a fibrate compound, active *in vitro*, fenofibric acid. Transcripts for CYP4A, strongly regulated by PPARα and those for thiolase, which are weakly regulated by PPARα, were analysed in rat and rabbit hepatocyte primary cultures treated for 24 h (Figure 3A). In this experiment, agonists were used at the single dose of 100 μM. As previously reported fenofibric acid markedly increased CYP4A transcripts and less markedly increased thiolase transcripts, but only in rat hepatocytes. LGD1069 significantly increased CYP4A transcripts in only rat cultures. Regarding CYP4A, messenger induction ratios were 27.3 and 7.5 for fenofibric acid and LGD1069 respectively. No regulation of thiolase transcripts by rexinoids was noted. No changes in transcript levels were observed in rabbit hepatocytes for all treatments.

To test a different range of concentrations, chemicals were used at 10 μM in experiments to compare rat to human hepatocytes. The pyruvate dehydrogenase kinase 4 (PDK4) messenger RNA levels are shown in Figure 3B. This transcript has been previously shown to be regulated by PP (Sugden *et al*., 2001a) and to display a PPARα-dependent induction by fasting (Wu *et al*., 2001; Sugden *et al*., 2001b). Fenofibric acid and LGD 1069 substantially increased the abundance of PDK4 transcripts in rat hepatocytes while no statistically significant effect was noted in cultures of human origin even when exposed to 100 μM fenofibric acid.

This first set of experiments was entirely compatible with PPARα-mediated biochemical and molecular actions of LGD1069 both *in vivo* and *in vitro*. To formally establish these putative rexinoid pathways, we compared LGD1069 and fenofibrate administration in wild type and PPARα-deficient mice. Figure 4A shows that fenofibrate-induced hepatomegaly in wild type mice was not reproduced in PPARα-deficient mice. Conversely, LGD1069 exposure resulted in equivalent enlargements of liver in both genotypes.

In wild type mice, PPARα-upregulated genes, cytochrome P450 4A, acyl CoA oxidase and thiolase, were induced by LGD1069 and fenofibrate treatments (Figure 4B). In PPARα-deficient mice, these effects were abolished for both treatments with the exception of a small increase in thiolase transcripts, after LGD1069, in knockout animals, a result previously reported following clofibrate and Wy14,643 treatments (Lee *et al*., 1995). A recently documented negative transcriptional regulation was additionally studied. Transcripts for serine protease inhibitor (Anderson *et al*., 1999) were substantially decreased following LGD1069 and fenofibrate treatment in wild type mice liver. These observations were not reproduced in PPARα-null mice (Figure 4C).

It has been extensively documented that fenofibrate is a potent TG-lowering agent, but the action of rexinoids on circulating TG is not completely clear. Numerous conflicting data have been collated (Davies *et al*., 2001). Figure 5A presents serum cholesterol and TG levels following LGD1069 and fenofibrate treatment of wild type mice with identical doses. The marked TG-lowering effect of fenofibrate was not reproduced by LGD1069. None of the tested treatments affected serum cholesterol levels. Considering that fibrate hypolipidaemic action is mediated *via* PPARα, we studied rexinoid effects on serum cholesterol and triglyceride levels in the PPARα-knockout model. As previously reported the

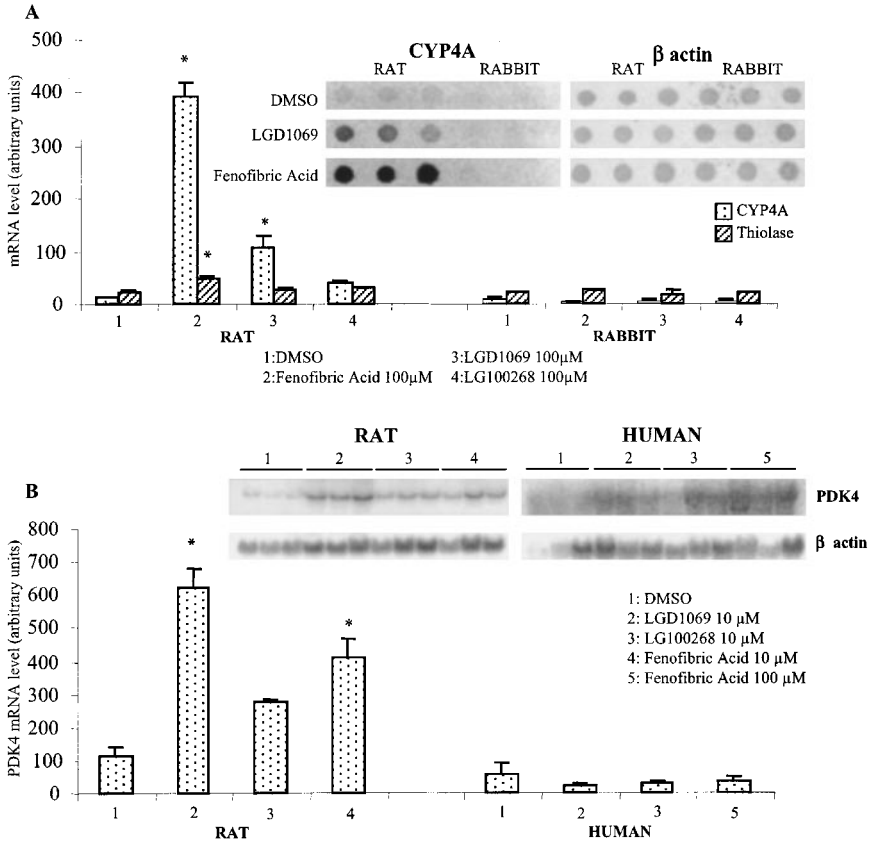

Figure 3 Species-specific effects following rexinoids and fenofibric acid treatments. (A) Twenty-four hours after plating, rat and rabbit primary cultured hepatocytes were treated for 24 h with 100 μM of rexinoids (LGD1069 and LG100268) or fenofibric acid. Total RNA was subjected to Northern blot analysis using CYP4A and thiolase radioabelled cDNA probes (5 μg of total RNA per spot, each spot corresponding to a culture dish). CYP4A hybridization is shown as a representative blot, while the densitometric quantification presents results obtained for both target genes. Graph bar represents the average ($n=3$) obtained following normalization of absolute values by the β actin level. (B) Effects of rexinoids or fenofibric acid in rat primary cultured hepatocytes are compared to those in cryopreserved human hepatocytes. Treatments, Northern blot analysis and quantification were performed as described below, except for the human hepatocyte treated group which received 100 μM of fenofibric acid. Asterisks (*) indicate that, within a given species, the observed value for the group of treated dishes of hepatocytes is statistically different from the value obtained from the group receiving DMSO alone.

cholesterol level was clearly higher in PPARα-deficient mice, but no effect on this parameter was observed following either treatment. The lowering of TG levels induced by fenofibrate and observed in wild type mice, was not reproduced in PPARα-deficient mice. Of particular interest is the effect of LGD1069 on circulating TG. While no effect was observed in wild type mice, a marked increase in TG was noted following administration of LGD1069 to PPARα-deficient mice. In this experiment, LGD1069 exposure of wild type mice resulted in a slight increase in the circulating cholesterol concentration.

Considering these observed modifications in circulating lipid levels, we analysed plasma lipoproteins in wild type male and female mice exposed to both compounds. LGD1069 and fenofibrate significantly decreased apolipoprotein B levels in males and females while both treatments elicited a rise, but only in females, in apolipoprotein A1 (22% and 39% for LGD1069 and fenofibrate, respectively) (Figure 5C).

In addition to the liver, the kidney is reported as a prototypical target organ regarding PP molecular signalling. Indeed, PPARα-dependent up-regulation of the PDK4 renal transcripts by fasting has recently been shown (Sugden *et al*., 2001b). Interestingly, in the heart, an organ where the influence of PPARα is less documented but with substantial RXRα expression, fasting also regulates PDK4 expression. Therefore, this gene was a relevant target to test two parameters: (i) the influence of different cellular environments (kidney and heart) regarding a putative regulation by rexinoids and (ii) the role played by PPARα in mediating this effect. In wild type mice LGD1069 treatment elicited a marked increase of PDK4 transcripts in the kidney and heart. In knockout animals, i.e., in the absence of PPARα, the positive regulation of PDK4 by LGD 1069 was totally abolished in the kidney but surprisingly it was fully preserved in the heart (Figure 6A). This PPARα-independent up-regulation of PDK4 cardiac transcripts by LGD1069 has been reproduced in an independent experiment (Figure 6B). The levels of LGD1069 regulation were comparable in wild type and knockout animals (3.7 and 3.9 times, respectively). Additionally, we observed the absence of any effect of fenofibrate on cardiac PDK4 transcripts between wild type and PPARα-deficient mice (Figure 6B).

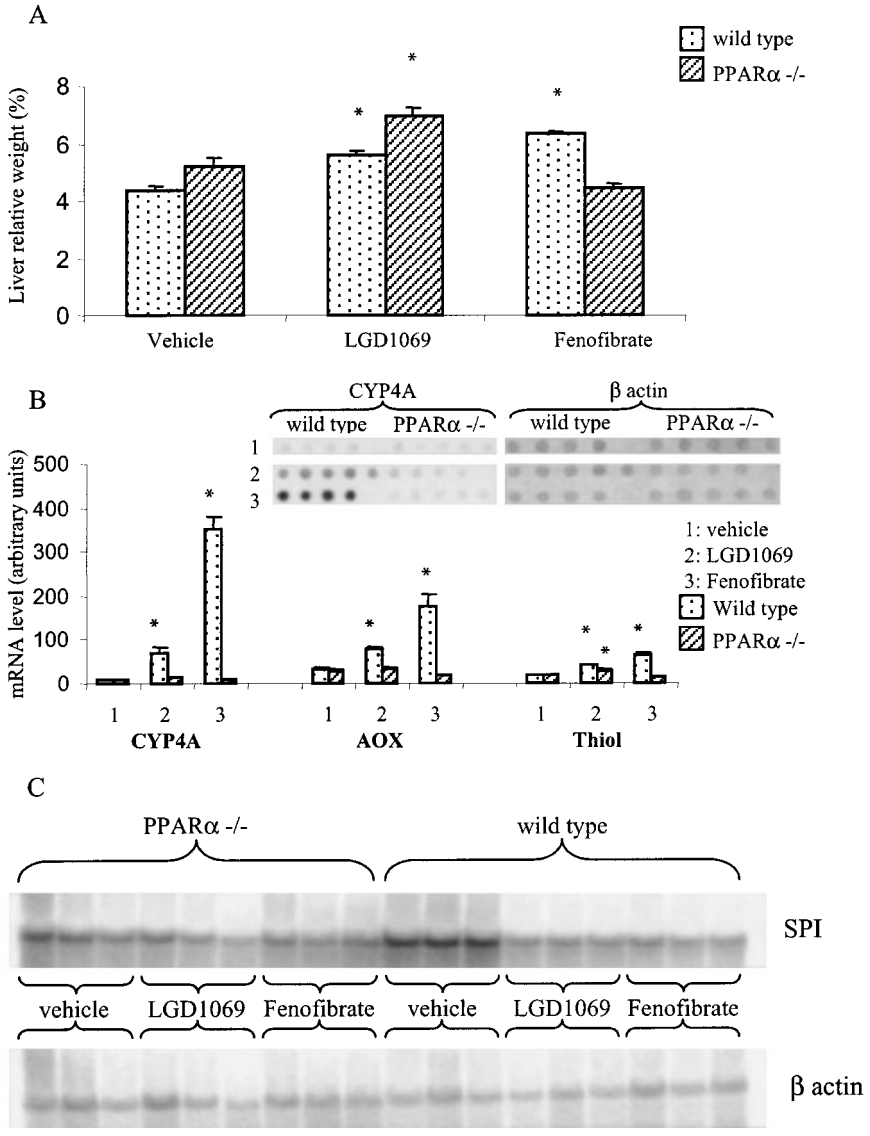

Figure 4 Hepatic effects of PPARα mobilization by rexinoids. (A) Effect of treatments on liver weight. Wild type and PPARα−/− mice were treated by LGD1069 (100 mg kg^{-1} day^{-1}) or fenofibrate (100 mg kg^{-1} day^{-1}) for 8 days. Values are ratios (liver weight/body weight) × 100, ± s.e.mean ($n=4$ for wild type mice treated with vehicle or fenofibrate and for PPARα−/− mice treated with LGD1069 whereas $n=5$ for all the other groups). (B) CYP4A, acyl CoA oxidase (AOX) and thiolase (Thiol) transcript levels were studied in liver of wild type and PPARα−/− treated mice. Following Northern blot and densitometric analysis, values were normalized by the β actin level. Graph bars represent mean ± s.e.mean. Serine protease inhibitor (SPI) hybridization is shown as an illustrative blot of rexinoid-induced mRNA down regulation in liver. Asterisks (*) indicate that the observed value for the treated group of mice is statistically different from the value obtained from the group of the same genotype receiving the vehicle alone.

Discussion

Several hormonal and micronutrient signalling pathways (e.g. thyroid hormone, vitamin D) rely on class II nuclear receptors that also mediate the therapeutic action of several families of drugs (e.g. fibrates, thiazolididiones, rexinoids). Remarkably, RXR holds an unique position among nuclear receptors since, following their chemical activation, each member of this class undergoes a dimerization with RXR that leads to the trancriptionally active form of the complex. A novel pharmacological class of RXR-activators, rexinoids, which overcome the side effects associated with the recruitment of RAR by retinoid drugs, has been developed. The rexinoid molecule LGD1069 (Targretin® capsules, Bexarotene® gel) is used in clinical practice to treat CTCL (Hurst, 2000; Talpur *et al*., 2002). Nevertheless, the molecular signal transduction pathway(s) activated by rexinoids is (are) not fully elucidated. In a receptor-rich environment, like the rodent liver, the sole administration of rexinoids sustains the biological and molecular effects (i.e. hepatomegaly, mRNA transcript regulation) that strongly evoke the hepatic activation of the PPARα signalling pathway (Standeven *et al*., 1997; Mukherjee *et al*., 1998). Our study was designed to assess the effectiveness and limits of such a molecular cross talk.

We analysed two relevant end points of PP signalling in rodents: (i) liver enlargement which is associated with the

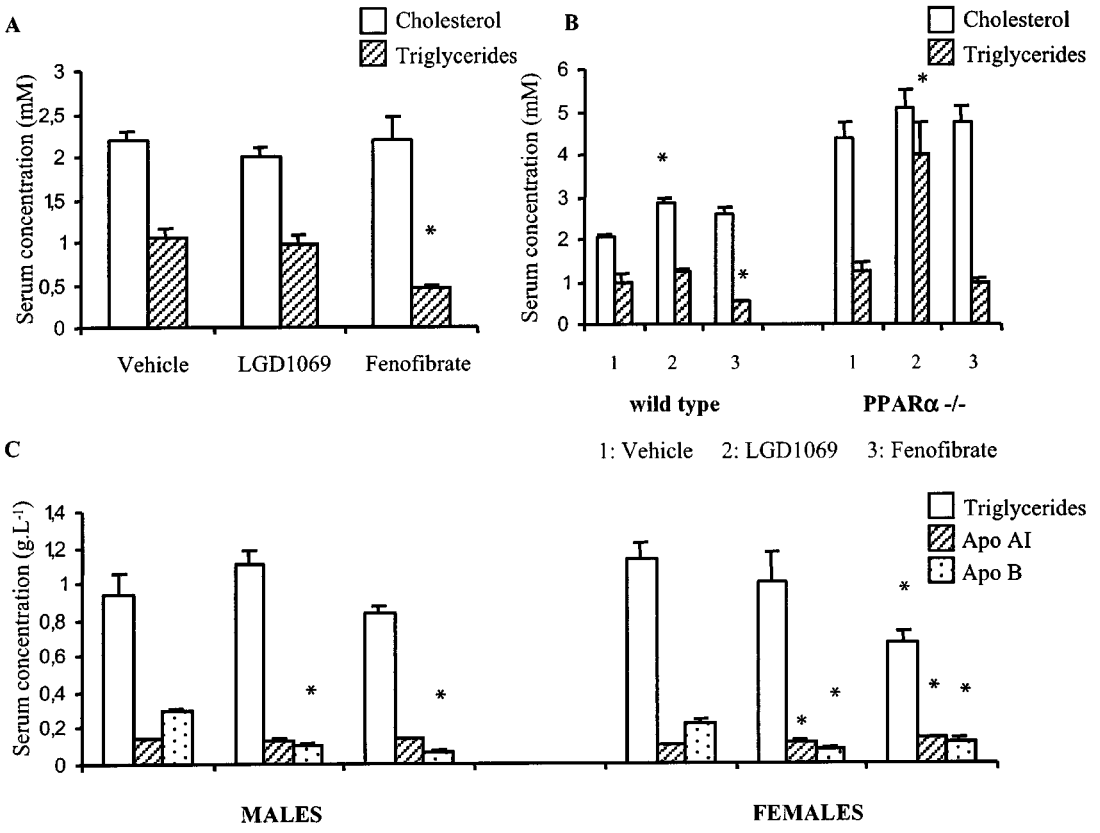

Figure 5 Comparison of LGD1069 or fenofibrate effects on serum lipids. (A, B) LGD1069 or fenofibrate (100 mg kg^{-1} day^{-1}) were administered to male mice for 8 days. Two hours before blood collection, mice were fasted and treated for the last time. (A) Male wild type mice were used ($n=8$). (B) Male wild type and PPARα−/− mice were used ($n=4$ or 5). (C) Male and female C57 BL/6J mice received either LGD1069 (100 mg kg^{-1} day^{-1}) or fenofibrate for 8 days ($n=4$ or 5). Two hours after the last dose, blood samples were collected and serum concentrations of TG, apolipoproteins AI and B were determined (see Methods). Asterisks (*) indicate that the observed value for the treated group is statistically different from the value obtained from the group receiving the vehicle alone.

hepatic proliferation of peroxisomes and (ii) the rise in CYP4A hepatic transcripts which results from the recruitment of PP response elements located in the promoter of this gene (Aldridge *et al*., 1995). Although with a lower magnitude, the effect of LGD1069 on both parameters paralleled accurately those of the PP fenofibrate, in wild type mice (Figure 1). In our experimental conditions, both molecules displayed reversible effects, with similar kinetics. To further delineate the involvement of PPARα in the molecular signalling of rexinoids, we tested the species selectivity of some of their transcriptional regulations. Indeed, like fenofibrate, LGD1069 failed to raise the level of CYP4A and PDK4 transcripts in hepatocytes from non-rodent species (i.e. rabbit, human) while both were active in rat hepatocytes. Again, we observed that the behaviour of the rexinoid molecule matches those of a prototypical PP which is a potent gene regulator in rodents (Dirven *et al*., 1993) but loses this potency within the lagomorpha order (rabbits) and in primates (Roberts *et al*., 2000). It is noteworthy that, contrary to CYP4A, a larger increase in PDK4 transcript level was observed by treating rat hepatocytes with LGD1069 rather than with fenofibric acid (Figure 3B). Although it has formally been established that the PDK4 gene is a downstream target of PPARα, in the liver (Sugden *et al*., 2001a; Huang *et al*., 2002), the original observation above suggests its promoter could possibly contain particular features compared to a conventional PP-responsive promoter (i.e. CYP4A gene) thus explaining the apparent higher induction of PDK4 by a rexinoid.

Considering that the RXR-selective activator LGD1069 displayed several prototypical characteristics of PP, we tested the hypothesis of the direct involvement of PPARα in this pathway. Clearly, the vast majority of PP actions are abolished in PPARα-deficient mice (Lee *et al*., 1995; Peters *et al*., 1998). Using these genetically engineered mice, we unambiguously established that LGD1069 exerts its *in vivo* modulating action on hepatic messengers for CYP4A, acyl CoA oxidase and serine protease inhibitor, in a way strictly dependent on the presence of the active PPARα. While this clearly sustains the functional link between rexinoid signalling and the PPARα transduction pathway, we noticed a biochemical discrepancy. Unexpectedly, while fenofibrate promoted a liver enlargement in a strictly PPARα-dependent manner, LGD1069 treatment resulted in hepatomegaly in both wild type and deficient animals (Figure 4A). This suggests the recruitment of separate pathophysiological pathways by rexinoids and PP. Indeed, co-administration of both molecules to wild type mice resulted in an exacerbation of effects (data not shown) thus sustaining the hypothesis of separate pathways with additive effects. Further investiga-

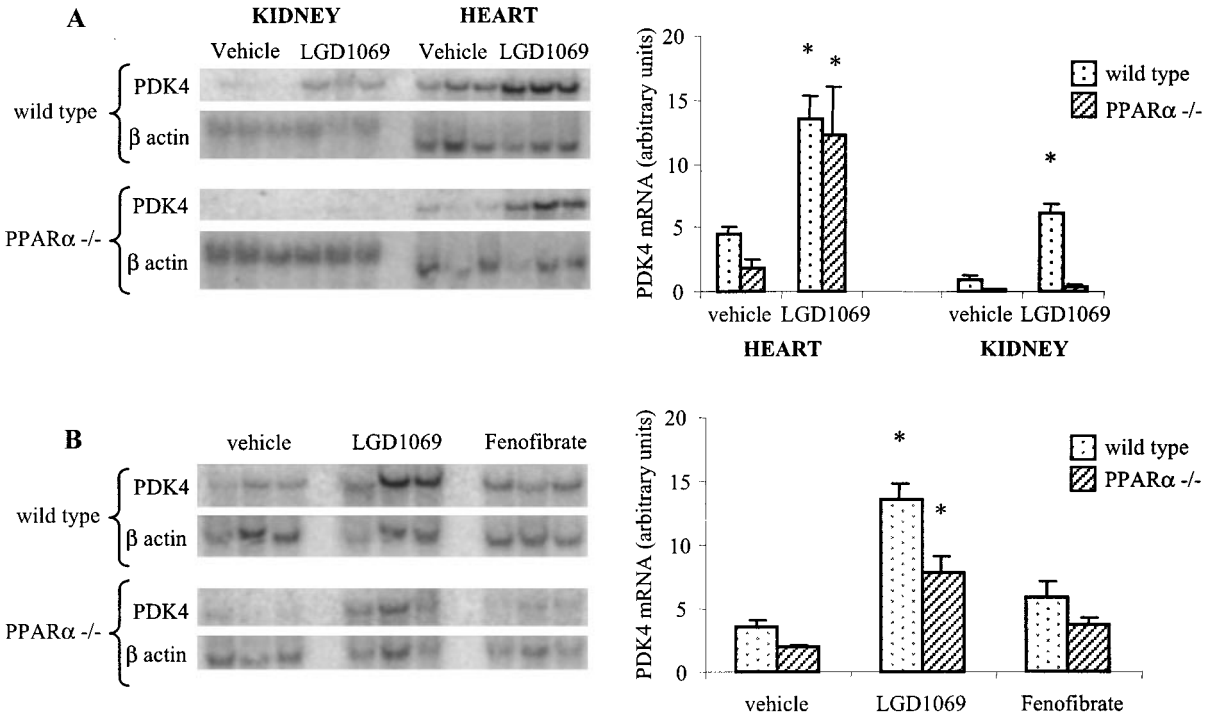

Figure 6 PDK4 mRNA level in the kidney and heart of control, LGD1069 and fenofibrate-treated mice. (A) Northern blot and densitometric analysis of PDK4 mRNA expression in the kidney and heart of LGD1069-treated mice. Absolute values were normalized by the β actin densitometric level. (B) Total RNA samples from LGD1069 and fenofibrate-treated mice were compared for PDK4 mRNA abundance in the heart. Absolute values were normalized by the β actin densitometric level. Asterisks (*) indicate that the observed value for the treated group is statistically different from the value obtained from the group of the same genotype receiving the vehicle alone.

tions will be conducted, in both genotypes, to substantiate this hypothesis.

The results concerning the influence of rexinoid exposure on circulating lipid parameters in wild type mice were inconsistent all through this investigation, reflecting the variety of conflicting results (Davies *et al*., 2001; Standeven *et al*., 1996; 2001; Mukherjee *et al*., 1997; Lenhard *et al*., 1999). In PPARα-deficient mice, LGD1069 consistently raised the circulating TG. The hypothesis of a RXR-driven rexinoid signalling through two separate pathways could possibly accommodate all observed results. One pathway would raise the TG in a PPARα-independent manner; a second one would decrease TG through the documented PPARα-associated pathway. Therefore, the net effect on TG levels in wild type animals would depend on the ratio between both pathways in a given animal, thus displaying a relative inter-individual and inter-experimental variability. In PPARα−/− mice, the sole PPARα-independent and TG-raising pathway would account for the observed TG increase.

This study was extended to the kidney and the heart to document the influence of different organ environments on the RXR molecular signalling. PPARα expression in proximal tubule cells of the kidney (Braissant *et al*., 1996), sustains its status of a prototypical PP-responsive organ (Peters *et al*., 1996). Despite the reports of PPARα messenger expression (Kliewer *et al*., 1994) and some PP-driven transcript regulations in the rodent heart (Djouadi *et al*., 1999), this organ is not considered as a clinically relevant pharmacological target of fibrate drugs. RXRα, which is expressed in the adult myocardium, plays a major role in the morphogenesis of the heart (Kastner *et al*., 1994). As previously reported in studies using another PP, the Wy14,643, (Sugden *et al*., 2001b; Holness *et al*., 2002a), no cardiac regulation of PDK4 was observed in normal mice treated with fenofibrate. Interestingly, we observed the positive regulation of the PDK4 transcript, by LGD1069, in both organs. But remarkably, while this renal regulation depends on PPARα expression, it is PPARα-independent in the heart. This dedicated kinase catalyses the inactivating phosphorylation of the E1 component of the pyruvate dehydrogenase complex thus controlling the partitioning of the pyruvate flux between oxidation and the gluconeogenic pathway. This represents the first report that, in the heart, the PDK4 gene behaves as a downstream target of RXR. This suggests that, in the heart, a pharmacological influence on the pyruvate dehydrogenase complex is theoretically achievable with a subsequent influence on fuel partitioning in this organ (carbohydrate versus fat) (Sugden *et al*., 2001c). Our results are consistent with recent studies which established that starvation raises cardiac PDK4 protein independently of PPARα expression (Holness *et al*., 2002a) and also that in the murine soleus, a model of slow oxidative fibre muscle (like the heart), starvation but not PP (Wy14,643) controls PDK4 expression (Holness *et al*., 2002b).

Elucidating the molecular basis of this cardiac regulation will require identifying if it occurs through either the RXR homodimer or the RXR heterodimers. Noticeably, it has been established that tri-iodothyronine administration to rats

raises their cardiac expression of PDK4, regardless of PPARα co-activation (Holness *et al*., 2002a). An interaction between TR and RXR, following rexinoid-activation of RXR, appears plausible since their concomitant cardiac expression has been shown (Klein & Ojamaa, 2001; Kastner *et al*., 1994; Sucov *et al*., 1994). This hypothesis is further supported by a recent report of the functional modulation of the TR/RXR heterodimer by a RXR physiological activator (i.e. 9-*cis*-RA) (Li *et al*., 2002). This argues against the non-permissive former status of this receptor dimer and the role of a silent partner previously attributed to RXR (Aranda & Pascual, 2001; Forman *et al*., 1995; Mangelsdorf & Evans, 1995).

Our results have clearly established that rexinoid molecules display a dual potency. In several organs, they undoubtedly recruit PPARα to regulate a specific set of genes, while they additionally display PPARα-independent effects (i.e. liver enlargement, cardiac transcript regulation). The molecular cross talk established between rexinoids and fibrates signal transduction pathways exemplifies the putative role of rexinoids in recruiting and/or synergizing other pharmacological pathways. This could initiate a valuable reassessment of the rexinoids therapeutic uses by thoroughly investigating their capabilities in nuclear receptor-expressing organs. The net effect of administering rexinoids should be considered as the sum of numerous, intricate, organ-specific actions that remain to be globally assessed using large scale technologies to record and compare the transcriptional signatures elicited by these molecules.

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