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Research paper

Peroxisome proliferator-activated receptorα (PPARα) agonists down-regulate α2-macroglobulin expression by a PPARα-dependent mechanism☆

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ABSTRACT

Fibrates are peroxisome proliferator-activated receptor alpha (PPARα) ligands used to normalize lipid and glucose parameters and exert anti-inflammatory effects. The acute-phase response (APR) is an important inflammatory process. One of the most important acute-phase proteins in rats is α2-macroglobulin (A2Mg). Whereas normal adult rats present low serum levels, pregnant rats display high amounts. Therefore, we used pregnant rats to detect the effect of fenofibrate on hepatic A2Mg expression by RT-PCR and Northern blot. Virgin rats were used as controls. The expression of other APR genes, a known fibrate-responder gene, gamma-chain fibrinogen (γ-Fib), and one gene from the same family as A2Mg, complement component 3 (C3), were also measured in liver. In order to determine whether the fibrate-effects were mediated by PPARα, wild-type mice and PPARα-null mice were also used and treated with WY-14,643 (WY) or di-2-ethylhexyl phthalate (DEHP). Fenofibrate depressed A2Mg expression in virgin rats, but expression was decreased more sharply in pregnant rats. Expression of C3 and γ-Fib was diminished after treatment only in pregnant rats. On the other hand, WY, but not DEHP, reduced A2Mg and γ-Fib expression in the livers of wild-type mice, without any effect in PPARα-null mice. WY or DEHP did not affect C3 expression. Therefore, A2Mg expression is modified by PPARα agonists not only in pregnant rats under augmented APR protein synthesis, but also in virgin rats and mice under basal conditions. Interestingly, our results also identify A2Mg as a novel PPARα agonist-regulated gene.

1. Introduction

Fibrates have been effectively used to reduce plasma triglyceride levels under conditions of hypertriglyceridemia [1]. The molecular basis for the action of fibrates on lipid metabolism involves the activation of transcription factors, known as peroxisome proliferator-activated receptors (PPAR), principally the PPARα subtype expressed in liver [2,3]. Fibrates decrease apolipoprotein C-III, and increase both acyl-coenzyme A oxidase [4] and fatty acid translocase [5] gene expression in rodent liver.

PPARα ligands also display other effects, not directly related to the lowering of plasma lipids, including the modulation of immune and inflammatory responses. The molecular mechanisms involved in the interaction of PPARα with the inflammatory signalling pathway have recently been reviewed [6]. The acute-phase response (APR) is an important inflammatory process for the initiation of defence mechanisms, that may become deleterious if chronic activation is reached. Interleukin-6 (IL-6) and IL-1 stimulate the production of acute-phase proteins such as C-reactive protein (CRP), fibrinogen, and serum amyloid A (SAA), which are markers of cardiovascular disease [6]. Fibrates are shown to be negative regulators of acute-phase proteins [7,8]. Thus, the expression of acute-phase proteins is down-regulated by PPARα activators, and accordingly fenofibrate treatment of dyslipidemic patients decreases the plasma concentrations of fibrinogen, SAA, IL-6, and CRP, whereas levels of albumin, a negative APR protein, are augmented [9,10].

Abbreviations: PPAR, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; A2Mg, α2-macroglobulin; γ-Fib, gamma-chain fibrinogen; C3, complement component C3; Apo, apolipoprotein; ACO, peroxisomal acyl-CoA oxidase; α2u, α2 urinary globulin; DEHP, di-(2-ethylhexyl)phthalate; WY, WY-14,643; APR, acute-phase response; RT-PCR, reverse transcriptase polymerase chain reaction; A.U., arbitrary units.

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One important acute-phase protein in rats is α2-macroglobulin (A2Mg) [11], a protein synthesized in the liver [12]. Although A2Mg levels in serum are normally low in adult rats, they are markedly increased in serum of the rat fetus, neonates and late-term dams, as well as in rats with acute inflammation [12, and references therein]. In mice, plasma levels of A2Mg are augmented after acute or chronic inflammatory stimuli [13]. Although some authors do not consider A2Mg as an acute-phase protein in humans [11,14], other authors have proposed that A2Mg clearly contributes to the APR [9,15] as a positive acute-phase protein. In fact, although fibrates lower plasma A2Mg levels in hyperlipidemic patients [9], the effect of fibrates on A2Mg gene expression has been shown to be controversial [16,17].

Since the high A2Mg serum levels in pregnant rats can serve as a model to study the influence of PPARα agonists on hepatic gene expression, present work was addressed to investigate the effect of fibrates on A2Mg expression in pregnant rats. Furthermore, in order to determine whether the effect of fibrates on A2Mg gene expression was mediated by PPARα, wild-type and PPARα-null mice were also used. We compared the results to that of γ-fibrinogen (γ-Fib), a positive acute-phase protein known to be modulated by PPARα agonists [7,9] and another positive acute-phase protein, complement C3 [18] belonging to the same family as A2Mg [19], but whose expression has not been previously examined for regulation by PPARα agonists.

2. Materials and methods

2.1. Animals, drug administration, and collection of the samples

Study I: Female Sprague–Dawley rats weighing 180–210 g were fed *ad libitum* standard rat chow (B&K Universal, Barcelona, Spain) and housed under controlled light and temperature conditions (12-h light–dark cycle; 22 ± 1 °C). The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU, Madrid, Spain. Half of the animals were mated, and day 0 of pregnancy was determined by the appearance of spermatozoids in vaginal smears, whereas the other half were kept virgin. From day 16 of gestation, rats were given by oral gavage two daily doses of 0, 100 or 200 mg of fenofibrate (from Sigma, USA)/kg of body weight, one at 8.00 h and the other at 18.00 h, suspended in 2% Tween-80 or Tween-80 alone. On the morning of the 20th day of pregnancy (after 4 days of treatment), corresponding to 14 h after receiving the last treatment, rats were decapitated and blood collected into tubes containing Na_2-EDTA. Liver was immediately removed, placed in liquid nitrogen and kept at −80 °C until analysis. Virgin rats received the same treatment and were studied in parallel. There were 5–6 animals per group.

Study II: Male SV129 wild-type mice were purchased from Taconic (Germantown, NY), and male SV129 PPARα-null mice [20] were a kind gift from Frank Gonzalez (National Cancer Institute, Bethesda, MD). Control and treated mice ($n = 2$–5) were provided with NIH-07 rodent chow (Ziegler Brothers, Gardner, PA, USA) and deionized, filtered water *ad libitum*. This study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Chemical Industry Institute of Toxicology Institutional Animal Care and Use Committee (NC, USA). Lighting was on a 12-hr light/dark cycle. Male SV129 wild-type and SV129 PPARα-null mice were fed the diet supplemented with either WY-14,643 (WY) (0.1%) or di-(2-ethylhexyl)phthalate (DEHP) (0.6%), or a control diet for 3 weeks. WY and DEHP were selected because of their different structural properties and uses. DEHP is considered a weak PPAR activator compared to WY. At the designated time after treatment, animals were deeply anesthetized by isofluorane anesthesia or pentobarbital injection and killed by exsanguination. The livers were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen, and stored at −70 °C until analysis.

2.2. Total RNA preparation and analysis

Study I: Rat total hepatic RNA was isolated by a modification of the guanidium isothiocyanate method, using Ultraspec RNA according to the manufacturer's instructions (Biotecx Labs, Houston, USA). Total RNA concentration was determined by absorbance measurement at 260 nm. The 260/280 absorption ratio of all samples was between 1.8 and 2.0. Total RNA-genomic DNA-free samples were used to analyse the expression of APR genes: α2-macroglobulin (A2Mg), complement component 3 (C3), gamma-chain of fibrinogen (γ-Fib), and GAPDH as endogenous control, by semi-quantitative RT-PCR, according to the following protocols. Briefly, total RNA (2.5 μg) was digested with 5 U RNase free-DNaseI (Roche, USA) for 20 min at 37 °C to remove traces of genomic DNA. The DNase was inactivated at 64 °C for 10 min and cDNA was synthesized from total RNA by oligo(dT)-primed reverse transcription with Superscript II (Invitrogen, USA), according to the manufacturer's instructions. PCRs were performed in a 25 μL reaction mix containing 20 pmol of both forward and reverse primer, 10 mmol/L of each deoxyribonucleoside triphosphate, appropriate dilutions of the cDNA stock, 2.5 μL of PCR 10X buffer, and Accu Taq-polymerase (Sigma, USA). Table 1 defines primers and their references, along with PCR conditions (size of PCR products, number of cycles and primer annealing temperatures) for each gene tested. All reactions were performed in a PTC-100 Thermocycler (MJ Research, USA) in which samples underwent a 3-min initial denaturing step, followed by the number of cycles indicated (Table 1) of 45 s to 1 min at 94 °C, 45 s at the annealing temperature indicated in Table 1, and a primer extension step at 72 °C for 45 s to 1 min. The final extension step was 10 min at 72 °C. The PCR products were analysed by agarose gel electrophoresis and DNA was visualized by ethidium bromide staining and using a UV-light box. The intensities of the bands on the images were determined by quantitative scanning densitometry (GS-700 Imaging Densitometer, BioRad, California, USA). To determine the linear range of the PCR, dilutions of the cDNA preparations were previously used for each gene and experimental group of rats. Results for the expression of specific mRNAs were always expressed relative to the expression of the control gene (GAPDH).

Table 1
Sequences of oligonucleotide primers and PCR conditions.

Gene name	Size (bp)	5′-oligonucleotide	3′-oligonucleotide	Annealing (°C)	Cycles	Reference
A2Mg (rat)	617	GAGCTCCGGGTCACTGCTCAA	GAAAATGTGCCCGAAGAACG	54	35	[21]
A2Mg (mouse)	200	TCACCAAAGGGTCAGGATCAGGATG	CATGTTGGAGCTGGGTCGTTCTCC	68	35	Atlas RT-PCR Primer Sequences (Clontech, CA, USA)
γFib (rat, mouse)	394	GTTTCATTCTATGCTGGGCGCT	TCATGGGTTAGTAACAGTGCTT	57	30	[22]
C3 (rat, mouse)	230	GACCTGCGACTGCCCTACTCT	CTGATGAAGTGGTTGAAGACGG	65	30	[23]
GAPDH (rat, mouse)	450	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGT	57	35	[24]

Rat primer sequences were used in RT-PCR analysis and mouse primer sequences were used in Northern blot experiments.

Study II: Total RNA was isolated from mouse livers by a modification of the guanidinium isothiocyanate method using RNAzol according to manufacturer's instructions (Tel-Test, Friendswood, TX). Twenty μg of denatured total RNA was separated on 1.2% agarose gels and transferred to nylon membranes in 20x SSC. The DNA probes for Northern blot analysis were labeled with [α-^{32}P]dCTP using the random primer DNA labelling kit provided by Amersham. Probes used were: 1.1 kb PstI fragment of rat peroxisomal acyl-CoA oxidase (ACO) cDNA (kindly supplied by Dr. H. N. Sorensen, University of Oslo, and with permission of Dr. T. Osumi, Humeji Institute of Technology, Hyogo, Japan), the rat alpha-2u globulin (α2u) cDNA fragment spanning nucleotides +43 to +138, and the PCR products generated as indicated in Table 1, and using rat cDNA as a template (except for A2Mg, that mouse cDNA was used). All reactions were performed in a PTC-100 Thermocycler (MJ Research, USA) in which samples underwent a 3-min initial denaturing step, followed by the number of cycles indicated (Table 1) of 45 s to 1 min at 94 °C, 45 s to 1 min at the annealing temperature indicated in Table 1, and a primer extension step at 72 °C for 45 s to 1 min. The final extension step was 10 min at 72 °C. The probes were sequenced (ABI PRISM 377 Perkin Elmer DNA sequencer), and the sequences obtained were compared to Gene Bank sequences to confirm the accuracy of the probes used. Blots were prehybridized at 42 °C for 2 h and hybridized overnight at the same temperature. Washing conditions were 0.1x SSC, 0.1% SDS at 53 °C for 15 min three times and membranes were exposed to appropriate screens (Imaging Screen K, BioRad, California, USA) at 4 °C from 1 h to three days and the images analyzed (Personal Molecular Imager FX, BioRad, California, USA). Filters were stripped of label at 75–80 °C for 1 h with 0.1× SSC, 0.5% SDS, 0.1% tetrasodium pyrophosphate and then rehybridized.

2.3. Statistical analysis

Results were expressed as means ± S.E. Treatment effects were analyzed by one-way analysis of variance (ANOVA). When treatment effects were significantly different ($P < 0.05$), means were tested by Tukey multiple range test. When necessary, the Mann–Whitney *U* test was used instead. Differences between the two groups were analyzed by using the Student *t* test.

3. Results

3.1. Effect of fenofibrate on A2Mg and other positive acute-phase proteins expression in pregnant and virgin rats

The effect of fenofibrate on acute-phase protein mRNA gene expression was studied. As shown in Fig. 1A, hepatic A2Mg mRNA levels appeared higher in pregnant than in virgin rats not receiving any treatment. Such finding is in accordance with the high serum levels of A2Mg in late gestation found in previous studies [12]. In virgin rats, hepatic A2Mg mRNA levels decreased by treatment with fenofibrate in a dose-independent manner, suggesting that the lowest dose of fenofibrate used here was sufficient to maximally influence the expression of A2Mg gene in non-pregnant rats. Interestingly, A2Mg mRNA levels in pregnant rats were sharply decreased by fenofibrate in a dose-dependent manner (Fig. 1A), but never reached the low levels observed in virgin rats.

The γ-Fib mRNA levels also appeared higher in pregnant than in virgin rats not receiving the drug (Fig. 1B). Such a result is in accordance with the high plasma levels of fibrinogen observed in mammals at the time of parturition [25]. While fenofibrate treatment did not affect this variable in virgin rats, γ-Fib mRNA levels were reduced in pregnant rats (Fig. 1B) independently of the dose used, reaching the values found in non-pregnant rats.

There were no differences in C3 expression between untreated virgin and pregnant rats. In non-pregnant rats, the drug did not affect C3 gene expression (Fig. 1C), whereas in pregnant rats, hepatic C3 mRNA levels decreased due to treatment in a dose-independent manner (Fig. 1C).

3.2. Requirement for PPARα in fibrate regulation of A2Mg and other positive acute-phase gene expression

Because PPARα has been shown to mediate several fibrate-inducible responses in the liver, we examined the dependence of fibrate-induced decreases in A2Mg and positive acute-phase gene regulation on PPARα expression. Wild-type mice and mice that lacked a functional form of PPARα (PPARα-null mice; [20]) were fed a control diet or the same diet supplemented with either WY (0.1%) or DEHP (0.6%) for 3 weeks. As shown in Fig. 2, when wild-type mice were fed WY, there was a decrease in the liver expression of A2Mg mRNA, whereas treatment with DEHP did not change the levels of A2Mg mRNA. Treatment of PPARα-null mice with WY or DEHP resulted in no changes in A2Mg gene expression. Moreover, A2Mg expression appeared slightly higher in PPARα-null mice in comparison to that found in wild-type mice, for all the experimental groups (Fig. 2).

The expression of γ-Fib mRNA also decreased after feeding WY, but not after DEHP, in wild-type mice, without effect in PPARα-null mice (Fig. 2). Moreover, γ-Fib expression appeared higher in PPARα-null mice than in wild-type mice, for all the experimental groups. Treatment of either wild-type or PPARα-null mice with WY or DEHP did not modify the levels of C3 mRNA (Fig. 2).

As a control of PPARα agonist-induced repression, we also examined the expression of α2u-globulin, which has already been reported to be negatively regulated by PPARα activation [5,8,26]. When wild-type mice were fed WY, there was a decrease in the expression of α2u mRNA (Fig. 2), whereas this effect was not found in PPARα-null mice. Here again, treatment with DEHP did not result in any changes in the levels of α2u mRNA in wild-type or PPARα-null mice (Fig. 2). Moreover, α2u expression appeared higher in PPARα-null mice in comparison to that found in wild-type mice, for all the experimental groups. As a control of PPARα agonist-induced up-regulation, we also examined the expression of ACO mRNA. As expected [27,28], ACO mRNA expression was enhanced by WY or DEHP treatments in wild-type mice but not in PPARα-deficient mice (Fig. 2). As a negative control, GAPDH mRNA levels remained constant under all conditions (Fig. 2).

On the other hand, to evaluate the kinetics of A2Mg gene suppression after short-term WY exposure and to compare it to other genes well-known as being regulated by PPARα, F344 male rats were given a single gavage dose of WY at 50 mg/kg body weight and killed 3, 6, 12, or 24 h after treatment. We measured the mRNA levels by macroarray technology (Atlas Nylon Arrays, Clontech, BD Biosciences, Palo Alto, CA, USA) and we observed that γ-Fib and C3 expression were reduced after acute administration of WY. Unfortunately, A2Mg expression was extremely low in normal rats and it was not possible to detect any change produced by the fibrate-treatment (unpublished results).

4. Discussion

We found both in pregnant and virgin rats, that fenofibrate treatment repressed liver A2Mg mRNA expression, the effect being more evident in pregnant animals; where basal levels of A2Mg mRNA were higher than in virgin rats, in agreement with previous reports [12]. These findings are interesting since they emphasize the anti-inflammatory effect of PPARα agonists independently of whether the A2Mg levels are elevated, as in gestation, or low as in

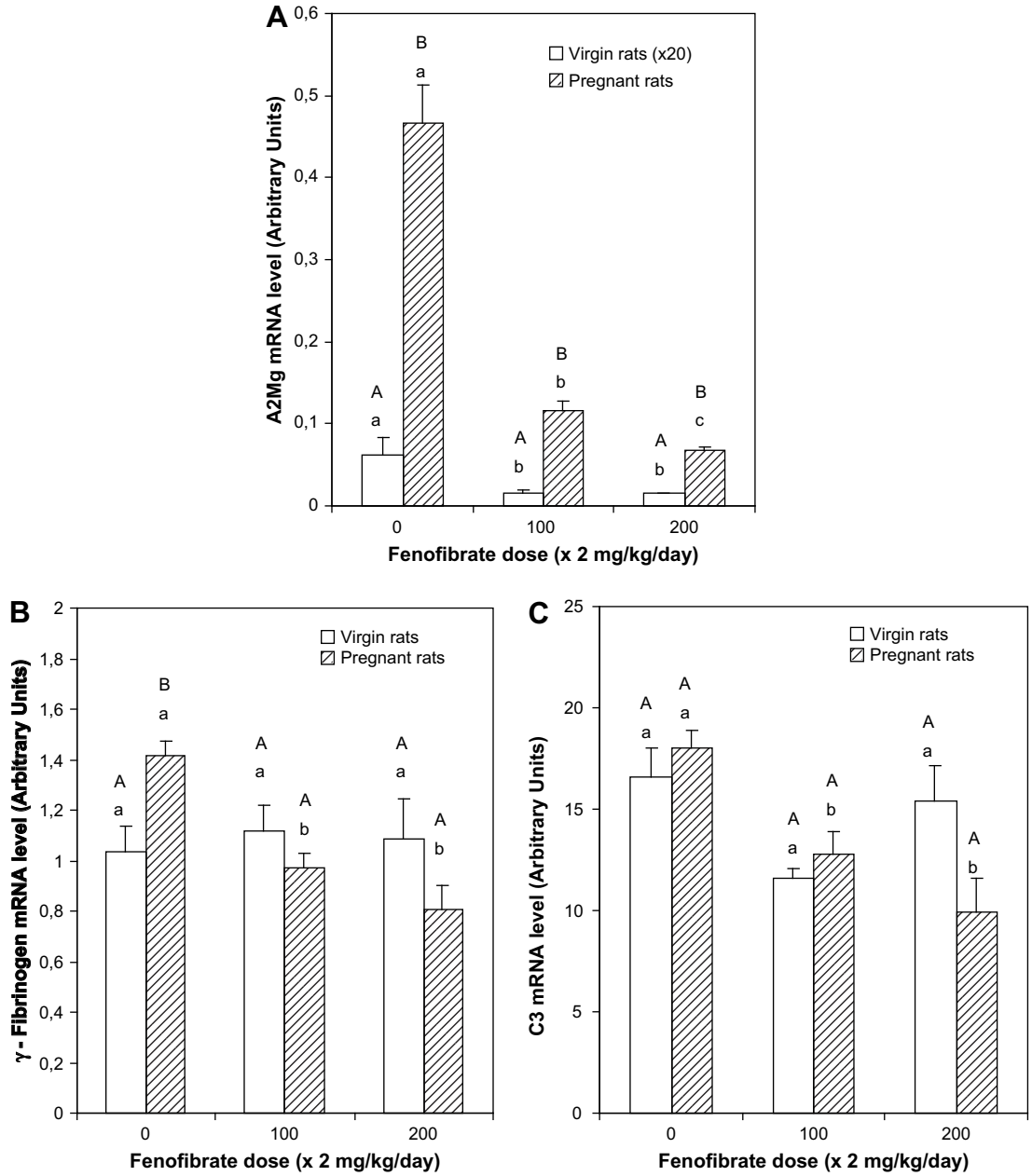

Fig. 1. Fenofibrate down-regulates APR gene expression in pregnant rats. Relative amount of mRNA of: A) liver α-2 macroglobulin (A2Mg), B) gamma-chain fibrinogen (γ-Fib), and C) complement component 3 (C3), from virgin and pregnant rats treated or not with fenofibrate, measured by semi-quantitative RT-PCR. Values were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and were represented using arbitrary units. The capital letters correspond to the statistical comparisons between pregnant and virgin rats receiving the same treatment. The small letters correspond to the statistical comparisons between rats receiving different drug doses. Values not sharing a common letter are significantly different at $P < 0.05$. Each value represents the mean ± standard error of five animals. Values for A2Mg expression in virgin rats were previously multiplied by a factor of 20 before being reported.

non-pregnant rats. These results are consistent with those previously reported [7,8,9,29] for different APR proteins, such as α_1-acid glycoprotein (AGP), β-fibrinogen, ceruplasmin, haptoglobin, SAA, and CRP. IL-6 is the most potent cytokine inducing hepatic APR genes expression as part of the inflammatory response [30]. In fact, IL-6 is a major inducer of A2Mg in rats [31], and also stimulates APR and hypertriglyceridemia [32]. In late gestation there is hyperlipidemia [33], and the synthesis of acute-phase proteins is induced [34,35,36]. The possibility exists that these two parameters are co-induced in pregnancy by IL-6, when serum levels are increased [37,38]. Thus, the effect of fenofibrate on A2Mg expression found here would support the ability of PPARα agonists to interfere in IL-6 induction of APR gene expression. In fact, in hyperlipidemic patients, fenofibrate treatment decreases the plasma concentrations of IL-6, fibrinogen and CRP [39] and fenofibrate decreases basal and IL-6-induced expression of the fibrinogen-alpha, -beta, and -gamma genes [9]. However, we found here that both γ-Fib and C3 expression were sensitive to fenofibrate in pregnant, but not in virgin, rats.

We also examined the promoter region of A2Mg from rats for transcription factor binding sites with BioBase Biological Databases using default settings. Although no classical PPAR binding sites (i.e., direct repeat-1) were identified, STAT3 and RelA-p65 sites were located within the ~10 kB promoter region. There is evidence that

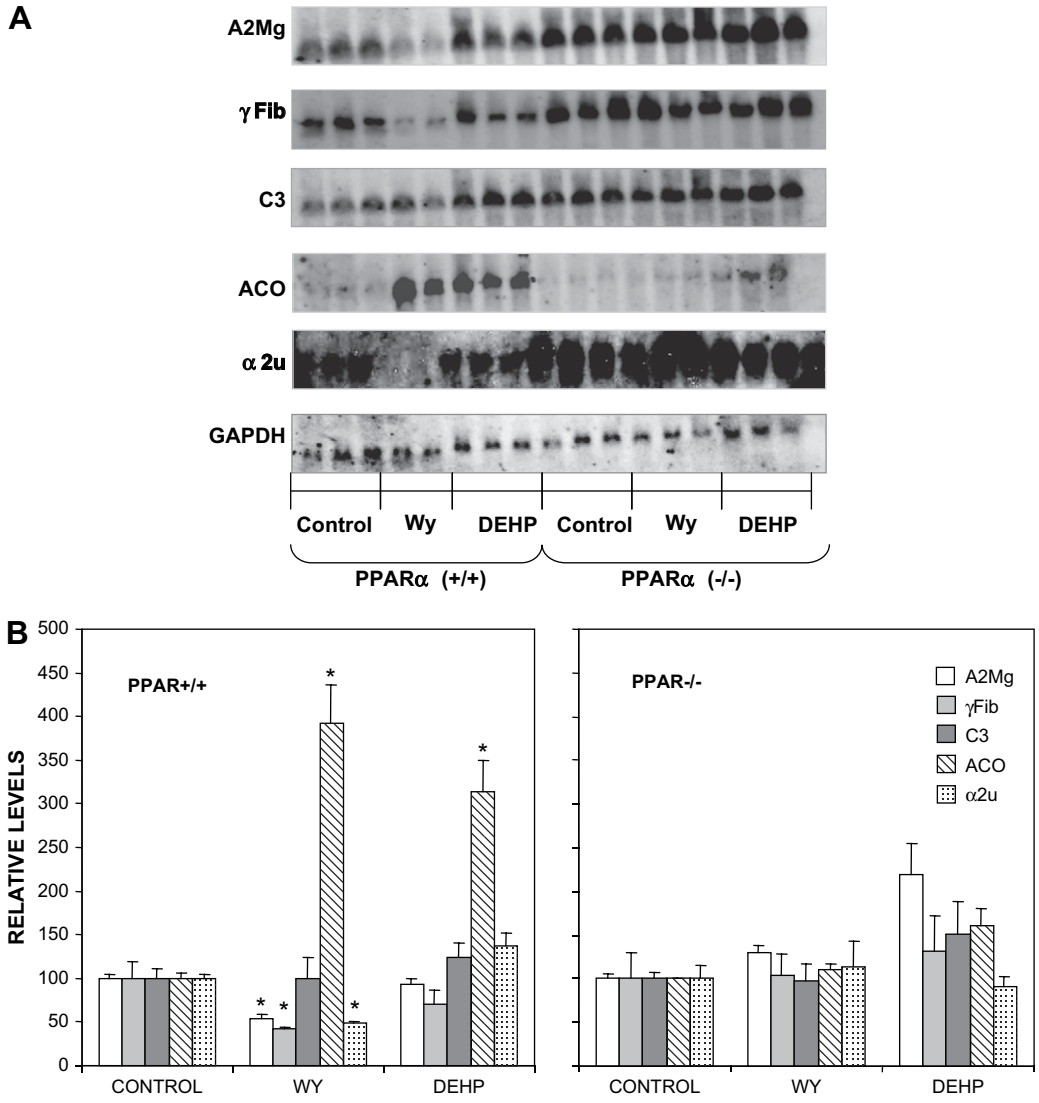

Fig. 2. Down-regulation of APR gene expression by WY is dependent on PPARα. Wild-type SV129 mice (+/+) or SV129 mice that lack PPARα (−/−) were fed a control diet (*Control*) or a diet containing WY (0.1%) or DEHP (0.6%) for 3 weeks. Total RNA isolated from the livers were separated by 1.2% agarose, transferred to nylon and analysed by Northern blot by using probes for A2Mg, γ-Fib, C3, ACO, α2u, and GAPDH as a control. The Northern autoradiograms (A) were densitometrically scanned, and expression was normalized to GAPDH expression and showed by histograms (B). Each value represents the mean ± standard error of two-three animals. *, Significantly different from control ($p < 0.05$).

both STAT3 and NF-kB are negatively regulated by PPAR subtypes [6,9,18,40,41]. Thus, PPAR may be negatively regulating A2Mg through negative regulation of STAT3 or NF-kB.

Rat A2Mg is a major acute-phase protein that under normal conditions exhibits low serum levels [12]; serum levels are increased after inflammatory stimuli [13]. In mice, in spite of the high levels observed in normal conditions, the concentration of A2Mg in plasma has been shown to increase during acute and chronic inflammatory reactions [13]. In our hands, fibrates efficiently reduced A2Mg expression in wild-type mice, but not in mice lacking PPARα. These findings are in accordance with those reported for other acute-phase proteins in rodents [8,9,29] and humans [39], as well as that reported for A2Mg in plasma of hyperlipidemic patients [9] and in human and rat hepatocytes after treatment with the PPARδ activator, GW501516 [18]. By contrast, in isolated hepatocytes from rats, mice, and humans, 72 h treatment with clofibrate diminished A2Mg expression in rat hepatocytes only [16]. In female Fischer rats treated with ciprofibrate for 60 days, A2Mg expression was augmented in apparent contradiction with our studies [17]. In contrast, we found here that activation of PPARα with WY, but not with DEHP, down-regulates A2Mg expression in wild-type mice *in vivo*, whereas no effect was seen in PPARα-null mice. Therefore, our data show that the decrease in A2Mg gene expression by PPARα ligands was dependent on PPARα agonist potency and mediated by PPARα. Moreover, since PPARα-null mice presented higher A2Mg mRNA levels than wild-type mice, PPARα regulates the constitutive expression of A2Mg. Similar findings have been previously related for other acute-phase proteins [9,29], suggesting that PPARα exerts a basal inhibitory effect on the expression of APR genes.

Similarly to the results found for A2Mg in mice, γ-Fib was decreased by WY, but not by DEHP, in wild-type but not PPARα-null mice. PPARα-null mice expressed higher levels of γ-Fib. Taken together, these observations and previous results [8,29], confirm the important role that PPARα plays in the inflammatory response, since when PPARα is lacking, the levels of acute-phase proteins increase: A2Mg and fibrinogen (present work and [8,9]), and antitrypsin, ceruloplasmin and haptoglobin [29].

In contrast to the results observed for A2Mg and γ-Fib, the liver expression of C3 was not affected by PPARα activation. Although C3 is a member of the same family as A2Mg [19], present findings

show that it is not affected by PPARα agonists under the same conditions. Such a divergent response between acute-phase proteins, species [29], and even PPARα activators [5], is not surprising and it has been attributed to several possibilities: a) the changes in acute-phase proteins are often species-specific with regard to the magnitude and direction of change [29,42]; b) differences in the duration of PPARα agonist treatment [29,43]; c) the PPARα activator itself, with regard to its different potency and the different spectrum of action that fibrates can display [44]; and d) the diverse molecular pathways and mechanisms that explain the interactions of PPARα with several levels of inflammatory signalling pathways [6]. In fact, the APR may differ according to the inducing agent employed and factors and cytokines involved [45].

Late pregnancy is a hyperlipidemic state that is comparable to that normally seen in type 2 diabetic patients. The reductions in hepatic A2Mg expression found here in pregnant fenofibrate-treated rats, along with the reductions of plasma A2Mg found in hyperlipidemic patients treated with fenofibrate [9], underlie the possible clinical relevance of A2Mg. This possibility is reinforced by the fact that A2Mg expression is modified by PPARα agonists not only in pregnant rats under augmented acute-phase protein synthesis, but also in virgin rats and mice under basal conditions. Since A2Mg is a protein of the APR, these findings highlight the anti-inflammatory potency of PPARα agonists. Interestingly, our results also identify A2Mg as a novel PPARα agonist-regulated gene.

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