Evidence for a new human CYP1A1 regulation pathway involving PPAR-α and 2 PPRE sites

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Evidence for a new human CYP1A1 regulation pathway involving PPARα and two PPRE sites.

Abbreviated title: PPARα regulates CYP1A1 expression

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Key words: CYP1A1, PPARα, PPARγ, PPRE, AhR, cytochrome P450
Abstract

Background & aims: Cytochrome P450 1A1 (CYP1A1) catalyzes the degradation of endobiotics (estradiol, fatty acids...) and the bioactivation of numerous environmental procarcinogens such as arylamines and polycyclic aromatic hydrocarbons that were found in food. Several peroxysome proliferators and arachidonic acid derivatives enhance CYP1A1 activity, but the mechanisms involved remain unknown. The aim of this work was to study the role of PPAR receptors in CYP1A1 gene induction.

Methods: The role of PPAR transcription factors in CYP1A1 induction was assessed by means of Enzymatic activities, QRT-PCR, gene reporter assays, mutagenesis and EMSA.

Results: We demonstrate that PPARα agonists (WY-14643, bezafibrate, clofibrate and phthalate) induce human CYP1A1 gene expression, while 2,4-thiazolidinedione, a specific PPARγ agonist, represses it. The induction of CYP1A1 transcripts by WY-14643 was associated with a marked increase of EROD activity (10-fold at 200 µM). Transfection of PPARα cDNA enhanced CYP1A1 mRNA induction by WY-14643 although WY-14643 failed to activate XRE sequences. Two PPRE sites were located at the positions −931/−919 and −531/−519 of CYP1A1 promoter. Their inactivation by directed mutagenesis, suppressed the inductive effect of WY-14643 on CYP1A1 promoter activation. EMSA and chromatin immunoprecipitation assay experiments demonstrated that the two CYP1A1 PPRE sites bind the PPARα/RXRα heterodimer.

Conclusions: We describe here a new CYP1A1 induction pathway involving PPARα and two PPRE sites indicating that PPARα ligands, which are common environmental compounds, may be involved in carcinogenesis.
**Introduction**

Cytochromes P450 (CYPs) are hemoproteins involved in the metabolism of some endogenous compounds (estradiol, fatty acids) and numerous xenobiotics. CYPs are the main enzymes involved in the bioactivation of environmental procarcinogens. CYP1A1 catalyzes the bioactivation of polycyclic aromatic hydrocarbons (PAHs) and arylamines. PAHs are ubiquitous compounds found in tobacco smoke, industrial workplace, and food. Arylamines are pyrolysis derivatives of proteins. PAHs and arylamines are mainly involved in the etiology of smokers’ lung cancers and colorectal cancers, respectively (1, 2). Moreover, CYP1A1 overexpression was associated with predisposition to various human cancers (3).

CYP1A1 is constitutively expressed in human enterocytes at relatively low level. However, it should be borne in mind that its expression is highly enhanced after exposition to chemical inducers, modulating cellular susceptibility to procarcinogens.

CYP1A1 gene expression is mainly regulated by the Aryl hydrocarbon Receptor (AhR) which is activated by a variety of xenobiotic compounds including dioxins, PAHs (4-6), flavonoids, indole and benzimidazole derivatives (7-9). The AhR is present in cytoplasm as an inactive form associated with two hsp90 and another protein, named AIP, involved in the stability of the complex AhR-hsp90. The ligand binding on AhR induces the dissociation of this complex and activates the receptor. After its activation, AhR is able to translocate in the nucleus, where it dimerizes with its partner, the aryl hydrocarbon receptor nuclear translocator (Arnt). This new complex binds specifically to enhancer DNA sequences within the CYP1A1 promoter called XRE (Xenobiotic Responsive Element).
In spite of the fact that AhR plays a central role in CYP1A1 gene regulation, the activation of this transcription factor cannot always explain the observed CYP1A1 induction. For instance, carbaryl which is not an AhR ligand was found to induce CYP1A1 (10) by a mechanism not yet characterized.

AhR excepted, the other transcription factor involved in CYP1A1 induction was RAR but the level of induction reached was relatively faint (11).

It has been previously demonstrated that induction of CYP1A1 activity can be obtained by cell culture agitation, with a level comparable to those obtained with exposure to 0.1 nM TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxine) (12). Increasing shear stress leads to an increase of arachidonic acid level (13, 14) and arachidonic acid metabolites are PPARα ligands (15). Using arachidonic acid and different phospholipid metabolism inhibitors, Mufti et al. (12) demonstrated that arachidonic acid and derivatives induce CYP1A1 gene activity and that cell stress induces phospholipase D, resulting in the formation of phosphatidic acid which activates phospholipase A2, resulting in the release of arachidonic acid. The metabolism of arachidonic acid probably resulted in a metabolite that, by an as yet unknown mechanism, induced CYP1A1 (12).

Moreover, it was demonstrated that peroxisome proliferators, such as phthalates, can increase the DNA adducts induced by the bioactivation of benzo(a)pyrene which is mainly catalyzed by CYP1A1 (16). The mechanism involved in this increase of DNA adducts was not characterized. So, phthalates appeared to be inducers of CYP1A1 activity. To date, it has been demonstrated that some phthalate derivatives are potent agonist ligands of PPARα and to a lesser extent of PPARγ (17). We hypothesized that the potentiation of benzo(a)pyrene DNA adducts by phthalates could be related to CYP1A1 induction via the PPAR pathway.
The aim of this study was to evaluate the effect of PPARα ligands such as WY-14643 (a specific PPARα ligand), Bezafibrate (BZF), Clofibrate (CF), Mono(2-ethylhexyl)phthalate (MEHP) or specific PPARγ ligand such as Thiazolidinedione (TZD) on CYP1A1 expression in CaCo-2 cells. We demonstrated that CYP1A1 is highly inducible by PPARα ligands. This induction specifically involves the transcription factor PPARα and requires 2 PPRE sites located within the CYP1A1 promoter (positions –931/-919 and –531/-519). Our data characterize a new PPARα-mediated CYP1A1 regulation pathway. This may be the basis of carcinogenesis by many environmental compounds acting as PPARα ligands.


**Materials and Methods**

**Chemicals** WY-14643 were purchased from VWR (France), 2-4, thiazolidinedione, 3-methylcholanthrene (3-MC), clofibrate (CF), bezafibrate (BZF) and dimethylsulfoxide were purchased from SIGMA (France) and Mono(2-ethylhexyl)phthalate (MEHP) from TCI Europe (Belgium).

**Cell culture and treatments** Human colic adenocarcinoma CaCo-2, the hepatoma HepG2, the adenocarcinoma A549 cells and the primoculture of human keratinocytes were used. As soon as the CaCo-2 cells reached confluence, the culture medium was changed and 24 h later, they were further treated during 6 h with either the PPARα agonists such as WY-14643 (10, 30, 100, 200, and 400 µM). 100 µM MEHP, 50 µM clofibrate, 50 µM bezafibrate or the PPARγ agonist 2, 4-Thiazolidinedione (200 µM) or the AhR ligand 3-MC (1 µM). In the same conditions, HepG2, keratinocytes and A549 were treated with 200 µM of WY-14643 and 1 µM 3-MC. 3-MC was used as control CYP1A1 inducer.

**QRT-PCR experiments** Total RNA was isolated from cells using Nucleospin RNAII (Macherey Nagel, France). One µg of total RNA was reverse-transcribed in 30 µl using GibcoBRL M-MLV reverse-transcriptase (Life Technologies, Cergy Pontoise, France) in its own buffer and random primers at 37°C for 1 h. CYP1A1 mRNA expression normalized to β2-microglobulin expression was determined using the LightCycler System (Roche Diagnostic, France) and the Faststart DNA master SYBRGreen I Kit (Roche diagnostics). The primers for CYP1A1 and β2-microglobulin were as follow:
CYP1A1-S: 5’AAGAGGAGCTAGACACAGT3’
CYP1A1-AS: 5’GAAACCGTTCAGGTAGGA3’
β2m-S: 5’CCGACATTGAAGTTGACTTAC3’
β2m-AS: 5’ATCTTCAAACCTCCATGATG3’

PCR was performed with 5 mM MgCl₂, 0.25 µM of each primer and LightCycler FastStart DNA Master SYBR Green I mix in a total volume of 16 µl. Cycling conditions were as follows: 10 min denaturation at 95°C, followed by 45 cycles of 10s denaturation at 95°C, 8 s primer annealing at 55°C and 8 s of fragment elongation at 72°C. The melting curve was analyzed with the LightCycler software and quantitation was done with RelQuant software (Roche Diagnostic, France).

Three independent experiments were at least realized in triplicate.

**EROD activity** CaCo-2 cells were either untreated or treated with different concentrations of WY-14643 (10, 30, 100 and 200 µM). Ethoxyresorufin O-Deethylase (EROD) activity, mainly supported by CYP1A1, was determined according to the method of Burke et al. (18), slightly modified by Kennedy et al. (19). The fluorescence of resorufin produced during the reaction was measured using a CYTOFLUOR Multi-well plate reader series 400 (PerSeptive Biosystems) with 530 nm excitation and 590 nm emission wavelengths. Fluorescence values were converted to picomoles using a calibration curve of resorufin fluorescence and EROD activity was expressed as pmol of resorufin/min/mg total cellular protein.

**Cloning of CYP1A1 XRE sequences** Sense and antisense oligonucleotides containing two CYP1A1 XRE sites were synthesized with addition of Hind III and Xba I restriction sites at the 5’ and 3’ ends respectively. After hybridization and their
digestion by Hind III and Xba I, these two oligonucleotides were cloned in the corresponding sites of the pBLCAT2 vector containing a thymidine kinase promoter (TK). This construction was named XRE-TK-CAT. The oligonucleotide sense used is:
5’CCGCCCAAGCTTCCTCCCCCTCCGCGTGACTGCGAGGGGAAGGAGGGCGTG
CCACACGTCTAGACTAGCT3’. The XRE sites are in bold letters and the Hind III and Xba I restriction sites are in italic characters and underlined.

**CAT assays** The chimeric construction pRNH25c containing the –1140/+80 region of CYP1A1 gene driving chloramphenicol acetyltransferase (CAT) gene expression was used. Cells in 6 well-plates were transiently transfected with 1 µg of pRNH25c using lipofectin (Life Technologies, France). The transfection was performed as specified by the supplier. 24h after the end of the transfection, cells were treated during 48 h with 200 µM WY-14643 or 1 µM 3-MC. CAT expression was then evaluated by the amount of CAT protein using the CAT Elisa System (Roche Diagnostic, France).

A similar experiment was realized with a construct coding PPARα. Transfected cells with 1 µg of cDNA or untransfected cells were then either untreated or treated with 200 µM WY-14643 during 48 h. CYP1A1 mRNA level was determined as described above.

Three independent experiments were at least realized in triplicate.

**Site-Directed mutagenesis of the CYP1A1 promoter** The PPRE mutations of pRNH25c were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, France) to obtain the pRNH25c(ΔPPRE1), the pRNH25c(ΔPPRE2) and the pRNH25c(ΔPPRE1+2). The primers sense and antisense were used for
mutagenesis. The primers sens were as follows (the PPRE core was underlined and bold letters represent mutated nucleotides compared to the wild type sequence (Wt)):

Wt 1: 5’GGACGGGGCCGCTGACCTCTGCCCCCTAGAGGGATGTCG3’

PPRE1 muts : 5’GGACGGGGCCGCTGACCTC GATCCCCTAGAGGGATGTCG3’

Wt 2: 5’ GGCCTTCCGCCCCGTGACCTCAGGGCTGGGGTCGACG 3’

PPRE2 mut-s : 5’GGCCTTCCGCCCCGTG A T CAGGGCTGGGGTCGACG 3’

Presence of the mutations was verified by DNA sequencing. Cells were transfected by either the pRNH25c or pRNH25c(ΔPPRE1) or pRNH25c(ΔPPRE2) or pRNH25c(ΔPPRE1+2) and after a 48 h-treatment with WY-14643 (200 µM) the CAT expression was evaluated as described above.

**In vitro translation and electromobility shift assays** Electromobility shift assays were performed using PPARα and RXRα prepared by in vitro translation using a coupled transcription-translation system (Promega, France). Proteins were incubated for 20 minutes at room temperature with 50,000 cpm of T4 polynucleotide kinase-labeled oligonucleotides in 10 mM Tris (pH8.0), 100 mM KCl, 10% glycerol, 1 mM dithiothreitol, 1 µg poly (dIdC) and 0.5 µg of salmon sperm. The mixture was then submitted to electrophoresis on a 4% polyacrylamide gel in 45 mM Tris Base, 45 mM boric acid, 1 mM EDTA. The following oligonucleotides were used either as radiolabeled probes or as competitors (the sense strand is shown, with core sequence underlined and mutation in bold face): PPRE Cons, 5’-CCGCCAAGCTTGCTCCGCCCCAGGTCACAGGTCACTAG; CYP1A1-PPRE1, 5’-GGACGGGGCCGCTGACCTCTGCCCCCTAGAGGGATGTCG; CYP1A1-PPRE2, 5’-GGCCTTCCGCCCCGTGACCTCAGGGCTGGGGTCGACG; CYP1A1-PPRE1 mutant, 5’-GGACGGGGCCGCTGACCTC GATCCCCTAGAGGGATGTCG; CYP1A1-
PPRE2 mutant, 5'-GGCCTTCCGGCCGATGACATTAGGGCTGGGGTCGCAGC.

Anti-PPARα antibody (Santa Cruz Biotechnology) was used for the “supershift” assays. Autoradiography was carried out by exposing the dried gel to Kodak X-AR film.

**Chromatin ImmunoPrecipitation assay (ChIP)** Confluent CaCo-2 cells (4 x 100mm dish) were treated with or without WY-14643 200 µM for 1 hour. Cells were then fixed with formaldehyde at 37°C for 15 min. The reaction was then stopped by addition of 1ml 1 M glycine and incubation for 15 min at room temperature. Cells were harvested, and pooled. Soluble chromatin prepared with a chromatin immuno-precipitation assay kit (Upstate Biotechnology Inc) was precleared for 1 h with salmon sperm DNA-BSA/Protein A–Sepharose. Precleared chromatin was then incubated for 16 h with 10 µg of anti-human PPARα antibodies (N-19 from Santa Cruz Biotechnology, or MIA-822 from Affinity BioReagents) or 10 µg of control mice IgG antibody (Sigma). Immune complexes were collected with 50 µl of Protein G-Sepharose (Santa Cruz Biotechnology) supplemented with salmon sperm DNA (200 µg/ml) and BSA (1 mg/ml) and purified according to manufacturer’s recommendations (Upstate Biotechnology Inc). Purified DNA samples (phenol-chlorophorm-isoamyl alcohol and further on Qiagen columns) were used as template for PCR performed for 40 cycles (Pfx Taq DNA polymerase, Invitrogen). Similarly purified DNA fragments from the chromatin extracts (input) were used as control (PCR performed for 30 cycles). Cycling conditions were as follows: 3 min denaturation at 93°C, followed by 30 or 40 cycles of 30 s denaturation at 91°C, 30 s primer annealing at 55°C and 1 min of elongation at 72°C. Primers used for proximal CYP1A1 PPREs containing promoter region amplification were
5’-GCCTCCGGTGCTCTCACAC-3’ and 5’-CGCTACAGGCCTACCAGGACT-3’ (-1000 to –413 upstream of the transcriptional start site), while primers used for distal CYP1A1 promoter region (harboring no PPRE sites) were 5’-CACAACTTGCACGTGCCCTTA-3’ and 5’-CCAACAGGCTCATTGAGAAGG-3’ (-4053 to -3780 upstream of the transcriptional start site).

**Statistical analysis** Statistical analysis were performed using the Newman-Keuls test. Data were considered statistically significant at a p<0.05. Significant results were noted with * or ** or *** for a p<0.05, p<0.01 and p< 0.001 respectively.

**Results**

**Induction of CYP1A1 gene by PPAR ligands**

CaCo-2 cells were treated during 6 h with increasing concentrations of WY-14643 (10-400 µM), or 50 µM BZF, or 50 µM CF, or 100 µM MEHP, or 200 µM TZD or 1 µM 3-MC. CYP1A1 mRNA was then evaluated by quantitative real-time (QRT-PCR) analysis. The results presented in Fig.1A show that TZD does not increase and even decreased CYP1A1 gene expression. In contrast, CYP1A1 mRNA levels increased after treatments with the PPARα ligands such as WY-14643 or BZF or CF or MEHP. The WY-14643 CYP1A1-mediated induction was dose dependent and reached the plateau at 200 µM (6-fold).

The results presented in Figure 1B also show that CYP1A1 expression was induced after 200 µM WY-14643 treatment of various human cell lines other than CaCo-2 including HepG2 (hepatoma), in a primary culture of human keratinocytes.
and A549 (lung adenocarcinoma). However, the CYP1A1 inductions obtained in these cells were lower than in CaCo-2.

**Induction of CYP1A1 EROD activity by WY-14643**

The EROD activity was analyzed after treatment of CaCo-2 cells with increasing concentrations of WY-14643 (10-200 µM). The results, shown in Fig. 2, indicate that the CYP1A1 EROD activity was dose dependently induced by WY-14643 (10-fold at 200 µM) and that the induction obtained with 200 µM WY-14643 was similar to those obtained with 1 µM 3-MC (12.6-fold).

**WY-14643 did not stimulate the XRE sequence**

By means of CAT assay analysis, we investigated whether WY-14643, a potent PPARα ligand, was able to stimulate the XRE target sequences of AhR. Cells were treated either with 1 µM 3-MC, or 200 µM WY-14643 after transient transfection with a XRE-TKCAT construct. Figure 3A shows that 3-MC, a classic AhR ligand, activated CAT expression 2.1-fold, while WY-14643 did not increase CYP1A1 gene expression via the AhR signaling pathway.

**Transfection with PPARα cDNA increases the CYP1A1 induction by WY-14643**

To strengthen the implication of PPARα in CYP1A1 induction by WY-14643, PPARα transfected CaCo-2 cells were treated with WY-14643. Fig. 3 B shows that PPARα transfection doubled CYP1A1 mRNA induction by 200 µM WY-14643 (10-fold) as compared to cells receiving the reporter gene alone (6-fold).
Two PPRE sequences located within the CYP1A1 gene promoter are involved in the CYP1A1 induction by WY-14613

CYP1A1 promoter gene analysis, using Mathinspector (20), revealed that two putative PPRE sites are located within the promoter at positions −931/−919 (CYP1A1PPRE1) and −531/−519 (CYP1A1PPRE2) respectively.

To confirm that PPARα ligands were able to induce CYP1A1 mRNA by activation of its promoter, we transfected CaCo-2 cells with either the pRNH25c construct containing the wild type CYP1A1 promoter or with the pRNH25c construct where either the PPRE1 (pRNH25cΔPPRE1) or the PPRE2 (pRNH25cΔPPRE2) or the PPRE1+2 (pRNH25cΔPPRE1+2) sequences were mutated. Cells were then treated with either 200 µM WY-14643, or 1 µM 3-MC for 48 h. Figure 4 shows that WY-14643 and 3-MC efficiently induced the transactivation of the CYP1A1 wild type promoter pRNH25c (2.4-fold with 200 µM WY-14643 and 4.6-fold with 1 µM 3-MC). The mutation of one of the two CYP1A1 PPRE sites led to a decrease of CYP1A1 promoter activation by WY-14643. Indeed, the induction of CAT expression was lower using the pRNH25cΔPPRE1 or pRNH25cΔPPRE2 constructs (1.6-fold and 1.4-fold respectively) as compared to the pRNH25c construct (2.4-fold). When the two sites were mutated (pRNH25cΔPPRE1+2) the decrease of WY-14643 effect was higher (1.1-fold at 200 µM WY-14643).

The two CYP1A1 PPRE sites were able to bind PPARα

We first checked the binding of an in vitro translated PPARα-RXR heterodimer to the two CYP1A1 putative PPRE sequences by gel shift assay (Fig. 5). As expected, a specific retarded band was observed when PPARα and its partner RXR were incubated together with the target oligonucleotide (lane 5 and 11), but not when
these receptors were incubated separately (lanes 3, 4 and 9, 10). We confirmed that the shift obtained using the CYP1A1 PPRE1 and PPRE2 was the consequence of the binding of PPARα using a specific PPARα antibody which produced a super shift shown in lanes 6 and 12.

In addition, the specific PPARα/RXRα-PPRE complex was repressed in a dose-dependant manner when incubated in the presence of 10-fold (Fig. 6 lanes 7, 9, 11) or 50-fold (lanes 6, 8, 10) excess of unlabeled consensus PPRE or unlabelled CYP1A1-PPRE1 or CYP1A1-PPRE2 sequences.

In the same conditions, a 50-fold excess of mutated PPRE1 or PPRE2 CYP1A1 sequences did not affect the binding of PPARα/RXRα on the PPRE consensus core (Fig 6 lanes 12 and 13 respectively). A supershifted band appeared using a PPARα antibody (lane 14).

These observations showed that the in vitro translated heterodimer PPARα/RXRα binds to the two PPRE1 and PPRE2 sites within the CYP1A1 gene promoter.

To strengthen our demonstration, ChIP analysis was performed using CaCo-2 cells untreated or treated with 200 µM WY-14643 for 1 h. We showed (Figure 7) that the PPARα/RXRα heterodimer binds to the PPRE sites located within the proximal CYP1A1 gene promoter.
Discussion

We characterized a new CYP1A1 regulation pathway involving the binding of PPARα transcription factor on two PPRE sequences located within the 5’ untranslated region of CYP1A1 gene.

The effects of agonists of PPARα (WY-14643, CF, BZF and MEHP) and PPARγ (TZD) on CYP1A1 mRNA expression levels were evaluated in Caco-2 cell line by real time QRT-PCR analysis. WY-14643 induced CYP1A1 mRNA in a dose dependent manner, up to 6-fold as soon as 200 µM. Similarly, CYP1A1 mRNA level was increased after 6h treatment with other PPARα ligands (CF, BZF, MEHP). The induction factors were lower than those observed with WY-14643, which is one of the most potent agonists of PPARα. These results confirmed that CYP1A1 is induced by different classes of PPARα ligands including the fibrates and phthalates. In contrast, TZD led to a marked decrease of CYP1A1 transcript. Such an antagonist regulatory effect of these two transcription factors (PPARα and PPARγ) has already been described for some genes, such as VCAM-1 (21, 22). However, the repressive effect of PPARγ on CYP1A1 expression remains to be clarified and is presently under investigation in our laboratory. Moreover, transfection of PPARα cDNA leads to a higher induction with WY-14643, strengthening the involvement in Caco-2 cell line of PPARα in CYP1A1 regulation.

The CYP1A1 induction by PPARα was not restricted to CaCo-2 cells as similar results were obtained with HepG2 hepatoma cells, A549 lung adenocarcinoma cells, and primary cultures of human keratinocytes. The effect of PPARα ligands on CYP1A1 induction appears to be a general phenomenon and not restricted to colon cell lines.
The induction of CYP1A1 mRNA by WY-14643 was associated with an increase of EROD activity. This induction of EROD activity was similar to that obtained with 1 µM 3-MC. Therefore, PPARα agonists may increase the genotoxic effect of procarcinogens bioactivated by CYP1A1, such as PAH or arylamines. Such a phenomenon was previously described with two peroxisome proliferators, silvex and di(2-ethylhexyl)phthalate, which enhance BAP-induced DNA adducts (16).

To date, PPARα ligands belonging to the fibrates class have been widely used as hypolipidemic drugs, and have never been implicated in human carcinogenesis, although they are well known hepatocarcinogens in rodents. The lack of such a side effect in humans may be related to the poor expression of PPARα in liver. Our data suggest that expositions to environmental PPARα ligands, including fibrate and chemical compounds such as phthalates or chlorophenoxyacetic pesticides, may increase CYP1A1 extrahepatic expression. Therefore, cells harboring a higher CYP1A1 expression would have a greater susceptibility to the genotoxic effect of CYP1A1 bioactivated procancerogens.

Since CYP1A1 is mainly regulated by the Ah receptor, it was logical to assume that WY-14643 could have a direct or indirect action on this transcription factor. However, using XRE-TK-CAT construct we showed that WY-14643 failed to activate XRE site, demonstrating that CYP1A1 induction by PPARα ligand did not involve AhR.

Sequence analysis of CYP1A1 promoter revealed the presence of two putative PPRE sites. Indeed, treatment with WY-14643 increased CAT expression when cells were transiently transfected with pRNH25c harboring the wild-type CYP1A1 promoter. The results obtained in Fig. 4 clearly demonstrate that the mutation of one PPRE site (ΔPPRE1 or ΔPPRE2) reduces the CAT induction while no induction
occurred with pRNH25c (ΔPPRE1+2). So, it appears that the two PPRE sites located within the promoter at positions −931/-919 (CYP1A1 PPRE1) and −531/-519 (CYP1A1 PPRE2) respectively were necessary for the CYP1A1 induction by PPARα ligands.

The direct role played by these 2 PPRE sites in the binding of PPAR transcription factor was evaluated first by EMSA. We found that even if the two sites diverge from the canonical DR1 sequence they are still able to bind PPARα with good efficiency. PPARα binding is no longer possible when mutations are introduced in these CYP1A1 PPRE sites. In order to demonstrate the in vivo relevance of these PPRE sites, we performed ChIP assays. We immunoprecipitated the human CYP1A1 gene promoter using two different anti-human PPARα antibodies, demonstrating that PPARα/RXRα heterodimer binds in vivo on the CYP1A1 PPRE sites. We actually showed that with or without WY-14643 treatment, PPARα/RXRα bound to the PPRE sites within the CYP1A1 promoter. This binding without any treatment has already been shown in CaCo-2 for the carnitine palmitoyltransferase II gene (23). Recently, with a ChIP assay performed on mouse liver extract, IJpenberg et al (24) did not observe any PPARα/RXRα binding on PPRE site of malic enzyme gene in untreated mice but had to treat the animals with WY-14643 to visualize such a binding. Inversely, Jia et al (25) demonstrated in mouse liver extract, that PPARα/RXRα was already bound to PPRE site of L-PBE gene. Mouse treatment with WY-14643 seemed to increase this binding, but it is generally accepted that ChIP technology is qualitative but not quantitative.

To date, it has not been clearly documented whether, without any ligand the PPARα/RXRα heterodimer is unbound or bound to PPRE sites in an inactive conformation as has been largely described for RAR (26).
of PPARα/RXRα on PPRE sites in the presence or absence of PPARα ligands is dependent on the nature of the tissue, the cell type and the gene studied.

The PPRE2 site appears to have an inverted orientation compared to a canonical consensus site while PPRE1 site displays a conventional orientation. Sequence alignment of CYP1A1 promoter (Figure 8) from various species demonstrates that PPRE1 and PPRE2 sites are highly conserved in human, rat and mouse species. Rat and mouse harbor the same PPRE1 and PPRE2 sequences and differ from humans in that they exhibit a unique base transition. Interestingly, the flanking regions are also conserved. Moreover, when sequence alignment was performed with CYP1A1 promoter from fish species, such as *Anguilla japonica* or *Microgadus tomcod*, we were unable to localize any PPRE sites, while several XRE sites are located within these promoters (data not shown). PPAR orthologs have already been described in fish species. Therefore the CYP1A1 regulation by PPARα occurred tardily in the evolution of species, contrary to the regulation of CYP1A1 by AhR.

Until now, only the CYP4A, involved in fatty acid biotransformation, was known to be induced by PPARα ligands within the CYPs superfamily, (27). Since CYP1A1, largely concerned in procarcinogens activation, is also implicated in fatty acid metabolism (28), it was logical to find its expression regulated through PPARα. Moreover, CYP1A1 modification level could have a physiological impact in terms of fatty acid metabolite levels.

Until now, CYP1A1 was known to be expressed and induced by the transcription factor AhR. This is the first time that another strong CYP1A1 induction pathway, apart from AhR, has been described. We demonstrated that CYP1A1 was highly induced by PPARα via two PPRE sites within the promoter. The upregulation
of CYP1A1 by PPARα ligands may be involved in human carcinogenesis, notably in colon through an increase of CYP1A1-bioactivated procancerogens present in diet, such as arylamines.
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Figure 1

A

B

CYP1A1 mRNA fold induction

CaCo-2
HepG2
Keratinocytes
A549

CYP1A1 mRNA (fold induction)
Figure 2
Figure 3

- PPARα cDNA

+ PPARα cDNA
Figure 4
Figure 5

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Figure 6
Figure 7

PCR – 4053 to -3780

PCR – 1000 to -413
CYP1A1 PPRE1

Human CYP1A1 (D10855) -940 a c g g c g c c c T G A C C T c T G C C C c t a g a g g g a T -908
Rat CYP1A1 (M14633) -970 a c a g g c a a c c C G A C C T c T G C C C c a g a g g a t g G -938
Mouse Cyp1a1 (AF210905) -942 a c a g g c a g c c C G A C C T c T G C C C c a g a g g a t g g -910

CYP1A1 PPRE2

Human CYP1A1 (D10855) -541 t c c g g c c c g T G A C C T c A G G G C T g g g t c g c a g -509
Rat CYP1A1 (M14633) -591 t c c t g c c c t g T G A C C T c T G G G C T g g g t c g c t g -559
Mouse Cyp1a1 (AF210905) -533 t c c t g t c c t g T G A C C T c T G G G C T g g g t c g t t g -501

Figure 8
Figure Legends

Figure 1. (A): CYP1A1 mRNA levels in CaCo-2 cells. CYP1A1 mRNA was evaluated by QRT-PCR after cell treatment during 6 h with either WY-14643 (10, 30, 100, 200, 400 µM), or 50 µM BZF, or 50 µM CF, or 100 µM MEHP; or 200 µM TZD; or 1 µM 3-MC. C: control. **: p<0.01 compared with control. ***: p<0.001 compared with control. (B): Effect of WY-14643 on CYP1A1 expression in different cell lines. CYP1A1 mRNA was evaluated by QRT-PCR after cell treatment during 6 h with 200 µM WY-14643 or 1 µM 3-MC respectively in HepG2, keratinocytes, and A549.

Figure 2. Evaluation of EROD activity. EROD activity was evaluated after CaCo-2 cells treatment during 48 h with different WY-14643 concentrations (10, 30, 100, 200 µM) or 1 µM 3-MC. C: control. **: p<0.01 compared with control.

Figure 3. (A): Effect of WY-14643 on XRE activation. CaCo-2 cells were transfected with a construct where XRE drives CAT expression. After transfection, cells were treated 48 h with 200 µM WY-14643 or 1 µM 3-MC. CAT expression was evaluated using the CAT Elisa System. *: p<0.05 compared with control. (B): CaCo-2 cells were either untransfected or transfected with cDNA coding the PPARα and untreated or treated with 200 µM WY-14643 during 48 h. CYP1A1 mRNA level was then evaluated by Q-PCR as described in material and methods. ***: p<0.001 compared with cells which have been transfected with the empty vector and treated with WY-14643.
Figure 4. Effect of PPRE mutations on CYP1A1 promoter activation. CaCo2 cells were transfected with the wild-type pRNH25c (A), or the pRNH25c(ΔPPRE1) (B), or the pRNH25c(ΔPPRE2) (C), or the pRNH25c(ΔPPRE1+2) (D). Cells were then either untreated (C) or treated with 200 µM WY-14643 during 48 h. CAT expression was evaluated by CAT ELISA System. *: p<0.05 compared with control. ***: p<0.001 compared with control.

Fig 5. EMSA using CYP1A1 specific PPRE 1/2 sequences. RXRα, PPARα or both were produced by reticulocytes and incubated with CYP1A1 PPRE1 (lanes 1-6), or CYP1A1 PPRE2 (lanes 7-12). Lanes 1 and 7: free probe; lanes 2 and 8: TNT; lanes 3 and 9: RXRα alone; lanes 4 and 10: PPARα alone; lanes 5 and 11: PPARα/RXRα heterodimer; lanes 6 and 12: PPARα/RXRα heterodimer + PPARα antibody. S: shifted band; SS: super shifted band; NS: unspecific band.

Figure 6. Effect of unlabelled probes on EMSA. RXRα, PPARα lanes 3, 4 or both lanes 5-14 were produced by reticulocytes and incubated with PPRE consensus sequence. Competition with unlabeled 50-fold or 10-fold PPRE consensus lanes 6, 7 or CYP1A1 PPRE1 lanes 8, 9 or CYP1A1 PPRE2 lanes 10, 11 or 50-fold of mutated CYP1A1 PPRE 1 lane 12 or CYP1A1 PPRE2 lane 13 were realized. Lane 1 corresponds to the free PPRE probe and lane 14 shows a super shift obtained with a specific PPARα antibody. S: shifted band; SS: super shifted band; NS: unspecific band.
Figure 7. Chromatin immunoprecipitation assays of the CYP1A1 promoter in CaCo2 cells. CaCo2 cells were treated with or without 200 µM WY-14643 for 1 h. Soluble chromatin was immunoprecipitated with mice IgG (lanes 5-6), or antibodies against human PPARα (N19, lane 3-4; or MIA-822, lanes 7-8). Immunoprecipitates were analyzed by PCR with specific primers for the distal (-4053 to –3780) or proximal (-1000 to –413) CYP1A1 promoter as indicated. PCR was performed with total chromatin input (lanes 1-2).

Figure 8. Sequence alignment of CYP1A1 promoter from human, rat and mouse. Sequence alignment was performed using LFASTA analysis (http://www.infobiogen.fr/services/analyseq/cgi-bin/lfastan_in.pl). Gene accession numbers are noted in brackets. PPRE sites are noted in bold capital letters, and mismatches are underlined.