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Clofibrate and perfluorodecanoate both upregulate the expression of the pregnane X receptor but oppositely affect its ligand-dependent induction on cytochrome P450 3A23

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Abstract

The pregnane X receptor (PXR) interacts with a vast array of structurally dissimilar chemicals and confers induction of several major types of drug metabolizing enzymes such as cytochrome P450s (CYP). We previously reported that the expression of PXR was markedly increased in rats treated with clofibrate and perfluorodecanoic acid (PFDA). The present study was undertaken to test the hypothesis that induced expression of PXR increases PXR ligand-dependent induction on CYP3A23. Rat hepatocytes were treated with clofibrate or PFDA individually, or along with PXR ligand pregnenolone 16α-carbonitrile (PCN), and the levels of PXR and CYP3A23 were determined by Western blots. Both clofibrate and PFDA markedly increased the expression of PXR with PFDA being more potent, and the induction was abolished by actinomycin D, an inhibitor for mRNA synthesis. As expected, PCN alone markedly induced the expression of CYP3A23. Interestingly, co-treatment with clofibrate enhanced the induction, whereas co-treatment with PFDA suppressed it. Clofibrate and PFDA represent multi-classes of chemicals called peroxisome proliferators including many therapeutic agents and industrial pollutants. The opposing effects of clofibrate and PFDA on the PCN-induced expression of CYP3A23 suggest that peroxisome proliferators likely increase the expression of PXR but differentially alter its ligand-dependent induction. The interaction between PXR inducer and ligand provides a novel mechanism on how functionally and structurally distinct chemicals cooperatively regulate the expression of xenobiotic-metabolizing enzymes and transporters.

Keywords: PXR; CYP3A23; Clofibrate; Perfluorodecanoic acid; Pregnenolone 16α-Carbonitrile; Induction

1. Introduction

The pregnane X receptor (PXR) is a key regulator of genes encoding several major types of xenobiotic-metabolizing enzymes and transporters [1–4]. Structurally PXR belongs to a superfamily of nuclear receptors with two functional domains that mediate DNA and ligand binding, respectively [2]. The DNA binding domain is highly conserved from species to species, whereas the ligand-binding domain is relatively diverse [2]. PXR heterodimerizes with retinoic X receptor-α and the resultant heterodimers bind specifically to DNA elements with a half site AGGTCA. The half site sequence varies slightly in some target genes and is arranged in various configurations (e.g., direct repeats spaced by three or four nucleotides, DR3 or DR4), resulting in the formation of varying DNA elements [2,5,6]. The ligand-binding domain interacts with a ligand, initiating dissociation with co-repressors, and simultaneously association with co-activators [7,8]. In contrast to other nuclear receptors, PXR has a rather large ligand-binding pocket, which is spherical in shape, extremely hydrophobic and expandable [9–11]. The structural features of the ligand-binding pocket allow PXR to interact with a vast array of chemicals with dissimilar structure [12–14], and the ability to bind to varying DNA elements enables PXR to have a wide range of target genes with distinct physiological functions [5,15,16]. Among them, the CYP3A23 gene is considered one of the most sensitive targets [2].

Abbreviations: CYP, cytochrome P450; DMSO, dimethyl sulfoxide; MDR-1, multidrug resistance-1; PCR, polymerase chain reaction; PFDA, perfluorodecanoic acid; PXR, pregnane X receptor; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WME, Williams' Medium E

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The expression of PXR itself, like its target genes, is drastically altered by certain xenobiotics as well as conditions that induce acute phase response (APR) [17–20]. The levels of PXR mRNA are rapidly decreased in both rats and mice treated with APR-inducing agent lipopolysaccharide. The decrease is more profound in mice than rats (96% versus 50%), even though mice receive less lipopolysaccharide (0.1 mg/kg versus 0.5 mg/kg) [21,22]. With human hepatocytes, pro-inflammatory cytokine interleukin-6 has been shown to markedly reduce the levels of PXR mRNA [18]. More importantly, suppressed expression of PXR is accompanied by reduced induction on the expression of PXR-regulated genes such as CYP3A23 [21,22]. In contrast to suppressed expression, several chemicals are shown to markedly increase PXR expression. In rats treated with hypolipidemic agent clofibrate or industrial additive perfluorodecanoic acid (PFDA), for example, the levels of PXR are markedly increased [23,24]. The increased expression appears to be regulated at the transcriptional level as the increase in PXR protein is correlated well with the increase in PXR mRNA. The induction shows no tissue-dependence and occurs in both hepatic and extrahepatic tissues [24]. The PXR protein is normally undetectable in the kidney, whereas it is readily detected in this organ from rats treated with clofibrate or PFDA [24].

The present study was undertaken to test the hypothesis that increased expression of PXR by clofibrate and PFDA enhances PXR ligand-dependent induction on the expression of CYP3A23. Rat hepatocytes were treated with clofibrate or PFDA individually, or along with PXR ligand pregnenolone 16α-carbonitrile (PCN), and the levels of PXR and CYP3A23 were determined. Both clofibrate and PFDA markedly increased the expression of PXR, and the induction was abolished by actinomycin D, an inhibitor for mRNA synthesis. Neither clofibrate nor PFDA alone caused changes on the basal expression of CYP3A23. However, both chemicals markedly altered PCN-induced expression of this enzyme. Clofibrate potentiated the induction whereas PFDA suppressed it. The opposing effects by clofibrate and PFDA on the expression of CYP3A23 provide an example on the complexity regarding the regulated expression of xenobiotic-metabolizing enzymes and transporters.

2. Materials and methods

2.1. Chemicals and supplies

Actinomycin D, clofibrate, dexamethasone, PCN, PFDA, puromycin, HBSS, WME and β-actin antibody were purchased from Sigma (St. Louis, MO). Insulin-transferrin-selenium G supplement (ITS) was from Invitrogen (Carlsbad, CA). Collagenase (Type I) was from Worthington Biomedical Corporation (Lakewood, NJ). The goat anti-rabbit IgG conjugated with horseradish peroxidase and chemiluminescent kit were from Pierce (Rockford, IL). Fetal bovine serum was from HyClone (Logan, UT). The CYP3A23 antibody was purchased from Research Diagnostics (Flanders, NJ). Unless otherwise specified all other reagents were purchased from Fisher Scientific. Male Sprague–Dawley rats (∼220 g) were purchased from Harlan Teklad (Madison, WI). The use of animals was approved by the Institutional Animal Care and Use Committee.

2.2. Hepatocyte culture and treatment

Primary cultures of hepatocytes were isolated from rats by a modified two-step collagenase digestion method, essentially as described previously [25]. Rat liver was perfused through the portal vein with calcium-free HBSS buffer containing 0.5 mM EGTA for 1–2 min at a flow rate of 28 ml/min until the liver was completely blanched (1–2 min), followed by perfusion for 5–8 min with WME media containing collagenase (150 U of collagenase activity/ml of medium). The liver was further perfused with calcium-free HBSS containing EGTA. Hepatocytes were dispersed from the digested liver in WME without collagenase and washed by low speed centrifugation (100–150 × *g* for 5 min) for three times. The resulting cell pellet was then suspended in WME media containing 10% fetal bovine serum, ITS supplement and dexamethasone [25], and viability was determined by trypan blue exclusion. Hepatocytes were then plated onto collagen coated culture plate at a density of 6×10^5 hepatocytes/per well (12-well plate). The cells were allowed to attach for 3–4 h at 37 °C in a humidified chamber with 95%/5% air/CO_2. Culture plates were then gently swirled and the medium containing unattached cells was then aspirated. Fresh WEM containing ITS supplement and dexamethasone (100 nM) was added to each well, and the cultures were returned to the humidified chamber. Unless otherwise specified, the cells were maintained for 48 h before initiating treatment with chemicals. Groups of cultures ($n = 3$ individual rats) were then treated for 24–72 h with clofibrate, PFDA, PCN, or in combined form with daily changes of medium containing appropriate chemicals or solvent (DMSO at a final concentration of 0.1%).

2.3. Western analysis

Hepatocytes were washed once with cold PBS and lysed in Tris–HCl buffer (50 mM, pH 7.4) containing SDS and Triton X-100 (0.1% each). The cell lysates were subjected to centrifugation at 12,000 × *g* for 15 min at 4 °C to remove insoluble precipitates. Cell lysates (usually 6 μg) were resolved by 7.5% SDS-PAGE in a mini-gel apparatus and transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA). After non-specific binding sites were blocked with 5% non-fat milk, the blots were incubated with an antibody against

PXR, CYP3A23 or β-actin. The anti-PXR antibody was raised against a synthetic peptide from rat PXR and recognized rat but not human PXR [24]. The anti-CYP3A23 antibody recognized a single protein band with a molecular weight of ~52 kDa in the lysates of hepatocytes. This antibody showed no cross-reactivity toward CYP3A2, a closely related enzyme of CYP3A23. The primary antibodies were subsequently localized with the goat anti-rabbit IgG conjugated with horseradish peroxidase [26]. Horseradish peroxidase activity was detected with a chemiluminescent kit. The chemiluminescent signal was captured by KODAK Image Station 2000 and the relative intensities were quantified by KODAK 1D Image Analysis Software.

2.4. Reverse transcription coupled polymerase chain reaction (RT-PCR)

The mRNA levels of CYP3A23 were determined by RT-PCR experiments with a ThermoScript I kit (Invitrogen) as described previously [27]. Total RNA (2 μg) was subjected to the synthesis of the first strand cDNA in a total volume of 30 μl with random primers and ThermoScript I reverse transcriptase. The reactions were incubated initially at 50 °C for 30 min, and then at 60 °C for 60 min after additional reverse transcriptase was added. The cDNAs were then diluted (100 ×) and subjected to PCR amplification (10 μl of the diluted cDNA). The cycling parameters were: 95 °C for 30 s, 52 °C for 30 s and 68 °C for 30 s for a total of 23 cycles. The primers for PXR amplification were: 5′-GATGTGTCAACCTACATGTTCAAG-3′ (forward) and 5′-CTGCTCCGTGAGATCTCCACTCAG-3′ (reverse), and the primers for β-actin amplification were: 5′-GATCTTGATCTTCATGGTGCTAGG-3′ (forward) and 5′-TTGTAACCAACTGGGACGATATGG-3′ (reverse). The PCR-amplified products were resolved by agarose gel electrophoresis and detected by Typhoon 9410.

2.5. Other analyses

Protein concentrations were determined with BCA assay (Pierce) with albumin as standard. Data are presented as mean ± S.D. of at least three separate experiments, except where results of blots are shown in which case a representative experiment is depicted in the figures. Comparisons between two values were made with Student's test at $p < 0.05$.

3. Results

3.1. Clofibrate and PFDA efficaciously induce the expression of PXR but not CYP3A23

We previously reported that the expression of PXR was markedly induced in rats treated with clofibrate or PFDA [23,24], two well-known peroxisome proliferators in rodents [28]. The aim of this study was to determine whether these chemicals alter the basal and induced expression of PXR target gene CYP3A23 [15]. The initial focus of the study was to determine the induction of PXR by clofibrate or PFDA as a function of concentrations and time of treatment. Rat hepatocytes were isolated and treated for 24, 48 or 72 h with clofibrate or PFDA at various concentrations. Total cell lysates were isolated and analyzed for the abundance of PXR by Western blots with a chemiluminescent kit. The chemiluminescent signal was captured by KODAK Image Station 2000 and the relative intensities were quantified by KODAK 1D Image Analysis Software. All data were collected from three independent experiments (three rats).

The results on the induction of PXR are summarized in Fig. 1. Treatment with clofibrate markedly increased the expression. However, the magnitude of induction varied depending on the concentrations and time of incubation. No induction was detected in the 24-h cultures regardless of the concentrations (Fig. 1A). In the 48-h cultures, a moderate induction (~2-fold) was detected in the hepatocytes treated at 0.1 mM, and a markedly higher induction (~4-fold) was observed in hepatocytes treated with 0.2 mM. Interestingly, further increasing concentrations (0.5 and 1 mM) caused no additional induction (Fig. 1A). In the 72-h cultures, no apparent concentration-dependent induction was detected with the concentrations from 0.1 to 1 mM (Fig. 1A). As a matter of fact, comparable induction (~4-fold) was detected with all concentrations except the highest concentration (1 mM), which was less effective (~3-fold). No induction on PXR target gene CYP3A23 was detected with clofibrate (data shown from the 48-h cultures) (Fig. 1A, bottom).

In contrast to clofibrate, PFDA induced PXR in a highly concentration and time-dependent manner (Fig. 1B). The maximum induction (~4.5-fold) was detected with the highest concentration and the longest incubation (Fig. 1B). Higher concentration or longer incubation resulted in higher induction. In the 24-h cultures, for example, induction was detected only in the hepatocytes that were treated with high concentrations (10 and 25 μM), whereas in the 72-h cultures, the induction was detected in all concentrations except 0.1 μM (the lowest concentration). On the other hand, PFDA, like clofibrate, caused no changes on the expression of PXR target gene CYP3A23 (Fig. 1B, bottom). No apparent toxicity was detected based on the release of lactate dehydrogenase. Very recently, we tested two PPAR-isoform specific agonists WY-14643 (PPARα) and ciglitazone (PPARγ) for the induction of PXR. Both chemicals increased the expression of PXR with WY-14643 being more efficacious. This PPARα agonist induced PXR to a comparably extent as clofibrate or PFDA based on the maximum induction (data not shown).

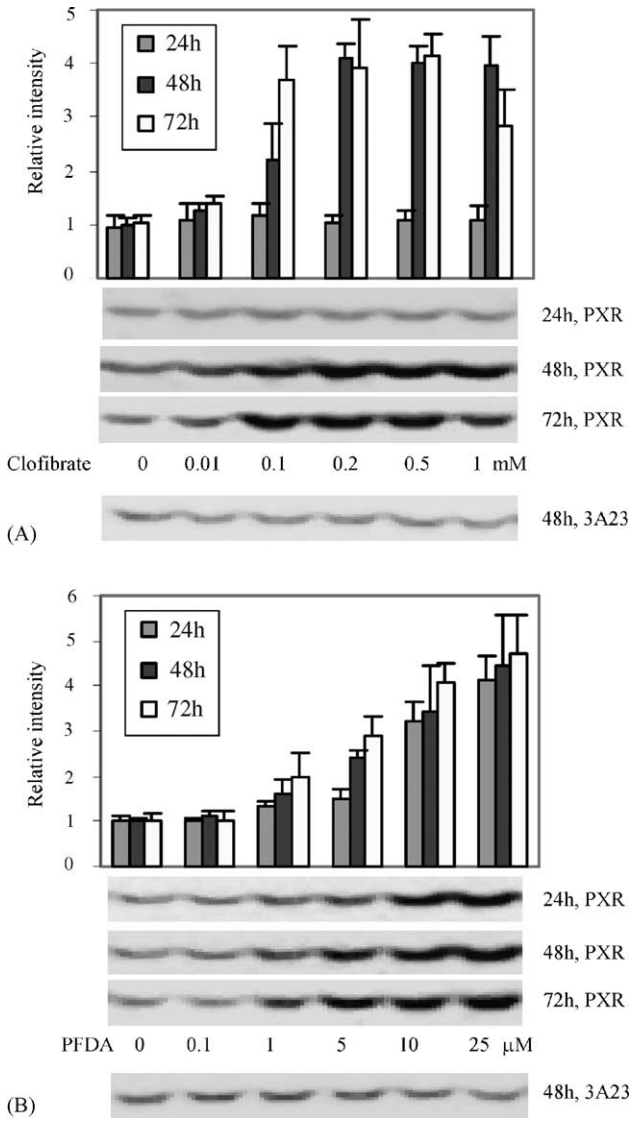
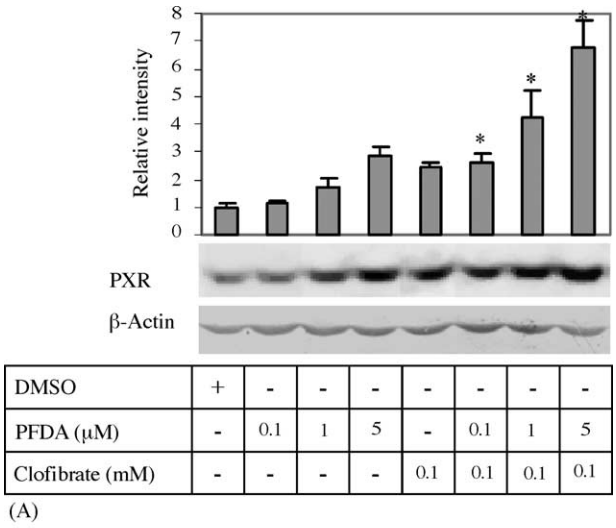


Fig. 1. Induction of PXR by clofibrate or PFDA as a function of concentrations and incubation time. (A) Induction of PXR by clofibrate: rat hepatocytes were cultured in WME and then treated with clofibrate (0.01–1 mM) or the same volume of DMSO (0.1%) for 24–72 h with a daily change of fresh medium and clofibrate. Cell lysates (6 μg) were analyzed by Western blots for the levels of PXR (top) or CYP3A23 (bottom) as described in Section 2. (B) Induction of PXR by PFDA: rat hepatocytes were cultured in WME and then treated with PFDA (0.1–25 μM) or the same volume of DMSO (0.1%) for 24–72 h with a daily change of fresh medium and PFDA. Cell lysates (6 μg) were analyzed by Western blots for the levels of PXR and CYP3A23. The chemiluminescent signal was captured by KODAK Image Station 2000 and the relative intensities were quantified by KODAK 1D Image Analysis Software. The data were collected from three independent experiments (three rats), normalized based on the signals from DMSO-treated hepatocytes, and expressed as mean ± S.D.

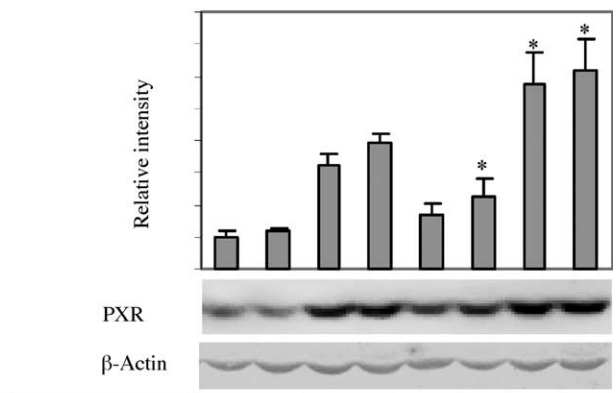
3.2. Clofibrate and PFDA co-operatively induce the expression of PXR

Clofibrate and PFDA caused a similar maximum induction of PXR with the concentrations used in this study. However, they varied markedly on the concentration–induction curves. For example, in the 72-h cultures, PFDA but not clofibrate showed a clear concentration-dependent manner (Fig. 1). The precise mechanism remains to be determined. In order to shed light on the underlying mechanisms, we performed an interaction study between clofibrate and PFDA. Hepatocytes were treated with various concentrations of clofibrate in the presence of constant PFDA or vice versa. Both chemicals were used at relatively low concentrations (based on the concentration-response



DMSO	+	-	-	-	-	-	-	-
PFDA (μM)	-	0.1	1	5	-	0.1	1	5
Clofibrate (mM)	-	-	-	-	0.1	0.1	0.1	0.1

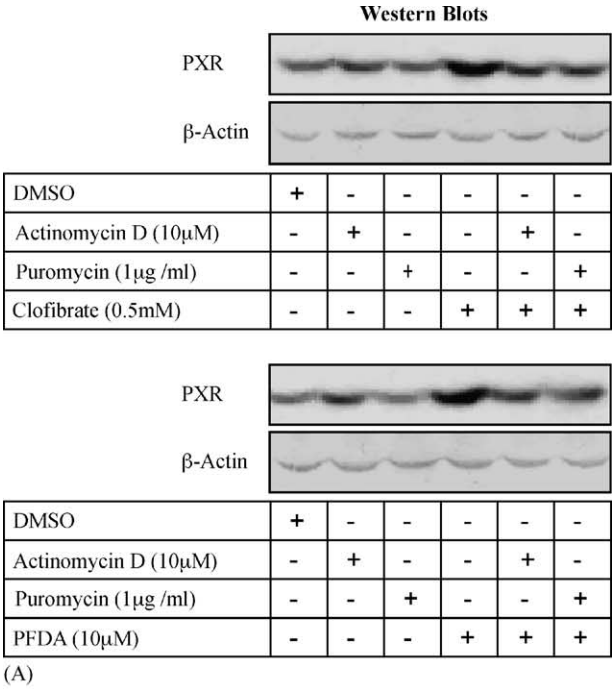
(A)



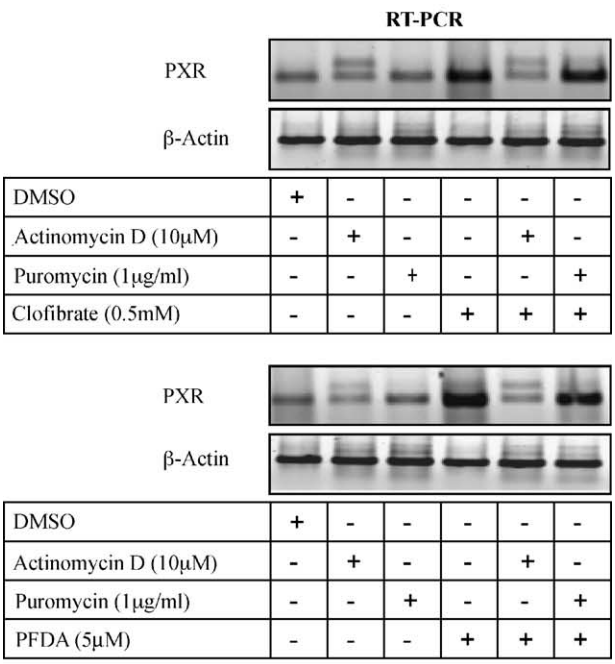
DMSO	+	-	-	-	-	-	-	-
Clofibrate (mM)	-	0.01	0.1	0.5	-	0.01	0.1	0.5
PFDA (μM)	-	-	-	-	1	1	1	1

(B)

Fig. 2. Cooperative induction of PXR by clofibrate and PFDA (A) Effect of clofibrate on PFDA-mediated induction of PXR: rat hepatocytes were treated for 48 h with PFDA at various concentrations (0.1–5 μM) in the presence or absence of clofibrate (0.1 mM). The lysates (6 μg) were analyzed for the abundance of PXR and β-actin by Western blots. (B) Effect of PFDA on clofibrate-mediated induction of PXR: rat hepatocytes were treated for 48 h with clofibrate at various concentrations (0.01–0.5 mM) in the presence or absence of PFDA (1 μM). The lysates (6 μg) were analyzed for the abundance of PXR and β-actin by Western blots. The data were collected from three independent experiments (three rats), normalized based on the signals from DMSO-treated hepatocytes, and expressed as mean ± S.D. (*) Significant difference between single and the corresponding co-treatment according to the Student's test ($p < 0.05$).



(A)



(B)

Fig. 3. Transcriptional and post-translational involvement in induced expression of PXR by clofibrate and PFDA (A) Western analysis of the effect of actinomycin D and puromycin on the induction of PXR by clofibrate or PFDA: rat hepatocytes were treated for 48 h with clofibrate (0.5 mM) or PFDA (10 μM) in the presence of actinomycin D (10 μM) or puromycin (1 μg/ml). Cell lysates were analyzed for the levels of CYP3A23 or β-actin by Western blots as described above. (B) RT-PCR analysis of the effect of actinomycin D and puromycin on the induction of PXR by clofibrate or PFDA: rat hepatocytes were treated for 48 h with clofibrate (0.5 mM) or PFDA (10 μM) in the presence of actinomycin D (10 μM) or puromycin (1 μg/ml). Total RNA was isolated and subjected to RT-PCR analyses with a ThermoScript I kit as described in Section 2. For PCR amplification, a master tube containing all common reagents was prepared and equally distributed to individual PCR reaction tubes (PXR and β-actin). PCR amplification was conducted with cycling parameters as follows: 95 °C for 30 s, 52 °C for 30 s and 68 °C for 30 s for a total of 23 cycles. The PCR-amplified products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Three independent experiments were performed but only representative data are presented.

curves in Fig. 1), and only a single time point (48 h) was conducted. These conditions were designed to detect interactions within the maximum induction (based on Fig. 1) and to prevent potential cytotoxicity. Similarly, total cell lysates were prepared and analyzed for the abundance of PXR and β-actin by Western blots.

Fig. 2A shows the results on the interaction study with PFDA assayed at various concentrations and clofibrate being constant. As expected, no induction was detected when PFDA was assayed at 0.1 μM, whereas a concentration-dependent induction was observed when higher concentrations (1 and 5 μM) were used (Fig. 2A). A moderate induction (~2-fold) was detected in hepatocytes treated with clofibrate alone at 0.1 mM. Interestingly, combined treatment resulted in even higher induction than the maximum induction (~4.5-fold) elicited by each chemical alone (Figs. 1 and 2A). For example, PFDA alone at 5 μM caused a 2.9-fold induction, but co-treatment with clofibrate resulted in an ~7-fold induction (Fig. 2A). The enhanced induction was also detected when clofibrate was assayed at various concentrations (0.01, 0.1 and 0.5 mM) in the presence of a constant amount of PFDA (1 μM) (Fig. 2B). For example, clofibrate alone at 0.5 mM caused an ~4-fold induction, in contrast, co-treatment with PFDA resulted in a 6-fold induction (PFDA at 1 μM caused little induction) (Fig. 2B).

3.3. Induction of PXR by clofibrate and PFDA is mediated by gene transcription

The enhanced induction between clofibrate and PFDA points to an important possibility: they induce PXR through distinct or overlapping mechanisms. In order to gain molecular insights, we examined whether induction of PXR by both chemicals is differentially inhibited by actinomycin D and puromycin, chemicals that selectively inhibit RNA synthesis and protein translation, respectively. Hepatocytes were treated with clofibrate or PFDA along with actinomycin D or puromycin, and cell lysates were analyzed for the abundance of PXR and β-actin or the corresponding mRNA. As expected, clofibrate or PFDA alone markedly increased the expression of PXR, however, the increased expression was abolished by actinomycin D and puromycin, excluding the possibility that the induction is achieved through increasing protein stability (Fig. 3A). Similarly, the levels of PXR mRNA were markedly increased in the hepatocytes treated with clofibrate or PFDA alone. However, the increased levels were abolished by actinomycin D but not puromycin (Fig. 3B), suggesting that transactivation is involved in the induction of PXR for

both chemicals and protein synthesis is not required for the transactivation. It should be emphasized that no apparent toxicity was observed with actinomycin D or puromycin at the concentrations assayed.

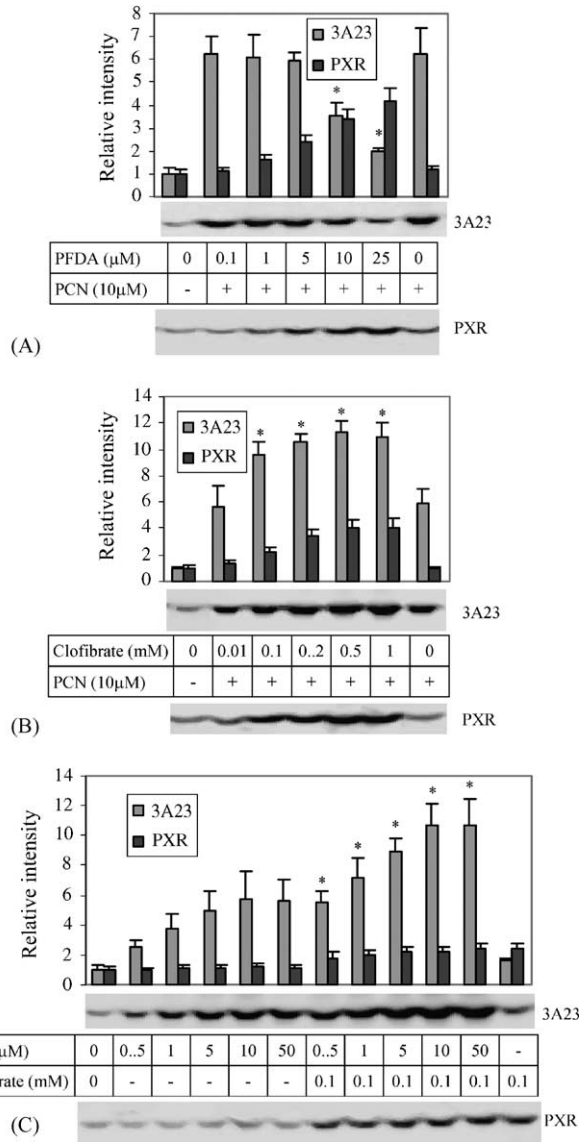


Fig. 4. PFDA suppresses PCN-mediated induction of CYP3A23 whereas clofibrate enhances it (A) PFDA suppresses PCN-mediated induction of CYP3A23: rat hepatocytes were cultured in WME and then treated for 48 h with PCN (10 μM) in the presence of PFDA at various concentrations (0–25 μM). Cell lysates (6 μg) were analyzed by Western blots for the abundance of CYP3A23 (top) or PXR (bottom). (B) Clofibrate enhances PCN-mediated induction of CYP3A23: rat hepatocytes were cultured in WME and then treated for 48 h with PCN (10 μM) in the presence of clofibrate at various concentrations (0–1 mM). Cell lysates (6 μg) were analyzed by Western blots for the abundance of CYP3A23 (top) or PXR (bottom). (C) Clofibrate-potentiated induction of CYP3A23 as a function of PCN: rat hepatocytes were cultured in WME and then treated for 48 h with PCN at various concentrations (0–50 μM) in the presence or absence of clofibrate (0.1 mM). Cell lysates (6 μg) were analyzed by Western blots for the abundance of CYP3A23 (top), PXR (bottom) or β-actin (not shown). Three independent experiments were performed. (*) Significant difference for the expression of CYP3A23 between PCN and the corresponding co-treatment according to the Student's test ($p < 0.05$).

3.4. Clofibrate enhances whereas PFDA suppresses PCN-mediated induction on CYP3A23

The experiments described above clearly established that clofibrate and PFDA are inducers of PXR, and the induction is achieved by increasing PXR mRNA. However, neither chemical altered the basal expression of CYP3A23 (Fig. 1), a target gene that is inducibly regulated by PXR in a ligand-dependent manner. Next, we examined whether clofibrate or PFDA alters PXR-induced expression of CYP3A23. PCN is an efficacious activator of PXR and a prototypical inducer of CYP3A23 [6,17]. Therefore, clofibrate and PFDA were tested for the ability to alter PCN-induced expression of CYP3A23. Two types of experiments were performed: (a) hepatocytes were treated with various concentrations of clofibrate or PFDA along with a constant amount of PCN; and contrarily (b) experiments with various concentrations of PCN but a constant amount of clofibrate were performed.

The results on the interaction study between PCN and clofibrate/PFDA are summarized in Fig. 4. As expected, PFDA caused a concentration-dependent induction of PXR (Fig. 4A, bottom), and PCN alone markedly induced CYP3A23 (∼6-fold). Surprisingly, the PCN-mediated induction on CYP3A23 was proportionally suppressed by increasing concentrations of PFDA (Fig. 4A). Similarly, clofibrate markedly induced the expression of PXR (Fig. 4B, bottom). In contrast to PFDA, clofibrate enhanced the PCN-mediated induction on CYP3A23 (Fig. 4B). For example, combined treatment with clofibrate (0.1 mM) and PCN (10 μM) caused a 10-fold induction of CYP3A23, whereas PCN alone caused only ∼6-fold induction. It should be emphasized that neither PFDA nor clofibrate alone caused any changes on the basal expression of CYP3A23 (Fig. 1). We next conducted the interaction study with clofibrate being assayed at a constant concentration (0.1 mM) and PCN at various concentrations. As expected, PCN caused a concentration-dependent induction of CYP3A23 with the maximum induction being ∼6-fold, and little changes were observed when clofibrate was used alone (Fig. 4C). However, co-treatment with clofibrate increased PCN-mediated induction on CYP3A23, and the relative enhancement appeared to be proportionally increased with increasing concentrations of PCN with an exception of 50 μM (Fig. 4C).

4. Discussion

The pregnane X receptor is a key regulator of genes encoding several major types of drug metabolizing enzymes (e.g., CYP3A) and transporters (e.g., MDR-1), and thus contributes significantly to drug–drug interactions [2]. Clofibrate and PFDA represent multi-classes of chemicals that induce proliferation of the peroxisome in rodents but less evidently in humans [28]. These chemicals

are manufactured for a variety of applications including pharmaceutical agents, food additives, industrial processors, herbicides and insecticides [28]. In this study, we report that both clofibrate and PFDA markedly induce the expression of PXR in rat hepatocytes. The induction is abolished by actinomycin D, suggesting that they regulate the expression of PXR through transactivation. More importantly, clofibrate potentiates PXR ligand-dependent induction on CYP3A23, whereas PFDA suppresses it. The differential effects provide additional complexity on the regulated expression of drug-metabolizing enzymes and transporters.

There are three types of PXR inducers based on their regulatory activity toward PXR ligand-dependent transactivation. The first type of inducers acts as both inducer and activator, and dexamethasone is an example in this category [7,19,20]. This synthetic steroid has been shown to increase the expression of both PXR and its target gene CYP3A23. Based on the concentrations to exert induction activity, the PXR gene is a more sensitive target than CYP3A23. Nanomolar dexamethasone effectively induces PXR, whereas micromolar concentrations are required to induce CYP3A23 [7,20]. The concentrations for CYP3A23 induction are consistent with the concentrations required to activate PXR [6]. The second type of PXR inducers increases the expression of PXR but has little effects on the expression of PXR-regulated genes, however, this type of inducers potentiates PXR ligand-dependent transactivation. In this study, we have shown that clofibrate markedly induces the expression of PXR and enhances PCN-mediated induction of CYP3A23, although this hypolipidemic agent alone does not alter the expression of this enzyme (Fig. 1A). The third type of PXR inducers is represented by PFDA, which is widely used as an industrial additive. Like clofibrate, PFDA induces the expression of PXR, but unlike clofibrate, PFDA suppresses PCN-mediated induction on CYP3A23 (Figs. 1B and 4A). It should be emphasized that only a single PXR-target gene CYP3A23 has been studied, whether the differential effects of clofibrate and PFDA on the regulated expression occur with other PXR targets remains to be determined.

In addition to the difference on the altered PXR ligand-dependent regulation, clofibrate and PFDA exhibit several additional differences in terms of regulating the expression of PXR. First, PFDA is significantly more potent than clofibrate. No induction is detected when clofibrate is used at 10 μM regardless of the incubation time (Fig. 1A). In contrast, PFDA at this concentration causes a 4-fold induction (Fig. 1B). Second, PFDA-mediated induction exhibits a broader range of dependence on the concentrations and incubation time, whereas clofibrate-mediated induction displays narrower ranges on both aspects (Fig. 1). For example, in the 72-h cultures, clofibrate causes no induction at 0.01 mM, whereas the maximum induction occurs at 0.1 mM (10 times). In contrast, the concentrations for PFDA to cause none to full induction display as many as a 250-fold difference (0.1–25 μM) (Fig. 1). And finally, clofibrate-mediated induction is delayed compared with that mediated by PFDA. No induction is detected in the 24-h cultures treated with clofibrate regardless of the concentrations, whereas PFDA-mediated induction is evident as early as 24 h (Fig. 1).

Although there are evident differences, it appears that both chemicals induce PXR through transcription activation. In support of this notion, actinomycin D, an inhibitor on mRNA synthesis, completely abolishes the induction in response to both chemicals (Fig. 3). Furthermore, puromycin, an inhibitor of protein synthesis, abolishes the induction of PXR at the protein but not mRNA level, suggesting that protein synthesis is not required for the transactivation in response to both chemicals (Fig. 3). Clofibrate and PFDA represent multi-classes of chemicals called peroxisome proliferators [28], and these chemicals exert their biological activities largely through the peroxisome proliferator activated receptors (PPARs) [29,30]. Therefore, it is likely that clofibrate and PFDA induce the expression of PXR through transactivation of PPARs, particularly PPARα. In support of this possibility, PFDA shows a higher affinity toward PPARα, thus has a relatively higher potency on the induction of PXR ([28], this study), PPARα specific agonist WY-14643 induces PXR to a much higher extent than PPARγ specific agonist ciglitazone (unpublished data), and motif search on the genomic sequences has revealed several perfect or slightly modified PPAR elements in the regulatory region of the rat PXR gene. However, several observations argue against the notion that the same mechanism (e.g., PPARα) is used by clofibrate and PFDA in the induction of PXR. In particular, clofibrate-mediated induction is delayed compared with that with PFDA (Fig. 1). The precise mechanism on the delayed induction remains to be determined. It is likely that PFDA is kinetically more favorable and achieves the steady level faster than clofibrate. Alternatively, metabolites rather than parent clofibrate efficaciously induce PXR. Interestingly, we have shown that clofibrate and PFDA synergize with each other on the induction of PXR (Fig. 2). These findings suggest that clofibrate and PFDA use overlapping mechanisms in PXR induction (assuming that PPARα plays a major role).

It appears that the induced expression of PXR by clofibrate is not sufficient to enhance PCN-mediated induction on CYP3A23. In this study, PFDA markedly induces the expression of PXR, however, the PCN-induced expression of CYP3A23 is reversely correlated with the increased expression of PXR by PFDA (Fig. 4A). Studies on the transactivation of CYP3A23 have demonstrated that several other transcription factors play roles in the induction of CYP3A23, notably chicken ovalbumin upstream promoter-transcription factors (COUP-TF), hepatocyte nuclear factor-4 (HNF-4) and complex B [7]. COUP-TF represses the expression of CYP3A23, whereas HNF-4 and complex B exert activation activity. It is conceivable that clofibrate

and PFDA differentially modulate the expression/activity of these proteins, leading to enhanced (clofibrate) or suppressed (PFDA) activity toward the PCN-mediated induction on CYP3A23. In support of this possibility, peroxisome proliferators or related compounds have been shown to alter the expression and modulate the activities of these factors (e.g., COUP-TF) [31,32], although it remains to be determined whether clofibrate and PFDA differentially regulate the expression of these factors and whether the altered expressions are the ultimate mechanisms for the observed differential effect on the induction of CYP3A23. Recently, we have found that both clofibrate and PFDA moderately suppress PCN-mediated stimulation on the CYP3A23 promoter in a reporter assay (CV-1 cells) (unpublished results), further supporting the notion that the opposing effects between clofibrate and PFDA on PCN induction of CYP3A23 are due to their differential effects on the expression of other transcription factors rather than PXR.

In summary, our work points to several important conclusions. First, both clofibrate and PFDA are inducers of rat PXR with PFDA being more potent, and the induction is achieved by transcription activation. Second, the transcription activation on the expression of PXR is likely mediated by overlapping mechanisms, based on the fact that both chemicals are PPAR ligands and the induction is synergistically enhanced by each other. Third, induction of PXR may result in enhanced or suppressed activity on PXR ligand-dependent regulation depending on an inducer. PXR is a key regulator of genes encoding several major types of xenobiotics-metabolizing enzymes and transporters. Clofibrate and PFDA represent multi-classes of chemicals and have a broad spectrum of its biological activities. The differential effects by clofibrate and PFDA on the expression of CYP3A23 provide an example on the complexity regarding the regulated expression of xenobiotic-metabolizing enzymes and transporters.

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