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AhR and PPARα: antagonistic effects on CYP2B and CYP3A, and additive inhibitory effects on CYP2C11

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Abstract
The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. The peroxisome proliferator-activated receptor alpha (PPARα) is a member of the nuclear receptor super-family of ligand-activated transcription factors and it functions as an obligate heterodimer with retinoid X-receptor alpha RXRα. The aim was to investigate whether the negative cross-talk recently proposed by the present authors between AhR and PPARα on CYP4A and CYP1A has any impact on other cytochrome P450 enzymes. Treatment of male Wistar rats with a PPARα ligand clofibric acid (CA) induced CYP2B1/2 and CYP3A proteins, activities, and the mRNA expression of CYP2B1, CYP2B2, CYP3A1 and CYP3A2, and suppressed CYP2C11 protein, activities and mRNA expression. AhR ligand Sudan III (S.III) treatment decreased basal and CA-induced CYP2B, CYP3A and CYP2C11 protein, activities and mRNA expression. To the best of the authors' knowledge, this is the first study showing the presence of mutual effects of AhR and PPARα on CYP2B and CYP3A and an additive inhibitory effect on CYP2C11 in the livers of male rats.

Keywords: *AhR, PPARα, CYP2B, CYP 2C11, CYP3A*

Introduction

The aryl hydrocarbon receptor (AhR) is an intracellular mediator of the xenobiotic signalling pathway and resides in the cytoplasm as a complex with HSP90 (Perdew, 1988) and XAP2 (Meyer and Perdew, 1999). Xenobiotics such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) bind to the AhR with an extremely high affinity, and the receptor complex

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subsequently translocates to the nucleus, where it switches its partner molecule from HSP90 to the AhR nuclear translocator (Arnt) protein (Heid et al., 1995). In the nucleus, the AhR/Arnt heterodimer formed binds to the xenobiotic-responsive element (XRE) sequences, which are enhancer DNA elements present in the 5′-flanking region of target genes CYP1A1/1A2 and binds to genes coding for a series of xenobiotic-metabolizing enzymes, cell cycle and growth-related factors (Weiss et al., 1996). It has been reported that HSP90-binding domain co-localizes with the ligand-binding domain of Ah receptor and has been suggested that the ligand may activate the receptor by displacing HSP90 (Coumailleau et al., 1995).

Peroxisome proliferator-activated receptors (PPARs) are members of the steroid nuclear receptor super-family, a large class of ligand-activated transcription factors regulating gene expression (Bocos et al., 1995). PPARs bind to peroxisome proliferator-responsive elements (PPREs) as a heterodimer with RXRα (Marcus et al., 1993). The transcriptional activity of this heterodimer is regulated in part by association with a family of proteins called co-activators and co-repressors that are known to regulate nuclear receptor functions (Krey et al., 1997). PPARα had been shown to form a complex with HSP90 and XAP2 (Sumanasekera et al., 2003). Fibrates are PPARα ligands that are widely used in the treatment of hyperlipidaemia (Staels et al., 1998).

Exposure of different rat strains to the AhR ligand, TCDD, produced up-regulation of AhR protein expression *in vivo* (France et al., 2001). Furthermore, the stability of the AhR-HSP90 complex is critical to the stability of the AhR protein (Chen et al., 1997). On the other hand, the PPARα mRNA and protein expression were increased in rat Fao cells by ciprofibrate (Passilly et al., 1999) and in rat liver by bezofibrate (Bonilla et al., 2001). CA is a stimulant of PPARα, a receptor reported to be associated with HSP90 and XAP2 (Sumanasekera et al., 2003). The induction of AhR expression could be expected to cause AhR to compete for HSP90 and XAP2 with PPARα, which could affect its stability. Moreover, the common co-activators such as SRC-1 and p300, which have been shown to co-activate PPARα (Zhu et al., 1996; Dowell et al., 1997), have been also shown to be necessary for AhR activation (Kobayashi et al., 1997; Ke et al., 2001).

The negative cross-talk between AhR and nuclear receptors has been well stated. Gierthy et al. (1987) reported that TCDD inhibited E2-induced secretion of tissue plasminogen activator activity, post-confluent focus production and proliferation of ER-positive MCF-7 cells. Furthermore, no effects were observed in ER-negative MDA-MB-231 cells (Gierthy et al., 1988). Moreover, subsequent several oestrogen-induced uterine/endometrial responses *in vivo* were inhibited in animals co-treated with E2 plus TCDD (Yang and Foster 1997). After treatment of MCF-7 cells with TCDD and other AhR agonists, there was a rapid decrease in nuclear ER binding and immunoreactive ER protein (Safe et al., 2000). In addition, HSP90 can regulate AhR activity *in vivo*, and that Ah-responsiveness is dependent upon cellular ER content through a mechanism that involves HSP90 (Caruso et al., 1999).

There are several lines of evidence suggesting the AhR as a negative regulator of adipocyte differentiation (Alexander et al., 1998). TCDD treatment prevented the increase of PPARγ expression in differentiating adipocytes (Vogel & Matsumura, 2003).

We have recently demonstrated the presence of mutual effects between AhR and PPARα manifested by down-regulation of AhR target genes by PPARα stimulation (Shaban et al., 2004a), and by down-regulation of PPARα target genes by AhR stimulation (Shaban et al., 2004b).

The PPARα ligand ciprofibrate has been reported to induce CYP2B in rat hepatocytes (Wortelboer et al., 1991; Zangar et al., 1995). Furthermore, CYP3A23 is induced by

dehedroepiandrosterone (DHEA), a PPARα ligand, in rat liver (Gonzalez et al., 1986). However, the induction of CYP3A23 with DHEA was less than CYP4A1 induction by DHEA (Singleton et al., 1999).

CYP2C11 has been reported to be suppressed after treatment with peroxisome proliferators (PPARs) (Corton et al., 1998) and DHEA treatment (Prough et al., 1995). Suppressive effect of AhR ligands on CYP2C11 has been also reported, as intraperitoneal injection of 3-methylcholanthrene decreased rat hepatic CYP2C11 mRNA levels to 40–50% of vehicle control levels (Jones & Riddick, 1996). The suppressive effect of the AhR ligand 3-methylcholanthrene on CYP2C11 has been also reported by Bhathena et al. (2002).

In the current study, it was assumed that these mutual effects would affect other genes that are affected in part by either of the two receptors. The paper presents results suggesting that the effects between AhR and PPARα occur only in the receptor-dependent CYP2B and CYP3A, but not in the receptor-independent CYP2C11.

Materials and methods

Materials

Bovine serum albumin (BSA), and Sudan III were from Sigma Chemical Co. (St Louis, MO, USA). Diamino-benzidine-tetrahydro-chloride was obtained from Kanto Chemical Co. (Tokyo Japan); clofibric acid (2-(*p*-chlorophenoxy)-2-methylpropionic acid) with 97% purity was from Pfaltz & Bauer, Inc. (NY, USA). NADPH, glucose 6-phosphate (G6P) and glucose 6-phosphatedehydrogenase (G6PDH) were from Oriental Yeast Co. (Tokyo, Japan). Polyclonal goat-anti rat CYP2B1, CYP2C11 and polyclonal rabbit-anti rat CYP3A2 were from Daiichi Pure Chemical Co., Ltd (Tokyo, Japan). Rabbit horseradish peroxidase-labelled anti-goat IgG was from Sigma Chemical Co. (St. Louis, MO, USA). Goat horseradish peroxidase-labelled anti-rabbit was from Santa Cruz Biotechnology (CA, USA). Other chemicals and solvents were of analytical grade.

Animals and treatments

Nine-week old male Wistar rats of body weight 220–240 g (SLC, Hamamatsu, Japan), divided into four groups of four rats each, were housed at $24 \pm 1^{\circ}C$ with a 12-h light and 12-h dark cycle, and given laboratory feed (MR stock, NOSA N Co.; Yokohama, Japan) and water *ad libitum*. Rats were given $80\,mg\,kg^{-1}$ S.III, $80\,mg\,kg^{-1}$ S.III + $300\,mg\,kg^{-1}$ CA, $300\,mg\,kg^{-1}$ CA or corn oil as a vehicle orally for 3 consecutive days. The choice of CA dose referred to the same dose used by Amacher & Schomaker (1998). The dose of S.III was used referring to Fujita et al. (1984). Twenty-four hours after the last dose, rats were killed with carbon dioxide and their livers removed. The microsomal fractions from livers were prepared according to the method of Omura and Sato (1964) with slight modifications. Livers were minced and homogenized in 3 vols ice-cold 1.15% potassium chloride solution with a Teflon homogenizer. Homogenized samples were centrifuged at 9000*g* at 4°C for 20 min. The supernatant fraction was centrifuged at 105 000*g* at 4°C for 70 min to attain a mitochondrial-free microsomal pellet. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer (KPB), pH 7.4, and divided into 1.5-ml tubes snap-frozen in liquid nitrogen and kept at −80°C until use. Microsomal protein concentrations were determined according the method of Lowry et al. (1951) using BSA as a standard.

Western blot analysis

Aliquots of 12 μg liver microsomal protein from treated and control rats were applied to 10−12% sodium dodecyl-sulfate (SDS) polyacrylamide gels and separated by electrophoresis using a Protean 2 mini 1-D cell (BioRad, Hercules, CA, USA). Western blot analysis was performed according to Towbin et al. (1979). The proteins were transferred electrophoretically to nitrocellulose membranes (Laemmli, 1970), blocked in 5% skimmed milk in phosphate-buffered saline (PBS) containing 1% Tween 20 for 2 h at room temperature, and probed with the polyclonal goat anti-rat CYP2B1, CYP2C11 or CYP3A2 antibodies as solutions in PBS containing 1% Tween 20 on a shaker for 2 h at room temperature. Horseradish peroxidase-labelled anti-goat IgG or anti-rabbit IgG were used as the secondary antibodies. Immunoreactive protein bands were revealed colorimetrically by oxidation of 0.025% 3,3-diaminobenzidine tetrahydrochloride with 0.0075% hydrogen peroxide and catalysed by peroxidase in 50 mM Tris-HCl (pH 7.6). Intensities of the immunoreactive bands were analysed densitometrically using the public domain NIH Image program (www.rbs.info.nih.gov/nih-image/).

Assays for CYP-mediated enzyme activities

Testosterone hydroxylation was assayed as described by Van der Hoeven (1981). The reaction mixture (1 ml) consisted of 100 mM potassium phosphate buffer (KPB, pH 7.4), 3 mM $MgCl_2$, 5 mM G6P, 0.5 mg microsomal protein and 0.25 mM testosterone. After pre-incubation at 37°C for 5 min, the reaction was started by the addition of 1 mM NADPH and 1 IU G6PDH, and was then allowed to continue at 37°C for 3 min. The reaction was halted by the addition of 1.5 ml ethyl acetate containing an internal standard (50 μM corticosterone, 10 μl). The mixture was centrifuged at 3000 rpm for 20 min, and an aliquot of the organic layer (0.9 ml) was dried and dissolved in HPLC mobile phase (180 μl) of methanol, water and tetrahydrofuran (35:55:10, respectively, pH 4.0).

The metabolites were assayed using a Shimadzu LC-6A liquid chromatograph with an SPD-6AV spectrophotometric detector (245 nm). The column was a SupelcosilTM LC-315 (5 ml, 250·4.6 mm) (Supelco, Bellefonte, PA, USA). The flow rate of the mobile phase was 1.1 ml min^{-1}.

RNA extraction

Total RNA was isolated from 50 mg liver using TRIzol reagent (Life Technologies, Inc., Grand Island, NY, USA). Briefly, liver tissue samples were homogenized in 1 ml TRIzol then 0.3 ml chloroform were added to the sample. The mixtures were then shaken for 30 s followed by centrifugation at 4°C and 12 500 rpm for 20 min. The supernatant layer were transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shaken for 15 s and centrifuged 4°C and 12 500 rpm for 15 min. The RNA pellets were washed with 70% ethanol. RNA was dissolved in DEPC water. The prepared RNA was checked by electerophoresis, and shown that the RNA integrity was fine; then it was further checked by measuring the optical density. The optical density of all RNA sample was 1.7−1.9 based on the 260/280 ratio.

RT-PCR

cDNA was synthesized as follows. A mixture of 5 μg total RNA and 0.5 ng oligo dT primer in a total volume of 24 μl sterilized ultra-pure water was incubated at 70°C for 10 min and then removed from the thermal cycler and made up to 40 μl with a mixture of 8 μl (5X) RT-buffer, 2 μl 10 mM dNTP, 2 μl DEPC water, and 2 μl reverse transcriptase (Toyobo Co., Ltd, Osaka, Japan). The mixture was then re-incubated in the thermal cycler at 30°C for 10 min, at 42°C for 1 h and at 90°C for 10 min to prepare the cDNA.

Semi-quantitative PCR

For CYP2C11 and β-actin mRNA measurement, the semi-quantitative PCR was performed as follows: 1 μl aliquots of the synthesized cDNA were added to 19 μl of a mixture containing sterilized ultra-pure water, 2 μl 10 × PCR buffer, 2 μl dNTP (2.5 mM), 0.3 μl sense and anti-sense primers (10μM), and 0.1 μl Taq polymerase (Takara, Kyoto, Japan). Amplification was initiated by denaturation by one cycle at 95°C for 1 min followed by, in each cycle, denaturation at 94°C for 1 min, and annealing at the proper temperature for 1 min, and then extension at 72°C for 1 min for 22 cycles for CYP2C11 and 23 cycles for β-actin mRNA using a DNA thermal cycler (BioRad). The samples were finally incubated for 7 min at 72°C after the last cycle of amplification. Amplified PCR products were separated by electrophoresis through 1.0−1.5% agarose gels. Bands of cDNA were stained with ethidium bromide and visualized by ultraviolet illumination. Photographic images were converted into computer files with an Epson colour-image scanner in combination with Adobe PhotoShop 6.0 software. The use of β-actin as a housekeeping gene was chosen referring to Kono et al. (2001) and Kovacs et al. (2001). The primer sequences and annealing temperature for each PCR reaction are shown in Table I.

Real-time quantitative PCR

Real-time quantitative PCR for rat CYP2B1, CYP2B1/2, CYP3A1, CYP3A2 and albumin mRNA levels were performed using an ABI PRISM 7700 and the SYBR green PCR kit (Giagen, Inc., CA, USA) following the instruction manual with the use of primer constructs according to Meredith et al. (2003). The reaction mixture (final volume 10 μl) for PCR was prepared with final concentration of 1× Master Mix reagents, 150 nM of each primer, 300 ng cDNA in 1 μl RNase-free water for each rat CYP and albumin, and the mixture was completed to the final volume by RNase-free water. The reaction was performed for 40 cycles: initial activation at 95°C for 15 min, denaturation at 95°C, annealing at 60°C

Table I. Primer sequences and conditions for the genes amplified by PCR.

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	PCR product (bp)
CYP2C11[a]	TGCCCCTTTTTACGAGGCT	GGAACAGATGACTCTGAATTCT	55.0	368
β-Actin[b]	ATGTACGTAGCCATCCAGGC	TCCACACAGAGTACTTGCGC	56.5	628

[a] Xu et al. (2001).
[b] Gene Bank Accession No. V01217.

for 1 min, extension at 72°C for 30 s. The measurements of each CYP enzyme and albumin were performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of albumin and was calculated relative to that of control.

Statistical analysis

All data are expressed as means ± SD. Statistical significances were evaluated using ANOVA and Fisher's protected least significant difference test. $p < 0.05$ were considered to be statistically significant.

Results

Effect of CA and/or S.III on CYP2B

CA treatment induced CYP2B protein, whereas S.III treatment decreased the basal and the CA-induced CYP2B protein expression (Figure 1A, B). To substantiate these observations, we also measured CYP2B-mediated testosterone-16β-hydroxylation activity. The induction with CA was eightfold of the control, whereas S.III co-treatment with CA reduced the activity into less than 40% of the CA-induced levels. Moreover, S.III treatment alone reduced the activity to less than 50% of the control (Figure 1C). To investigate the point at which CYP2B is affected by either CA or S.III, we measured CYP2B1 and CYP2B1/2 mRNA by real-time PCR, which, surprisingly, showed that CYP2B1 mRNA was induced with CA more than 130-fold of the control level, whereas this induction was reduced to only 40-fold when rats were co-treated with CA and S.III.

The basal CYP2B1 decreased without reaching statistical significance due to S.III treatment alone. CYP2B1/2 was also induced by CA to about 90-fold of control, whereas this induction was decreased to 30-fold when rats were co-treated with CA and S.III. Basal CYP2B1/2 was slightly induced with S.III without reaching significant levels (Figure 2). We have stated previously that PPARα mRNA was induced with CA treatment of male rats (Shaban et al., 2004b), while PPARα mRNA and protein expression were increased in rat Fao cells by ciprofibrate (Passilly et al., 1999) and in rat liver by bezofibrate (Bonilla et al., 2001). In addition, AhR protein expression was increased in rat's livers with S.III treatment (Shaban et al., 2004a), and the exposure of different rat strains to the AhR ligand, TCDD, produced up-regulation of AhR protein expression *in vivo* (Franc et al., 2001). These results show the induction of CYP2B by the PPARα ligand CA and the down-regulation of CYP2B by the AhR ligand Sudan III and suggest that the mutual effect on CYP2B might have occurred due to negative cross-talk between AhR and either PPARα or CAR.

Effect of CA and/or S.III on CYP3A

CYP3A protein expression was induced with CA treatment in rats without reaching a significant level (Figure 3A), whereas S.III treatment slightly decreased CYP3A protein but did not reach a significant level. When rats were co-treated with S.III and CA, S.III reduced the induction of CYP3A protein by CA. To confirm this mutual effect of the AhR ligand S.III and the PPARα ligand CA on CYP3A, we measured CYP3A-mediated testosterone 2- and 6β-hydroxylation. Testosterone hydroxylation mediated by CYP3A2 was consistent with the protein levels, but was more sensitive than protein measurement. CA induced the

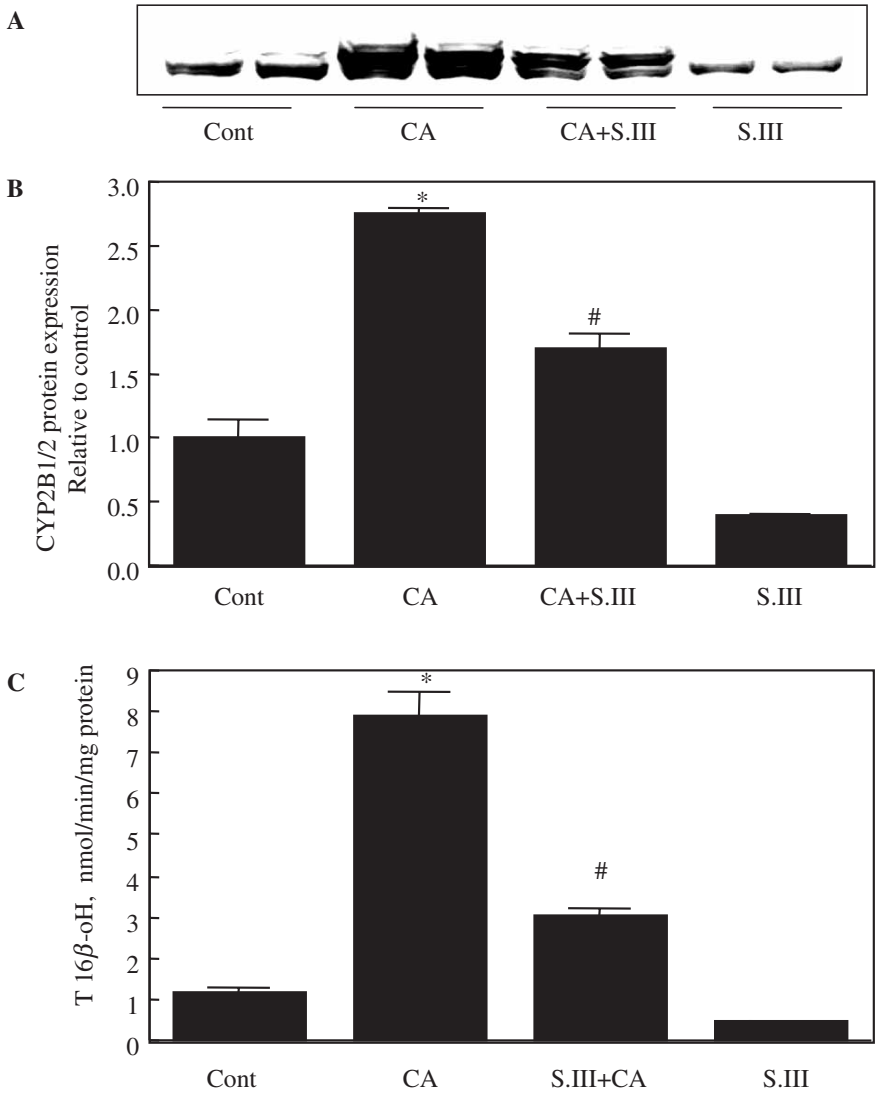

Figure 1. Effect of the PPARα ligand CA and the AhR ligand S.III on CYP2B protein and activity. Male Wistar rats were orally treated with, 80 mg kg^{-1} S.III, 80 mg kg^{-1} S.III +300 mg kg^{-1} CA, 300 mg kg^{-1} CA or vehicle for 3 days. (A) Microsomal protein samples (12 μg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP2B1 antibodies as described in the Materials and methods. (B) Analysis of CYP2B protein signals calculated relative to control. (C) CYP2B activity was evaluated by measuring CYP2B-mediated testosterone 16β-hydroxylation, as described in the Materials and methods, and was presented as the mean of three different measurements. $N = 4$ rats for each group. Data are means ± SD; *higher than control, #lower than CA-induced level, **lower than control. $p < 0.05$.

testosterone 2β- and testosterone 6β-hydroxylation significantly than control, whereas S.III treatment decreased it but did not reach a significant level in agreement with the effect of S.III treatment on the protein level. Importantly, S.III co-treatment with CA decreased the CA-increased testosterone hydroxylation activities. To explore this phenomenon more

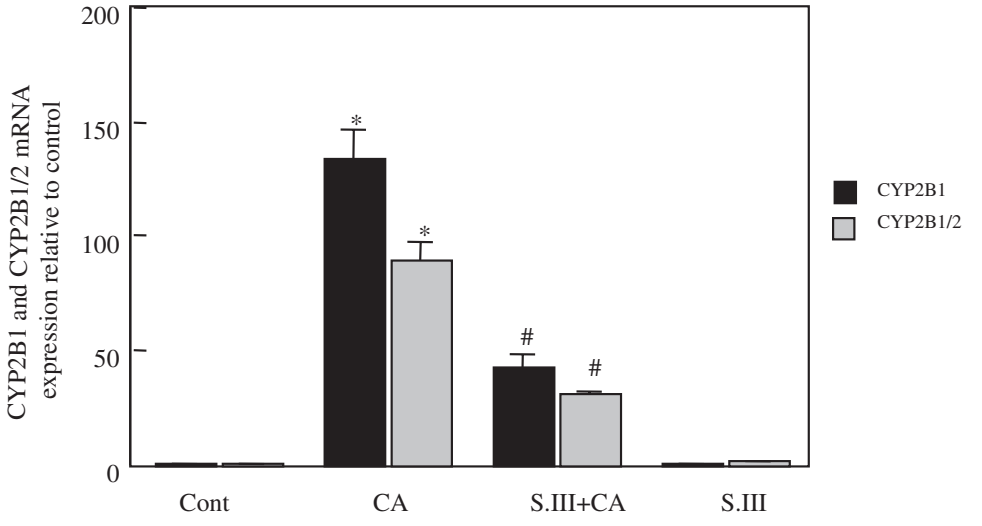

Figure 2. Effect of the PPARα ligand CA and the AhR ligand S.III on CYP2B1 and CYP2B1/2 mRNA. Male Wistar rats were orally treated with 80 mg kg^{-1} S.III, 80 mg kg^{-1} S.III + 300 mg kg^{-1} CA, 300 mg kg^{-1} CA or vehicle for 3 days. CYP2B1 and CYP2B1/2 mRNA were measured by real-time PCR as described in the Materials and methods. The results were analysed relative to control levels. $N = 4$ rats for each group. Data are means $\pm$ SD; *higher than control,$^{\#}$ lower than CA-induced level. $p < 0.05$.

thoroughly, we measured the CYP3A enzymes, namely CYP3A1 and CYP3A2 mRNA, using the highly sensitive method SYBR green kit and 7700 ABI PRISM (Figure 4). CYP3A2 mRNA was induced significantly with CA treatment but S.III had no effect, whereas induction with CA was reduced with S.III co-treatment with CA. CYP3A1 was induced by both CA and S.III treatments; however, it was reduced significantly compared with (CA-induced levels) when rats were co-treated with CA and S.III. As explained above, the results show the induction of CYP3A by the PPARα ligand CA and the down-regulation of CYP3A by the AhR ligand S.III, and suggest that that the mutual effect on CYP3A might have occurred due to negative cross-talk between AhR with either PPARα or CAR.

Effect of CA and/or S.III on CYP2C11

CYP2C11 protein expression was suppressed compared with the control in rats treated with either CA or S.III, whereas co-treatment of the rats with CA and S.III produced suppression of the CYP2C11 protein even more than either CA or S.III alone (Figure 5A, B). To confirm these effects on CYP2C11 protein expression, we measured CYP2C11-mediated testosterone-2α-hydroxylation activity. CA reduced the testosterone-2α-hydroxylation to 50% of the control level, and S.III treatment also reduced the testosterone-2α-hydroxylation to 40% of the control level, whereas S.III co-treatment with CA decreased the activity to less than 20% of the control (Figure 5C). To explore whether this additive inhibitory effect was at the level of pre- or post-transcription, we measured CYP2C11 mRNA by semi-quantitative RT-PCR, which showed that mRNA expression was suppressed by both CA and S.III treatment compared with the control, and was also highly suppressed with CA and

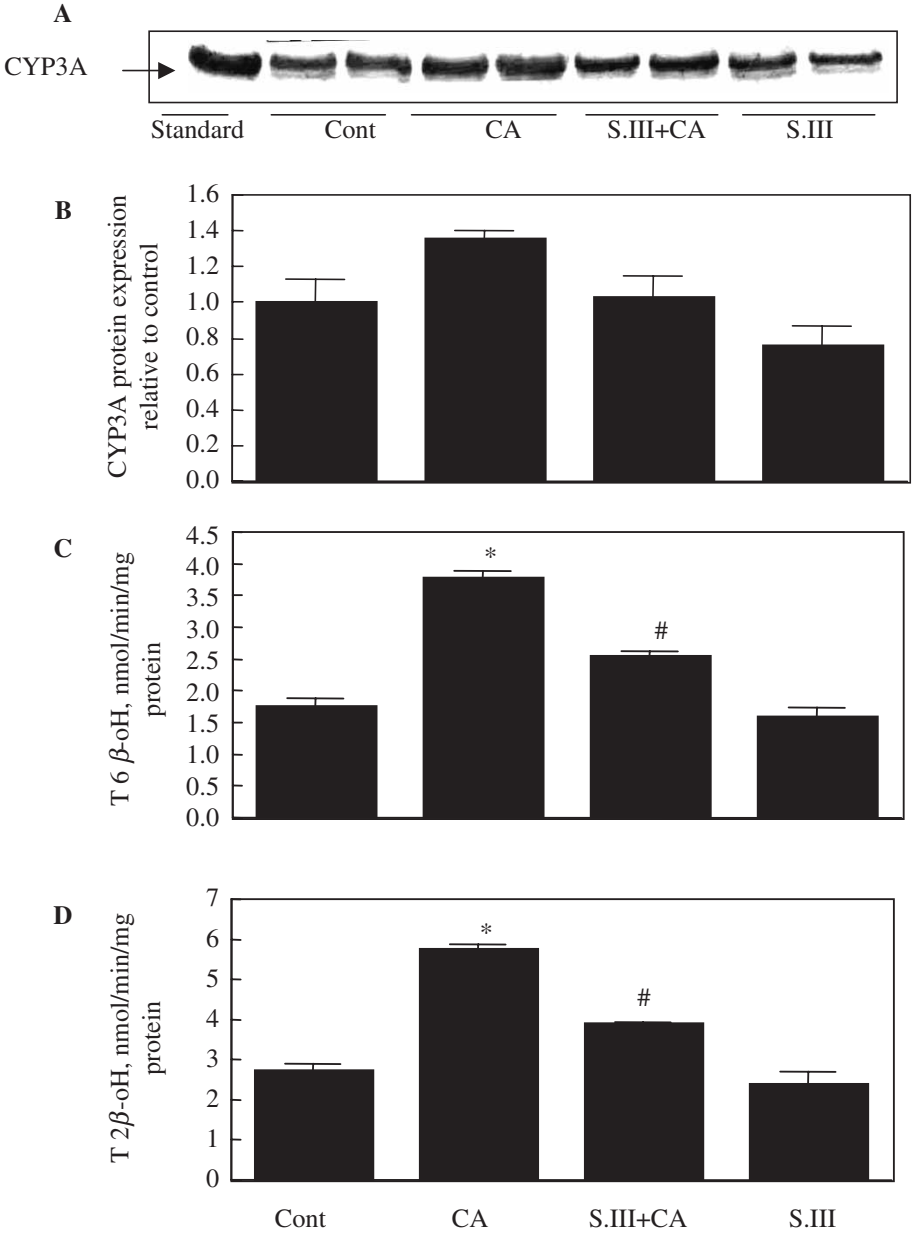

Figure 3. Effect of the PPARα ligand CA and the AhR ligand S.III on CYP3A Protein and activity. Male Wistar rats were orally treated with 80 mg kg^{-1} S.III, 80 mg kg^{-1} S.III + 300 mg kg^{-1} CA, 300 mg kg^{-1} CA or vehicle for 3 days. (A) Microsomal protein samples (12 μg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP3A2 antibodies as described in the Materials and methods. (B) Analysis of CYP3A protein signals calculated relative to control. (C) CYP3A2 activity was evaluated by measuring CYP3A2-mediated testosterone 2β-hydroxylation and 16β-hydroxylation, as described in the Materials and methods, and was presented as the mean of three different measurements. $N = 4$ rats for each group. Data are means ± SD; *higher than control, #lower than CA-induced level, $p < 0.05$.

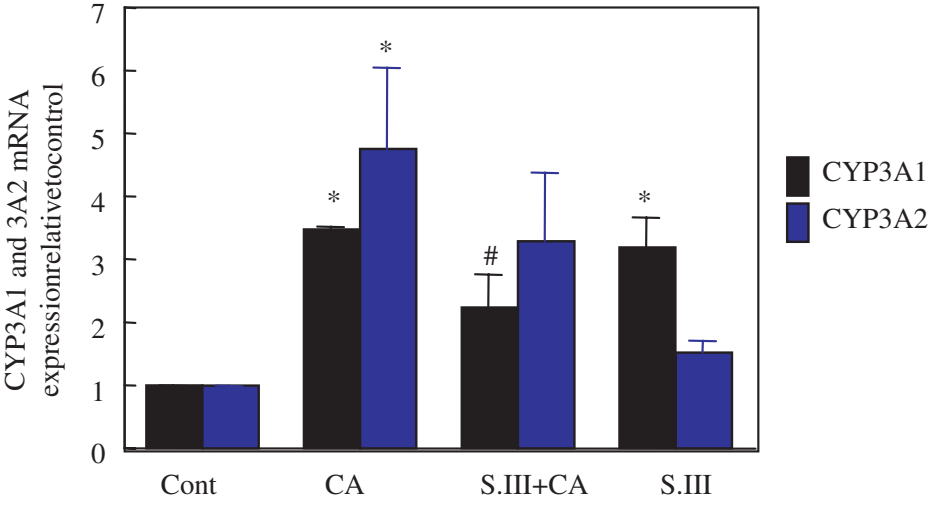

Figure 4. Effect of the PPARα ligand CA and the AhR ligand S.III on CYP3A1 and CYP3A2 mRNA: Male Wistar rats were orally treated with 80 mg kg^{-1} S.III, 80 mg kg^{-1} S.III + 300 mg kg^{-1} CA, 300 mg kg^{-1} CA or vehicle for 3 days. CYP3A1 and CYP3A2 mRNA were measured by real-time PCR as described in the Materials and methods. The results were analysed relative to control levels. $N = 4$ rats for each group. Data are means $\pm$ SD; *higher than control, #lower than CA-induced level. $p < 0.05$.

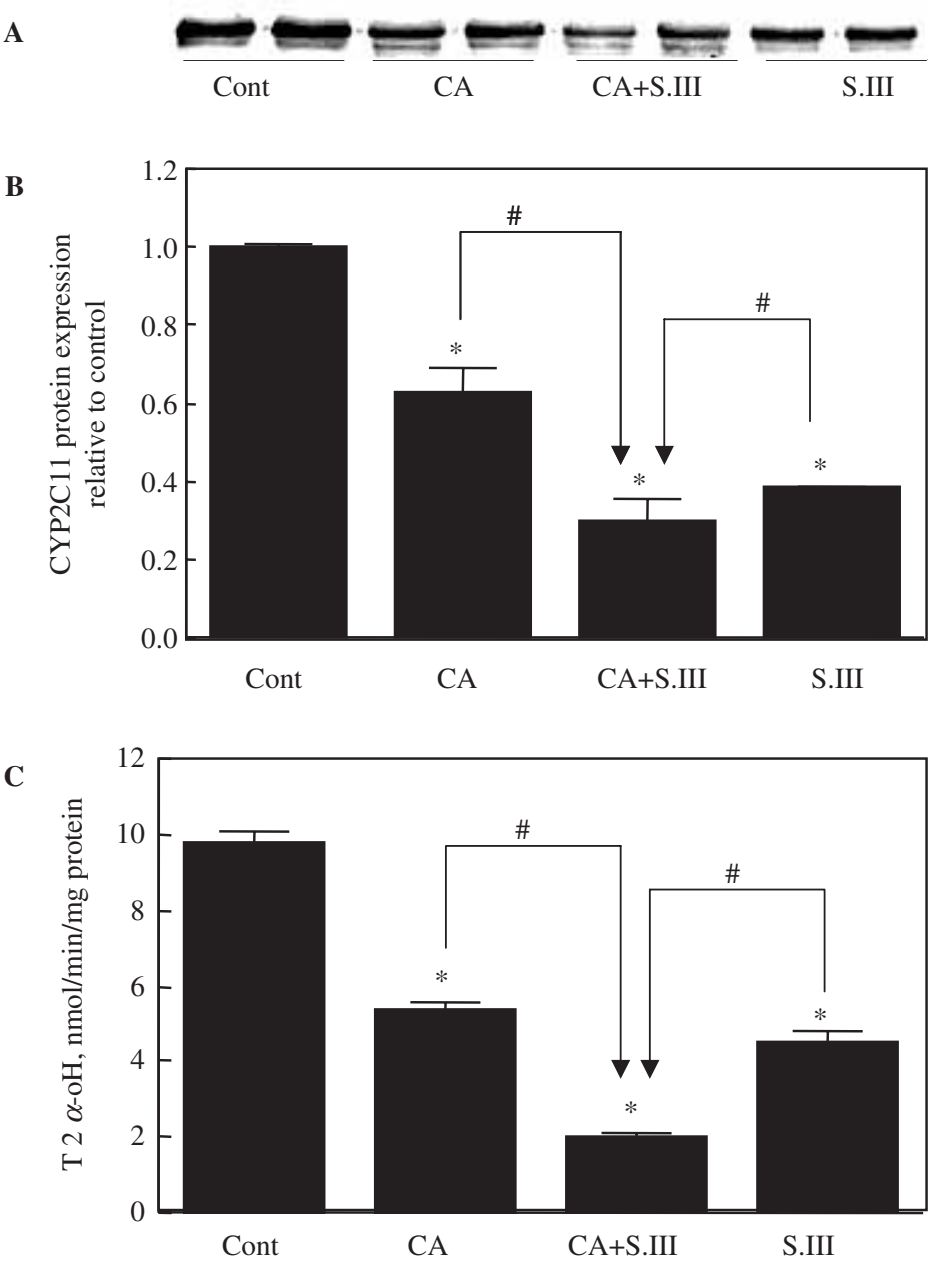

Figure 5. Effect of the PPARα ligand CA, and the AhR ligand S.III on CYP2C11 protein and activity. Male Wistar rats were orally treated with 80 mg kg^{-1} S.III, 80 mg kg^{-1} S.III + 300 mg kg^{-1} CA, 300 mg kg^{-1} CA or vehicle for 3 days. (A) Microsomal protein samples (12 μg/lane) were applied to 10% SDS-PAGE, transblotted onto nitrocellulose membranes and reacted with CYP2C11 antibodies, as described in the Material and methods. (B) Analysis of CYP2C11 protein signals. The results were analysed relative to control levels. $N = 4$ rats for each group. (C) CYP2C11 activity was evaluated by measuring CYP2C11-mediated testosterone 2α-hydroxylation, as described in the Materials and methods, and was presented as the mean of three different measurements. Data presented as mean ± SD; *lower than control, #lower than either CA or S.III-reduced levels, $p < 0.05$.

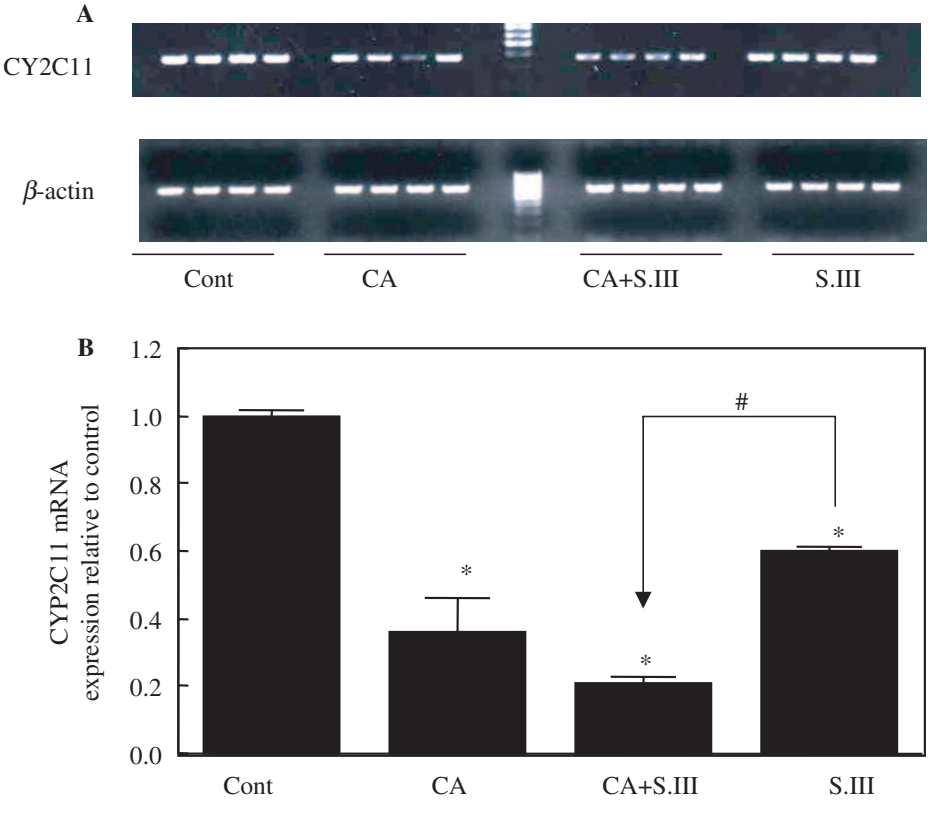

Figure 6. Effect of the PPARα ligand CA and the AhR ligand S.III on CYP2C11 mRNA. CYP2C11 mRNA was measured by semi-quantitative RT-PCR as described in the Materials and methods. cDNA samples were amplified at 25 cycles to amplify CYP2C11 mRNA (upper) and 23 cycles to amplify β-actin mRNA (lower). (B) Analysis of relative amounts of CYP2C11 mRNA by normalization to the corresponding bands of β-actin and calculated relative to the control level. Each treatment is represented by four rat samples. Data are means $\pm$ SD; *lower than control, $^{\#}$lower than either CA or S.III-reduced levels, $p < 0.05$.

S.III co-treatment (Figure 6). The results indicate that the PPARα ligand CA and the AhR ligand S.III have additive inhibitory effects on CYP2C11 in rat liver.

Discussion

This study hypothesized that the negative cross-talk between PPARα and AhR that was recently proposed (Shaban et al., 2004a) might have an impact on cytochrome P450 members that are partially modulated by each of the two receptors. The CA induction of CYP2B protein expression above that of control (Figure 1A, B) was confirmed by measuring CYP2B-mediated testosterone 16β-hydroxylation, which follows the same trend of the protein expression and was induced by CA to eightfold of the control value (Figure 1C). Moreover, the mRNA expression of both CYP2B1 and CYP2B1/2 was highly induced with CA (Figure 2). These results are in line with the previously reported induction of CYP2B1/2 with clofibric acid in rat primary hepatocytes (Bars et al., 1993, Wortelboer et al., 1991). The ability of the AhR ligand S.III to decrease CA-induced

CYP2B protein expression (Figure 1A, B) was confirmed by measuring CYP2B-mediated testosterone 16β-hydroxylation, which follows the same trend of the protein expression. S.III co-treatment with CA reduced the activity to less than 40% of the CA-induced activity. The suppressive effect of AhR stimulation on CYP2B was also confirmed by the results of CYP2B1 and CYP2B1/2 mRNA measurements, which showed that S.III caused a two-thirds reduction of the CA-induced CYP2B1 and CYP2B1/22 mRNA levels (Figure 2). The induction of CYP2B by CA is most likely to be through activation of PPARα since PPARα mRNA and protein expression are increased in rat Fao cells by ciprofibrate (Passilly et al., 1999) and in rat liver by bezofibrate (Bonilla et al., 2001). In addition, PPARα mRNA was induced in Wistar rats treated with the same dose of CA in a previous study in our laboratory (Shaban et al., 2004b) On the other hand, the suppressive effect of S.III on CYP2B is more likely to be through activation of AhR since exposure of different rat strains to the AhR ligand, TCDD, produced up-regulation of AhR protein expression *in vivo* (France et al., 2001). Furthermore, AhR protein and mRNA were induced in Wistar rats treated with the same dose of S.III in a previous study in our laboratory (Shaban et al., 2004a).

Currently there is no exact explanation of the mechanism by which PPARα ligand induces CYP2B. PPARα/RXRα heterodimers modulate gene expression by binding to sequences of DNA containing direct repeats of the hexanucleotide sequence AGGTCA separated by one nucleotide, a DR1 motif (Ijpeberg et al., 1997). PPARα/RXRα heterodimers can also modulate gene expression by a mechanism including protein–protein interactions with other transcription factors (Delerive et al., 1999). It is possible that the binding of PPARα/RXRα heterodimers to a specific binding region in the promoter of CYP2B is the mechanism by which PPARα stimulation activates CYP2B, but this speculation requires further investigation. The mutual effect between AhR and PPARα on their respective genes expression is possibly through protein–protein interactions as has been suggested for competition for the common pool of co-activators (Shaban et al., 2004a). However, the induction of CYP2B by PPARα either through direct binding to a peroxisome proliferator response element (PPRE) or through some other mechanism was suppressed by AhR stimulation in the present study. Consistent with our results, it has been reported that PCB156 caused induction of CYP2B in SD rats, whereas mutual effects were found due to TCDD co-treatment with PCB156 (Van Birgelen et al., 1994).

The induction of CYP2B by CA could be through the ability of CA to stimulate the constitutive androstane receptor (CAR), as it has been reported that ciprofibrate can induce the reporter assay of the CAR binding region on the CYP2B 5′ flanking when cells were transfected with a CAR expression vector (Kocarek et al., 2002). However, we found a PPARα binding sequence (AGGTCA) 29 bp upstream of the PBREM, which includes the CAR binding sequence used by Kocarek et al., (2002). Moreover, they showed that the transactivation of CYP2B response element with phenobarbital was enhanced by expression of wild PPARα and suppressed by co-expression of the dominant negative form. Their CYP2B response element contained PPAR, a half-binding site which we found. In this case, the inhibition of CAR-induced CYP2B by the AhR ligand could also indicate an ability of AhR to antagonize the function of CAR. In fact, a recent study states that the disruption of the AhR gene can lead to super-induction of CYP2B10 expression by phenobarbital in AhR-knockout mice (Noda et al., 2003). The ability of AhR to antagonize nuclear receptors is well known to be through competition for the common co-activators, such as SRC-1, as has been reported to be required for CAR activation (Muangmoonchai et al., 2001) as well as being required

for AhR activation (Kumar & Perdew, 1999). Furthermore, since RXRα acts as the obligate partner for CAR as well as for PPARα to mediate its function, the suppression of RXRα protein by AhR stimulation could be the mechanism by which AhR activation disturbs the induction of CYP2B (either via PPARα or CAR-dependent pathway) since it was found that S.III decreases RXRα protein expression in HepG2 cells (Shaban et al., 2004b). PPARα-mediated functions were altered in RXRα-deficient mice (Wan et al., 2000). In senescent male Fischer 344 rats, a 30% decline of the peroxisomal oxidation and catalase activities coincided with a similar reduction in the hepatic expression of the RXRα protein without significant alteration in PPARα expression (Chao et al., 2002).

The induction of CYP3A by CA treatment (Figures 3 and 4) is consistent with the previously reported induction of CYP3A23 by DHEA in rat liver (Gonzalez et al., 1986). However, peroxisome proliferators other than DHEA could not induce CYP3A23 in rats (Singleton et al., 1999). Furthermore, the induction of CYP3a11 was PPARα independent in mice (Ripp et al., 2002). In addition, it has been reported that the induction of CYP3A23 by DHEA is PXR dependent (Ripp et al., 2002). The induction of CYP3A1/2 by CA in rat in our experiments could be due to the activation of CAR since recent studies have demonstrated overlap in the genes regulated by CAR and PXR. For example, CAR can regulate *CYP3A* genes (Wei et al., 2002) and PXR can regulate *CYP2B* genes (Goodwin et al., 2001). Therefore, the down-regulation of the basal and CA-induced CYP3A (Figures 3 and 4) with S.III treatment may be due to the mutual inhibitory effect of AhR on CAR through the same mechanism mentioned above for the down-regulation of CYP2B.

The down-regulation of CYP2C11 protein expression when rats were treated with CA (Figure 5A, B) was confirmed by the suppression of CYP2C11-mediated testosterone 2α-hydroxylation (Figure 5C). This down-regulation is also consistent with the result of mRNA measured with semi-quantitative PCR (Figure 6) and is in line with the previously reported suppression by DHEA (Prough et al., 1995; Ripp et al., 2003) and by peroxisome proliferators (Corton et al., 1998). The suppression of CYP2C11 protein expression with AhR ligand, S.III (Figure 5A) is also consistent with the suppression of CYP2C11-mediated testosterone 2α-hydroxylation when rats were treated with S.III (Figure 5C), in agreement with previous results (Bhathena et al., 2002). In our study, we expected the negative cross-talk between AhR and PPARα to decrease the inhibitory effects of either of them, but surprisingly, it showed an additive inhibitory effect. This could be due to the different mechanisms of action of each as AhR down-regulates CYP2C11 by a negative transcriptional mechanism (Bhathena et al., 2002), but unfortunately this latter study failed to determine the exact negative DRE-like sequence to which AhR binds to and suppresses CYP2C11 expression. Hepatic CYP2C11 expression is regulated by the male pattern of growth hormone (GH) secretion through the Janus-kinase/signal transducer and activators of transcription proteins (JAK/STAT5b) signal transduction pathway (Badger et al., 2003). It has been reported that PPARα down-regulates STAT5b transcriptional activity, a mechanism whereby PPARα activators can suppress the expression of GH target genes including CYP2C11 (Shipley & Waxman, 2003). The additive inhibitory effect of both PPARα and AhR on CYP2C11 may indicate a completely different and separate mechanism of action for each of them, or may indicate that they have a common mechanism of action through which they compliment each other. It has been reported that STAT5b activity is not inhibited by the AhR ligand 3-MC (Bhathena et al., 2002). This excludes the ability of the stimulated AhR to synergies with PPARα to suppress CYP2C11 through an effect on STAT5b activity. However, since the exact mechanism of AhR suppressive effect on CYP2C11 is not yet clear, it is possible that there is a common mechanism through

which AhR can synergize with PPARα to produce the additive inhibitory effect on CYP2C11.

These results show that PPARα ligand CA and AhR ligand S.III have mutual effects on CYP2B expression also on CYP3A expression, whereas their inhibitory effects on CYP2C11 are additive. These mutual effects of CA and S.III on CYP2B and CYP3A are likely to be due to negative cross-talk between AhR and PPARα. However, the mutual effects of the AhR ligand S.III and the PPARα ligand CA on CYP2B and CYP3A and their additive inhibitory effect on CYP2C11 remain to be elucidated.

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