Down Regulation of Hepatic PPAR α Function by AhR Ligand

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ABSTRACT. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates a spectrum of toxic and biological effects of 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD) and related compounds. Peroxisome proliferator activated receptor alpha (PPAR α) is a nuclear receptor involved in the maintenance of lipid and glucose homeostasis. In this study we hypothesized that one of the possible mechanisms for the effect of TCDD and its related chemicals on fat metabolism could be through down regulation of PPAR α functions. We treated Wistar rats with an AhR ligand, Sudan III (S.III), and/or PPAR α ligand, Clofibrilic Acid (CA), for 3 days. We analysed the expression of one of the PPAR α -target gene products, CYP4A protein and its mRNA. We also tested HepG2 cells with the afore-mentioned treatments and evaluated their effects on PPAR α and RXR α protein. Treatment of Wistar rats with S.III was found to down regulates CYP4A protein expression and reduced its induction with CA. It also decreased mRNA expressions of CYP4A1, CYP4A2, CYP4A3 and PPAR α . In HepG2 cells, PPAR α and RXR α protein expression was decreased by S.III treatment in a dose dependent manner. Our results suggest that AhR has an inhibitory effect on PPAR α function and a new pathway by which AhR ligands could disturb lipid metabolism.

KEY WORDS: AhR, CYP4A, PPAR α rat, RXR α .

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Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear receptor deeply involved in the maintenance of lipid and glucose homeostasis [39] and is mainly expressed in liver, muscle and heart [3, 40]. Activation of PPAR α in liver, muscle and heart increases the expression and activity of lipoprotein lipase (LPL), an enzyme involved in lipolysis [35], and therefore increases the clearance of TG-rich lipoproteins, and circulating TG levels. PPAR α agonist, fibrates, inhibits ApoCIII, which act as a natural inhibitor of LPL activity [5]. Treatment of diabetic *db/db* mice with a PPAR α agonist normalizes circulating free fatty acids, TG, and glucose levels [2]. Moreover PPAR α influences the expression of numerous genes implicated in major pathways of amino acid metabolism, and fasting PPAR α -null mice have higher plasma urea concentration compared to wild type [19] PPAR α agonists, fibrates, have been in use for over 40 years for the treatment of dyslipidemia, mainly due to their actions of lowering TG levels, raising HDL [9], and decreasing levels of LDL [40]. The CYP4A sub-family members which have a role in fatty acid and prostaglandin hydroxylation, are abundantly expressed in the liver and kidney [38]. The members of this sub-family are well known to be regulated by PPAR α , which binds with RXR α to form heterodimers. These hetero- dimers then bind to enhancer elements PPRE on responsive genes including CYP4A family [17]. 20-hydroxyeicosatetraenoic acid is a major arachidonic acid metabolite of CYP4A isoforms in the microvasculature of the rat kidney and acts as a potent

vasoconstrictor and mediator of the myogenic response [24]. Collectively, PPAR α has an important role in lipid, glucose, and protein metabolism.

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 [37]. A hallmark of TCDD systemic toxicity involves an overall perturbation of energy homeostasis [31]. In rodents, low doses of TCDD result in diabetogenic effects, such as decreased glucose uptake, increased circulating triglycerides and insulin [34] and an overall loss of body fat and weight [25]. In a group of former TCDD workers, exposure to TCDD caused hyperlipidaemia, atherosclerotic plaques, ischaemic heart disease and neuropsychological disturbance, and a high percentage of them had elevated levels of triglycerides and/or cholesterol [29]. There are several lines of evidence suggesting the AhR as a negative regulator of adipocyte differentiation [1]. TCDD treatment prevented the increase of PPAR gamma expression in differentiating adipocytes [45]. Experimental animal exposure to TCDD, results in decreased levels of the glucose transporter (GLUT4) and reduced expression of many other adipocyte markers [22]. Recently, both cholesterol and triglycerides were reported to be increased shortly after intoxication with TCDD in two young women with extremely high blood TCDD levels [13]. Hyperlipidaemia is a well known predisposing factor for the development of Type 2 diabetes. A correlation between TCDD exposure and the development of type II diabetes mellitus has been suggested to be through opposition of PPARs mainly PPAR gamma [33], but to date there is no

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information regarding the effect of AhR activation on PPAR α expression and function. In the present study we investigated the effect of AhR ligand S.III on the expression of PPAR α , its partner RXR α and their target genes CYP4A. Our results show that AhR stimulation causes down regulation of PPAR α , RXR α , CYP4A protein and the mRNA expression of CYP4A1, CYP4A2, and CYP4A3, and suggest a role mediated by AhR in the down regulation of PPAR α function.

MATERIAL AND METHODS

Animal and treatments: All experiments using animals were performed under the supervision and approval of the Institutional Animal Care and Use Committee. Nine-week old Wistar rats (SLC., Hamamatsu, Japan) were divided into 4 groups of 4 rats each, housed at $24 \pm 1^\circ\text{C}$ with 12 hr light and 12 hr dark cycle, and given laboratory feed and water *ad libitum*. Rats were treated with either 80 mg/kg S.III (Sigma Chemical Co., St. Louis, MO, U.S.A.), 80 mg/kg S. III +300 mg/kg CA (Pfaltz & Bauer, Inc., NY, U.S.A.), 300 mg/kg CA, or vehicle, orally for 3 consecutive days. Twenty-four hours after the last dose, rats were sacrificed with carbon dioxide and livers were collected. The microsomal fractions from livers were prepared according to the method of Omura and Sato [28], with slight modifications. Livers were minced and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution using a Teflon homogenizer. Homogenized samples were centrifuged at $9,000 \times g$ at 4°C for 20 min. The supernatant fraction was centrifuged at $105,000 \times g$ at 4°C for 70 min to obtain a mitochondria-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 and snapped in liquid nitrogen and kept at -80°C until use. Microsomal protein concentrations were determined according to the method of Lowry *et al.* [23] using BSA (Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard.

Treatment of human hepatocarcinoma HepG2 cells: HepG2 cells, obtained from the American Type Culture Collection (Manassas, VA), were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, and antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin) at 37°C in a humidified incubator with 5% CO_2 in air. Cells were seeded in 60 mm collagen coated dishes and sub-cultured twice a week. HepG2 cells were grown to 80–90% confluence on 60 mm collagen-coated dishes. Full-length human PPAR α from human liver cDNA libraries (BD Biosciences Co., MA, U.S.A.) was amplified by polymerase chain reaction (PCR) and subcloned into pDEST12.2 mammalian expression vector using the Gateway system (Invitrogen, CA., U.S.A.). HepG2 Cells were transfected with the pDEST-h PPAR α full-length hPPAR α expression vector and/or the empty pDEST vector for equalization of the DNA amount, at concentration 1 $\mu\text{g}/\text{ml}$ by using Trans-It (Mirus, Madison, U.S.A.) according to the instruction manual. Twenty-four

hours later, cells were treated with either 0.1% DMSO (control), 500 μM CA or various concentrations of 0.01 to 10 μM S.III + 500 μM CA for a further 24 hr after which cells were scraped in ice-cold lysis buffer. The harvested cells were incubated on ice for 30 min, and the cell lysates were used as a whole for receptor measurement.

Western blot analysis: Aliquots of 12 μg of rat liver microsomal protein from treated and control rats, or of 100 μg of whole cell lysate were applied on 10–12% sodium dodecylsulfate (SDS) polyacrylamide gels and separated by electrophoresis as described by Laemmli [21] using a protean 2 mini 1-D cell (Bio Rad, Netherlands). Western blot analysis was performed according to Towbin [44]. The proteins were electrophoretically transferred to nitrocellulose membranes, blocked in 5% skim milk in phosphate buffer saline (PBS) containing 1% tween 20 for 2 hr in case of rat liver microsomal samples and for 6 hr in case of HepG2 cell lysate samples at room temperature, and probed with the polyclonal goat anti-rat CYP4A1 (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan), or rabbit-anti-human PPAR α or RXR α or goat anti-human β -actin Santa Cruz Biotechnology, CA., U.S.A.) as solutions in PBS containing 1% Tween 20 on shaker for 2 hr at room temperature. Horseradish peroxidase-labeled rabbit anti-goat IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.), or goat anti-rabbit (Santa Cruz Biotechnology, CA, U.S.A.) was used as secondary antibody. Immunoreactive protein bands were revealed colorimetrically either by oxidation of 0.025% 3,3 diaminobenzidine tetrahydrochloride (Kanto Chemical Co., Tokyo, Japan), with 0.0075% hydrogen peroxide and catalyzed by peroxidase in 50 mM Tris-HCL (pH 7.6), or by detection with a chemiluminescence Kit ECL-Plus (Amersham Life Science, Cleveland, OH, U.S.A.), with the use of Polaroid Camera. Intensities of the immunoreactive bands were densitometrically analysed using the Public domain NIH Image program (National Institutes of Health, MD, U.S.A.), available on the internet at (<http://rbs.info.nih.gov/nih-image/>).

Semi-quantitative RT-PCR: Total RNA was isolated from 50 mg of liver samples using TRIzol reagent (Life Technologies Inc, Grand Island, NY, U.S.A.), and dissolved in Diethyl pyrocarbonate (DEPC) water. cDNA was synthesized as follows: A mixture of 5 μg of 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. It was then made up to 40 μl with a mixture of 8 μl (5X) RT-buffer, 2 μl 2.5 mM dNTP, 2 μl DEPC water, and 2 μl of reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan). The incubation was done in the thermal cycler at 30°C for 10 min, 42°C for 1 hr, and 90°C for 10 min. For PCR, 1 μl aliquots of the synthesized cDNA were added to 20 μl of a mixture containing sterilized ultra-pure water, 2 μl of 10 X PCR buffer, 2 μl of 2.5 mM dNTP, 0.3 μl of sense and anti-sense primers (10 μM), and 0.1 μl of Taq polymerase (Takara, Kyoto, Japan). Amplification was initiated by a denaturation step 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 then extension at 72°C for 1 min for the proper number of

cycles for each gene using a DNA thermal cycler (Bio Rad, Hercules, CA, U.S.A.). The samples were finally incubated for 7 min at 72°C after the last cycle of amplification. The amplified PCR products were separated by electrophoresis through 1–2% agarose gel. Bands of DNA were stained with ethidium bromide and visualized by ultraviolet illumination. Intensities of the amplified DNA bands were densitometrically analyzed using the public domain NIH Image program. The primer sequences for CYP4A isoforms mRNA amplification were according to Wang *et al.* [46]. The primer sequences for rat PPAR α mRNA amplification were constructed from rat PPAR α gene sequence available in the Gene-Bank at accession number NM_013196, forward primer from 367–388: AACCATTCAACATGGTG-GACA and the reverse primer from 1781–1801: GTACATGTCTCTGTAGATCT. The primer sequences for rat β -actin mRNA amplification were constructed from rat β -actin gene sequence available in the Gene-Bank at accession number V01217,

Forward primer from 475–494: ATGTACGTAGC-CATCCAGGC,

Reverse primer from 1083–1102: TCCACACAGAG-TACTTGCGC.

Statistical analysis: All data were expressed as means $\pm$ SD. Statistical significances were evaluated using the Fisher's protected least significant difference test. An unpaired *t*-test was used to evaluate the comparisons between two means of two groups. *P* value less than 0.05 were considered to be statistically significant.

RESULTS

Down regulation of CYP4A by S.III treatment in rat liver: To investigate the effect of AhR activation on the PPAR α activity, Wistar rat were treated with S.III as an AhR ligand and CA as a PPAR α ligand. The PPAR α target gene CYP4A protein, was measured in liver microsomes by Western blotting. Rats treated with S.III showed a significant reduction of CYP4A protein compared to control (Fig. 1A and B). Treatment of rat with CA induced CYP4A protein six fold higher than control, while S.III co-treatment with CA, resulted in the reduction of CYP4A to only four and three fold depending on the dose of S.III treatment (Fig. 1C and D), indicating that S.III inhibit the ability of CA to induce CYP4A.

To explore the level at which AhR-activation causes down regulation of CYP4A, we measured the mRNA expression of CYP4A isoforms in all groups of rats. The increase of CYP4A isoforms mRNAs were increased linearly with the increase of the cycle number, we choose the lowest cycle number that shows clearly the difference between the different treated groups. As expected CYP4A1 mRNA was induced more than 7 fold with CA as compared with control, and was reduced with S.III to 50% of control, while S.III co treatment with CA reduced CYP4A1 mRNA to only 60% of the CA-induced levels. CYP4A3 mRNA follows the same trend of CYP4A1 mRNA. It was induced

4 fold with CA treatment compared to control, reduced with S.III treatment relative to control, and reduced by S.III co-treatment with CA compared to CA alone. However both the induction with CA and the reduction with S.III treatment were not so high as in the case of CYP4A1 mRNA. It is worth noting that while S.III produced reduction of CYP4A1 and CYP4A3 mRNA compared to control, it unexpectedly increased CYP4A2 mRNA higher than control levels and CYP4A2 was also induced with CA treatment to 4- fold higher than control. S.III co-treatment with CA completely abolished the induction of CYP4A2 by CA (Fig. 2 A and B). These results collectively show that AhR activation has an inhibitory effect on PPAR α function manifested by down regulation of its target genes CYP4A mRNA and protein.

Effect of AhR activation on PPAR α and RXR α protein expressions: To further investigate the nature of the inhibitory effect of AhR activation on PPAR α function, we examined the effect of AhR ligand on PPAR α and its necessary partner RXR α protein expression in a human model HepG2 cells. Transfection of HepG2 with 0.5 μ g/ml of hPPAR full-length PPAR α expressing vector increased the PPAR α expression to a highly detectable level compared to non-detectable in the non-transfected HepG2 cells. On the other hand treatment with 0.2 μ M S.III reduced the expression of the transfected PPAR α to less than 60% of the transfected control cells (Fig. 3 A and B). We further exposed other sets of PPAR α -transfected cells to increasing doses of S.III at concentrations from 0.01 to 10 μ M, which produced dose dependent decrease of PPAR α protein expression (Fig. 4 A and B). Treatment of the cells with S.III in the presence of CA also produced reduction of the receptor expression but not as much as S.III treatment alone (Fig. 4 C and D).

We also investigated the effect of AhR ligand on the important PPAR α -partner RXR α . Treatment of HepG2 cells with S.III showed reduction of protein expression of RXR α in S.III dose-dependent manner (Fig. 5 A and B). Since it was reported that PPAR α ligand stabilizes RXR α so we treated HepG2 with CA which showed no induction of its protein expression, and S.III co treatment with CA produced the same effect of dose dependent-reduction of RXR α protein expression (Fig. 5 C and D).

To make sure of the level at which AhR stimulation affects on PPAR α we measured the PPAR α mRNA expression by semi-quantitative RT-PCR in different treated and control groups of rats. Treatment of rats with CA increased PPAR α mRNA expression compared to control, and treatment with S.III decreased its expression compared to control, while S.III co-treatment decreased the CA-induced PPAR α mRNA (Fig. 6A and B). Moreover we measured RXR α mRNA also by the above method but we found no dramatic effects of either CA and/or S.III on RXR α mRNA expression (data not shown).

DISCUSSION

TCDD and dioxin-like chemicals are widespread and per-

Fig. 1. Suppression of CYP4A protein by AhR ligand treatment in rats. Male wistar rats were treated with, 80 mg/kg S.III, 80 mg/kg S.III + 300 mg/kg CA, 300 mg/kg CA or vehicle orally for 3 days. The microsomal protein samples (12 μ g/lane) were applied onto 10% SDS-PAGE, and reacted with CYP4A1 antibody as described in Materials and Methods. (A) Down-regulation of CYP4A protein in rats treated with S.III than control (C) S.III dose dependent reduction of the CA induction of CYP4A protein. Rats were treated with vehicle lanes (1,2), CA 40 mg/kg + S.III 80 mg/kg lanes (3,4) CA 40 mg/kg + S.III 40 mg/kg lanes (5,6), and CA 40 mg/kg lanes (7,8), N=4 rats for each group. Data were analysed and presented as Mean $\pm$ SD, # higher than control, * lower than control in (B), or lower than CA-induced levels in (D) $P < 0.05$.

sistent in the environment. In rodents, low doses of TCDD resulted in diabetogenic effects, such as decreased glucose uptake, increased circulating triglycerides and insulin [34] and an overall loss of body fat and weight [25]. The toxic and biological effects of TCDD and related compounds are suggested to be mediated by a ligand-activated transcription factor AhR [37] since AhR-knock out mice were reported to be resistant to TCDD-induced toxicity [10]. A large body of evidence has clearly shown that AhR plays a role in the negative regulation of adipose differentiation in 3T3-L1 cells and has a negative regulatory effect on PPAR gamma expression and function [37]. CYP4A, enzymes, the regulation of which are mediated by PPAR α act as fatty acid and prostaglandin hydroxylases that are abundantly expressed in

the liver and kidney [42]. They have been reported to metabolize arachidonic acids to 20-hydroxyeicosatetraenoic acid (20-HETE), a metabolite that has been implicated in diverse biological functions, including the regulation of vascular tone and renal tubular ion transport [32]. In this study we aimed to explore the effect of AhR on PPAR α function. S.III is an AhR ligand that has been reported to have affinity to AhR and is a CYP1A inducer [14]. Treatment of Wistar rats with S.III induced the AhR target genes CYP1A1/ CYP1A2 mRNA, protein and their metabolic activities EROD and MROD [36] as an indication for the activation of AhR.

The down regulation of CYP4A protein expression with S.III treatment of Wistar rats (Fig.1A and B) indicates that

Fig. 2. Effect of S.III treatment on CYP4A-isoform mRNA expression in rat liver. Male wistar rats were treated with 80 mg/kg S.III, 80 mg/kg S.III + 300 mg/kg CA, 300 mg/kg CA or vehicle orally for 3 days. Total RNA was isolated and RT-PCR was performed as in Materials and Methods. (A) DNA bands of CYP4A mRNA isoforms, cDNA samples were amplified at 24 cycles for CYP4A1 mRNA, 23 cycles for CYP4A2 mRNA, 25 cycles for CYP4A3 mRNA, and 23 cycles for β -actin. (B) Analysis of the relative amount of each CYP4A isoform mRNA by normalization to the corresponding band of β -actin and calculated in relation to control level. Each treatment is represented by 4 rat samples. The numbers 1, 2 and 3 indicate the column representing the mRNAs of CYP4A1, CYP4A2, and CYP4A3, respectively. Data presented as Mean $\pm$ SD. # higher than control, * lower than CA-induced level, and ** lower than control $P < 0.05$.

AhR activation has an inhibitory effect on PPAR α function. Moreover, the ability of AhR activation to decrease CYP4A induction with the PPAR α ligand CA when S.III was co-treated with CA (Fig. 1 D and C) confirmed the inhibitory effect of AhR on CYP4A. Furthermore, the reduction of the basal and the CA-induced expression of the mRNA of CYP4A1, and CYP4A3 with S.III treatment either alone or with CA (Fig. 2) suggests that AhR stimulation interferes with the transcription of all CYP4A isoforms. The induction of CYP4A2 mRNA with S.III (Fig. 2) would indicate the ability of AhR to stimulate some peroxisomal enzymes. Indeed, it was reported that TCDD and benz (a) anthracene (BA) stimulate the activities and increased the apoprotein levels of two peroxisomal enzymes, namely, acyl CoA oxidase and acyl CoA hydrolase, in intact C3H10T1/2 cells. This suggests a possible peroxisomal stimulatory effect of TCDD and BA [16]. However, the suppression of CYP4A2 induction in case of co-treatment with the PPAR α and AhR ligand (Fig. 2) strengthen our proposed hypothesis that

there is antagonistic cross-talk between the two receptors. This needs further investigation. The difference in the mode of regulation of CYP4A2 from that of CYP4A1 and CYP4A3 is well documented. CYP4A2 was reported to be male specific in the liver and kidneys [41]. There is a selective decrease in mRNA expression of CYP4A2 but not of either CYP4A1 or CYP4A3 in vitamin A-deficient rat's liver [27].

S.III was confirmed in our laboratory to have the ability to stimulate AhR in human hepatoma cells, HepG2, manifested by induction of CYP1A [36]. The reduction of PPAR α protein expression by activation of AhR with S.III treatment of HepG2 cells (Fig. 3 and Fig. 4) suggests that the effect of AhR activation on PPAR α is not only on the function but also on the expression of PPAR α . The ability of AhR stimulation with S.III to produce the same inhibitory effect on the expression of PPAR α protein, even in the presence of CA (Fig. 4 C and D) indicates that the activation of PPAR α with CA can not abolish the effect of AhR. The

Fig. 3. Efficiency of PPAR alpha transfection into HepG2 cells. HepG2 cells were grown to 80–90% confluence and either non-transfected or transfected with the full-length pDEST-hPPAR α expressing vector and/or the empty pDEST vector for equalization of the DNA amount, at 3 different concentrations (0.5 μ g, 1 μ g or 2 μ g /ml). Twenty-four hours later, cells were treated with CA 500 μ M and/or S.III 0.2 μ M for twenty-four hours after which cells were scraped in ice-cold lysis buffer. Harvested cells were incubated on ice for 30 min; aliquots of 100 μ g of whole cell lysate, were applied to 10–12% SDS-PAGE and protein was electroblotted onto nitrocellulose membrane. The membranes were reacted with rabbit anti-human PPAR α . Then the membranes were stripped and probed with goat anti-human β -actin (A). The expression of PPAR α proteins upper panel were normalized to the corresponding β -actin expression, lower panel and calculated in relation to the PPAR α expression in the control 0.5 μ g/ml-transfected HepG2 cells (B).

ability of AhR activation with S.III to down regulate PPAR α protein in HepG2 cells suggests that AhR has an effect on PPAR α expression and function in human as well as rat hepatocytes.

It was reported that PPAR α half-life is approximately 1 hr due to its rapid degradation by the proteasome, while ligand activation protects PPAR α from ubiquitination [4]. PPAR α has been shown to be in a complex with HSP90 and XAP2 in the cytoplasm [42]. At the same time, it was reported that AhR resides as a complex with HSP90 [30] and XAP2 [26] in the cytoplasm. Moreover, it was reported that exposure of different rat strains to the AhR ligand, TCDD, produced up-regulation of AhR protein expression [11], and mRNA [12]. It could be expected that the induction of AhR protein expression by its ligand activation causes the increased AhR to compete with PPAR α for the available amount of HSP90, which may affect the stability of PPAR α . SRC-1 and p300, which are co-activators for PPAR α [7, 48], have also been reported to be important for AhR activation [18, 20]. It is also possible that activation of AhR causes it to compete for these co-activators with PPAR α which may reduce its efficiency of binding to DNA, and renders it more sensitive to degradation. Furthermore,

the down regulation of PPAR α mRNA expression due to AhR activation (Fig. 6) could indicate that AhR activation affects PPAR α at transcriptional or post-transcriptional level by affecting the stability of PPAR α mRNA. In fact, TCDD was reported to decrease the PPAR gamma mRNA expression in 10T1/2 cells through activation of ERK [15]. However, the mechanism of suppression of PPAR α mRNA by AhR stimulation needs further investigation. Further examination of the effect of AhR activation on the PPAR α -partner, RXR α surprisingly showed that RXR α protein expression was also reduced by S.III in a dose-dependent manner (Fig. 5). The suppression of RXR α protein by AhR stimulation could be the mechanism by which AhR activation disturbs the function of PPAR α . Indeed it was found that PPAR α -mediated functions were altered in RXR α -deficient mice [47]. In the senescent male Fischer-344 rats, a 30% decline of the peroxisomal-oxidation and catalase activities was coincided with the similar decline in the hepatic expression of the RXR α protein [6]. However, there was no dramatic effect of either CA or S.III treatment on RXR α mRNA in our experiments, and therefore AhR stimulation may affect RXR α protein levels through enhancing its degradation.

Fig. 4. Effect of S.III treatment on PPAR alpha protein expression in HepG2 cells. HepG2 cells were grown on 60 mm collagen-coated dishes to 80–90%, and transfected with the pDEST-hPPAR α full-length expressing vector and or the empty pDEST, 24 hr later the media were washed out and the cells were treated with 0.1% DMSO (control), or S.III at different doses as shown under each lane in (A) or treated with CA 500 μ M, or CA 500 μ M + S.III at different concentrations as shown under each lane in (C). Aliquots of 100 μ g of whole cell lysate were resolved on 10% SDS-PAGE and proteins were transblotted onto nitrocellulose membranes. The membranes were reacted with rabbit anti-human PPAR α . Then the membranes were stripped and probed with goat anti-human β -actin. N=3 experiments. Data were analysed and presented as Mean $\pm$ SD. # higher than control, * lower than control in (B), or than CA induced level in (D) at P<0.05, ** lower than control in (B) or than CA induced level in (C) at P<0.01.

It was reported that di-(2-ethylhexyl) phthalate (DEHP) (a PPAR α ligand) decreased the TCDD-induced hyperlipidaemia and toxicity in F344 rats [43]. We have recently found that treatment of Wistar rats with PPAR α ligand down regulates AhR expression and function [36]. In the liver, muscle, and heart, activation of PPAR α increases the expression and activity of lipoprotein lipase (LPL), an enzyme involved in lipolysis [35], while in chickens, as in mammals, the TCDD-induced body wasting is accompanied by decreased LPL activity [8]. From our present and previous results, it seems that there is a mutual inhibitory effect

between AhR and PPAR α function and we hypothesize that this mutual inhibitory relationship is mediated by the two receptors mainly through competition for the common pool of the co-activators and, hence, affecting the stability of the receptor proteins. Also from the results of this study we present a new piece of evidence through which an AhR ligand could affect lipid metabolism and thereby possibly predispose animals to the development of Type2 diabetes.

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Fig. 5. Effect of S.III treatment on RXR alpha protein expression in HepG2 cells. HepG2 cells were grown on 60 mm collagen-coated dishes to 80–90%, and transfected with the pDEST-hPPARα full-length expressing vector and or the empty pDEST. Twenty four hours later, the media were washed out and the cells were treated with 0.1% DMSO (control), or S.III in different doses as shown under each lane in (A) or treated with CA 500 μM, or CA 500 μM + S.III at various concentrations as shown under each lane in (C). Western blotting was done as explained in Materials and Methods. The membranes were reacted with rabbit anti-human RXRα. Then the membranes were stripped and probed with goat anti-human β-actin. N=3 experiments. Data were analysed and presented as Mean ± SD. In (B) and (D), * lower than control at level P<0.05, ** lower than control at level P<0.01.

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REFERENCES

- Alexander, D.L., Ganem, L.G., Fernandez-Salguero, P., Gonzalez, F. and Jefcoate, C.R. 1998. Aryl-hydrocarbon receptor is an inhibitory regulator of lipid synthesis and of commitment to adipogenesis. *J. Cell. Sci.* **111**: 3311–3322.
- Aasum, E., Belke, D.D., Severson, D.L., Riemersma, R.A., Cooper, M., Andreassen, M. and Larsen, T.S. 2002. Cardiac function and metabolism in Type 2 diabetic mice after treatment with BM 17.0744, a novel PPAR-alpha activator. *Am. J. Physiol. Heart. Circ. Physiol.* **283**: 949–957.
- Auboeuf, D., Rieusset, J., Fajas, L., Vallier, P., Frering, V., Riou, J.P., Steels, B., Auwerx, J., Laville, M. and Vidal, H. 1997. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-[alpha] in humans: no alteration in adipose tissue of obese and NIDDM patients. *Diabetes* **46**: 1319–1327.
- Blanquart, C., Barbier, O., Fruchart, J.C., Staels, B. and Glineur, C. 2002. Peroxisome proliferator-activated receptor alpha (PPAR alpha) turnover by the ubiquitin-proteasome sys-

Fig. 6. Effect of treatment with CA and/or S.III on PPAR alpha mRNA expression in rat liver. Male wistar rats were treated with 80 mg/kg S.III, 80 mg/kg S.III + 300 mg/kg CA, 300 mg/kg CA or vehicle orally for 3 days. Total RNA was isolated and RT-PCR was performed as in Materials and Methods. (A) DNA bands of PPAR α mRNA was amplified at 28 cycles for bands of PPAR α mRNA, and 23 Cycles for β -actin. (B) Analysis of the relative expression of PPAR α mRNA, upper panel, by its normalization to the corresponding bands of β -actin, lower panel, and then calculated relative to control level. Each treatment is represented by 4 rat samples. Data presented as Mean $\pm$ SD. # higher than control, * lower than CA-induced level, and ** lower than control $P < 0.05$.

- tem controls the ligand-induced expression level of its target genes. *J. Biol. Chem.* **277**: 37254–37259.
- Bocher, V., Pineda-Torra, I., Fruchart, J.C. and Staels, B. 2002. PPARs: transcription factors controlling lipid and lipoprotein metabolism. *Ann. New York. Acad. Sci.* **967**: 7–18.
 - Chao, C., Youssef, J., Rezaiekhalegh, M., Birnbaum, L.S. and Badr, M. 2002. Senescence-associated decline in hepatic peroxisomal enzyme activities corresponds with diminished levels of retinoid X receptor alpha, but not peroxisome proliferator-activated receptor alpha. *Mech. Ageing. Dev.* **123**: 1469–1476.
 - Dowell, P., Ishmael, J.E., Avram, D., Peterson, V.J., Nevriy, D.J. and Leid, M. 1997. P300 functions as a coactivator for the peroxisome proliferator-activated receptor alpha. *J. Biol. Chem.* **272**: 33435–33443.
 - El-Sabeawy, F., Enan, E. and Lasley, B. 2001. Biochemical and toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin in immature male and female chickens. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **129**: 317–327.
 - Feher, M.D., Caslake, M., Foxton, J., Cox, A. and Packard, C.J. 1999. Atherogenic lipoprotein phenotype in type 2 diabetes: reversal with micronised fenofibrate. *Diabetes. Meta. Res. Rev.* **6**: 395–399.
 - Fernandez-Salguero, P.M., Hilbert, D.M., Rudikoff, S., Ward, J.M. and Gonzalez, F.J. 1996. Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetra-chloro dibenzo-p-dioxin-induced toxicity. *Toxicol. Appl. Pharmacol.* **140**: 173–179.
 - Franc, M.A., Pohjanvirta, R., Tuomisto, J. and Okey, A.B. 2001. *In vivo* Up-regulation of aryl hydrocarbon receptor expression by 2,3,7,8-tetra chlorodibenzo-p-dioxin-resistant rat model. *Biochem. Pharmacol.* **62**: 1565–1578.
 - Franc, M.A., Pohjanvirta, R., Tuomisto, J. and Okey, A.B. 2001. Persistent, low-dose 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: effect on aryl hydrocarbon receptor expression in a dioxin-resistance model. *Toxicol. Appl. Pharmacol.* **175**: 43–53.
 - Geusau, A., Abraham, K., Geissler, K., Sator, M.O., Stingl, G. and Tschachler, E. 2001. Severe 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) intoxication: Clinical and laboratory effects. *Environ. Health. Perspect.* **109**: 865–869.
 - Fujita, S., Suzuki, M., Peisach, J. and Suzuki, T. 1984. Induction of hepatic microsomal drug metabolism by azo compounds: a structure-activity relationship. *Chemico. Biol. Interact.* **52**: 15–37.
 - Hanlon, P.R., Ganem, L.G., Cho, Y.C., Yamamoto, M. and Jefcoate, C.R. 2003. AhR- and ERK-dependent pathways function synergistically to mediate 2,3,7,8-tetrachlorodibenzo-p-dioxin suppression of peroxisome proliferator-activated receptor-gamma expression and subsequent adipocyte differentiation. *Toxicol. Appl. Pharmacol.* **189**: 11–27.
 - Ikegwuonu, F.I. and Jefcoate, C.R. 1999. Evidence for the involvement of the fatty acid and peroxisomal beta-oxidation pathways in the inhibition by dehydroepiandrosterone (DHEA) and induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and benz(a)anthracene (BA) of cytochrome P4501B1 (CYP1B1) in mouse embryo fibroblasts (C3H10T1/2 cells) *Mol. Cell. Biochem.* **198**: 89–100.
 - Johnson, E.F., Palmer, C.N., Griffin, K.J. and Hsu, M.H. 1996.

- Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *FASEB J.* **11**: 1241–1248.
18. Ke, S., Rabson, A.B., Germino, J.F., Gallo, M.A. and Tian, Y. 2001. Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor- α and lipopolysaccharide. *J. Biol. Chem.* **276**: 39638–39644.
 19. Kersten, S., Mandard, S., Escher, P., Gonzalez, F.J., Tafuri, S., Desvergne, B. and Wahli, W. 2001. The peroxisome proliferator-activated receptor α regulates amino acid metabolism. *FASEB J.* **11**: 1971–1978.
 20. Kobayashi, A., Numayama Tsuruta, K., Sogawa, K. and Fujii-Kuriyama, Y. 1997. CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *J. Biochem. (Tokyo)* **122**: 703–710.
 21. Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**: 680–685.
 22. Liu, P.C. and Matsumura, F. 1995. Differential effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the “adipose-type” and “brain-type” glucose transporters in mice. *Mol. Pharmacol.* **47**: 65–73.
 23. Lowry, O.H., Rosebrough, N.J., FaRR, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
 24. Marji, J.S., Wang, M.H. and Laniado-Schwartzman, M. 2002. Cytochrome P-450 4A isoform expressions and 20-HETE synthesis in renal preglomerular arteries. *Am. J. Physiol. Renal Physiol.* **283**: 60–67.
 25. Matsumura, F. 1995. Mechanism of action of dioxin-type chemicals, pesticides, and other xenobiotics affecting nutritional indexes. *Am. J. Clin. Nutr.* **61**: 695S–701S.
 26. Meyer, B.K. and Perdew, G.H. 1999. Characterization of the AHR-hsp90-XAP2 Core Complex and the Role of the Immunophilin-Related Protein XAP2 in AhR.
 27. Murray, M., Sefton, R.M., Croft, K.D. and Butler, A.M. 2001. Differential regulation of endobiotic-oxidizing cytochromes P450 in vitamin A-deficient male rat liver. *Br. J. Pharmacol.* **134**: 1487–1497.
 28. Omura, T. and Sato, R. 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, Purification, and properties. *J. Biol. Chem.* **239**: 2379–2385.
 29. Pelclova, D., Fenclova, Z., Preiss, J., Prochazka, B., Spacil, J., Dubska, Z., Okrouhlik, B., Lukas, E. and Urban, P. 2002. Lipid metabolism and neuropsychological follow-up study of workers exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Int. Arch. Occup. Environ. Health* **75**: 60–66.
 30. Perdew, G.H. 1988. Association of the Ah receptor with the 90-kD a heat shock protein. *J. Biol. Chem.* **263**: 13802–13805.
 31. Potter, C.L., Menahan, L.A. and Peterson, R.E. 1986. Relationship of alterations in energy metabolism to hypophagia in rats treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Fundam. Appl. Toxicol.* **6**: 89–97.
 32. Rahman, M., Wright, J.T. Jr. and Douglas, J.G. 1997. The role of the cytochrome P450-dependent metabolites of arachidonic acid in blood pressure regulation and renal function. *Am. J. Hypertens.* **3**: 356–365.
 33. Remillard, R.B. and Bunce, N.J. 2002. Linking dioxins to diabetes: epidemiology and biologic plausibility. *Environ. Health Perspect.* **110**: 853–858.
 34. Rozman, K., Pfeifer, B., Kerecsen, L. and Alper, R.H. 1991. Is a serotonergic mechanism involved in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced appetite suppression in the Sprague-Dawley rat? *Arch. Toxicol.* **65**: 124–128.
 35. Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B. and Auwerx, J. 1996. PPAR α and PPAR β activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* **15**: 336–348.
 36. Shaban, Z., El-Shazly, S., Ishizuka, M., Kimura, K., Kazusaka, A. and Fujita, S. 2004. PPAR α -dependent modulation of hepatic CYP1A by clofibrate acid in rats. *Arch. Toxicol.* (in press).
 37. Shimba, S., Wada, T. and Tezuka, M. 2001. Arylhydrocarbon receptor (AhR) is involved in negative regulation of adipose differentiation in 3T3-L1 cells: AhR inhibits adipose differentiation independently of dioxin. *J. Cell. Sci.* **114**: 2809–2817.
 38. Simpson, A.E. 1997. The cytochrome P450 4 (CYP4) family. *Gen. Pharmacol.* **3**: 351–359.
 39. Smith, S.A. 1996. Peroxisome proliferator activated receptor (PPARs) Molecular targets for hypolipidaemic agents and insulin sensitizers. *Pharmacol. Res. Commun.* **8**: 57–64.
 40. Staels, B., Dallongeville, J., Auwerx, J., Schoonjans, K., Leitersdorf, E. and Fruchart, J.C. 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation* **98**: 2088–2093.
 41. Sumanasekera, W.K., Tien, E.S., Turpey, R., Vanden, Heuvel, J.P. and Perdew, G.H. 2003. Evidence that peroxisome proliferator-activated receptor α is complexed with the 90-kDa heat shock protein and the hepatitis virus B X-associated protein 2. *J. Biol. Chem.* **278**: 4467–4473.
 42. Sundseth, S.S. and Waxman, D. J. 1992. Sex-dependent expression and clofibrate inducibility of cytochrome P450 4A fatty acid omega-hydroxylases Male specificity of liver and kidney CYP4A2 mRNA and tissue-specific regulation by growth hormone and testosterone. *J. Biol. Chem.* **267**: 3915–3921.
 43. Tomaszewski, K.E., Montgomery, C.A. and Melnick, R.L. 1988. Modulation of 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in F344 rats by di (2-ethylhexyl)phthalate. *Chem. Biol. Interact.* **65**: 205–222.
 44. Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoresis transfer of proteins from Polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 4350–4354.
 45. Vogel, C.F. and Matsumura, F. 2003. Interaction of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with induced adipocyte differentiation in mouse embryonic fibroblasts (MEFs) involves tyrosine kinase c-Src. *Biochem. Pharmacol.* **66**: 1231–1244.
 46. Wang, M.H., Guan, H., Nguyen, X., Zand, B.A., Nasjletti, A. and Laniado-Schwartzman, M. 1999. Contribution of cytochrome P-450 4A1 and 4A2 to vascular 20 hydroxy-eicosate traenoic acid synthesis in rat kidneys. *Am. J. Physiol.* **276**: 246–253.
 47. Wan, Y.J., Cai, Y., Lungu, W., Fu, P., Locker, J., French, S. and Sucov, H.M. 2000. Peroxisome proliferator-activated receptor α -mediated pathways are altered in hepatocyte-specific retinoid X receptor α -deficient mice. *J. Biol. Chem.* **275**: 28285–28290.
 48. Zhu, Y., Qi, C., Calandra, C., Rao, M.S. and Reddy, J.K. 1996. Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor γ . *Gene. Expr.* **6**: 185–195.