Tissue-specific induction of 17 β -hydroxysteroid dehydrogenase type IV by peroxisome proliferator chemicals is dependent on the peroxisome proliferator-activated receptor α

L-Q Fan, R C Cattley and J C Corton

Chemical Industry Institute of Toxicology, PO Box 12137, Research Triangle Park, North Carolina 27709, USA

(Requests for offprints should be addressed to J C Corton, CIIT, 6 Davis Drive, PO Box 12137, Research Triangle Park, North Carolina 27709, USA)

Abstract

The 17 β -hydroxysteroid dehydrogenase (17 β -HSD) family of proteins regulates the levels of the active 17 β -hydroxy forms of sex steroids. The expression of 17 β -HSD type IV is induced by peroxisome proliferator chemicals (PPC) in rat liver. In order to characterize more generally the impact of PPC on 17 β -HSD expression, we determined (1) if expression of other members of the 17 β -HSD family was coordinately induced by PPC exposure, (2) the tissues in which 17 β -HSD was induced by PPC, and (3) whether the induction of 17 β -HSD by PPC was dependent on the peroxisome proliferator-activated receptor α (PPAR α), the central mediator of PPC effects in the mouse liver. The mRNA levels of 17 β -HSD I, II, and III were not altered in the liver, kidney, and testis or uterus of rats treated with PPC. The mRNA or 80 kDa full-length protein levels of 17 β -HSD IV were strongly induced in

liver and kidney, but not induced in adrenals, brown fat, heart, testis, and uterus of rats treated with diverse PPC. In liver and kidneys from treated rats, additional proteins of 66 kDa, 56 kDa, and 32 kDa were also induced which reacted with the anti-17 β -HSD IV antibodies and were most likely proteolytic fragments of 17 β -HSD IV. Treatment of mice which lack a functional form of PPAR α with PPC, demonstrated that PPC-inducibility of 17 β -HSD IV mRNA or the 80 kDa protein was dependent on PPAR α expression in liver and kidney. Our results demonstrate that 17 β -HSD IV is induced by PPC through a PPAR α -dependent mechanism and support the hypothesis that exposure to PPC leads to alterations in sex steroid metabolism.

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Introduction

The 17 β -hydroxysteroid dehydrogenases (17 β -HSD) are an important enzyme family which catalyze the inter-conversion of inactive (or less active) 17-keto and active 17 β -hydroxy forms of estrogen, androgen, and progesterone (reviewed in Andersson & Moghrabi 1997). Six types of 17 β -HSDs have been identified. 17 β -HSD type I, originally cloned from a human placental library is also expressed in the ovary and mammary gland (Martel *et al.* 1992, Ghersevich *et al.* 1994, Nokelainen *et al.* 1996). The type II enzyme, cloned from prostatic and placental cDNA libraries, is expressed in many tissues including liver, small intestine, endometrium, kidney, pancreas and colon in humans (Casey *et al.* 1994, Akinola *et al.* 1996). The expression of 17 β -HSD type III is primarily restricted to testes in humans and mice (Geissler *et al.* 1994, Sha *et al.* 1997). The 17 β -HSD IV, originally cloned from porcine endometrium (Adamski *et al.* 1995) is broadly expressed in rodents and humans (Mustonen *et al.* 1997, Carstensen *et al.* 1996). A human type V isozyme is expressed in liver and skeletal muscle (Zhang *et al.* 1995).

A 17 β -HSD type VI was cloned from rat prostate and is also expressed in liver (Biswas & Russell 1997). In keeping with their sex organ-specific expression, types I and III have been shown preferentially to catalyze the production of 17 β -hydroxy forms of estrogen and androgen respectively (Luu-The *et al.* 1995). In general, types II, IV, and VI inactivate the sex steroids by catalyzing the production of the keto forms in peripheral tissues.

The type IV enzyme possesses a number of unique features compared with the other 17 β -HSD family members. First, 17 β -HSD IV is found primarily in peroxisomes (Markus *et al.* 1995) whereas the other 17 β -HSD family members are located either in microsomes or in cytosol (summarized in Andersson & Moghrabi 1997). Secondly, 17 β -HSD IV is a multifunctional protein possessing separable 17 β -hydroxysteroid/fatty acyl-CoA dehydrogenase, fatty acyl-CoA-hydratase and sterol transfer activities (Leenders *et al.* 1996). 17 β -HSD IV has also recently been found to be involved in degradation of branched chain fatty acids (Dieuaide-Noubhani *et al.* 1997a) and the side chain of cholesterol (Dieuaide-Noubhani *et al.* 1997b). Lastly, 17 β -HSD IV expression is stimulated by

peroxisome proliferator chemicals in rat liver leading to the hypothesis that exposure to these chemicals could result in alteration of estrogen-dependent functions (Corton *et al.* 1996).

Peroxisome proliferator chemicals (PPC) are a large group of chemicals which include hypolipidemic drugs, plasticizers, and industrial solvents that induce hepatomegaly and hepatic peroxisome proliferation in rodents. Responses to PPC exposure are controlled by members of the nuclear receptor superfamily called peroxisome proliferator-activated receptors (PPAR). After binding PPC, these receptors heterodimerize with another member of the superfamily called retinoid X receptor (RXR). The PPAR–RXR heterodimer binds to DNA sequences called peroxisome proliferator response elements found in responsive genes resulting in either transcriptional activation or down-regulation (reviewed in Lapinskas & Corton 1998). Genes induced by PPAR include those that catalyze β - and ω -oxidation of fatty acids. Using a mouse strain which lacks a functional form of PPAR α (PPAR α -null mouse), Lee *et al.* (1995) demonstrated that most, if not all, of the effects induced by PPC in the liver are mediated by PPAR α .

To characterize more generally the impact of PPC on 17 β -HSD expression, we determined if tissues important in metabolizing steroids were sensitive to the induction of 17 β -HSD family members by PPC. Furthermore, we evaluated the mechanism of the PPC-mediated induction of 17 β -HSD by comparing inductive behavior in wild-type mice with mice which lack a functional PPAR α .

Materials and Methods

Animals

These studies were conducted under federal guidelines for the use and care of laboratory animals and were approved by the Chemical Industry Institute of Toxicology Institution Animal Care and Use Committee. In the first three studies, F344 (CDF(F344)/CrIBR) rats, 6–8 weeks of age were obtained from the Charles River Breeding Laboratories, Inc. (Raleigh, NC, USA). Control and treated rats or mice were provided with NIH-07 rodent chow (Ziegler Bros., Gardner, PA, USA) and deionized, filtered water *ad libitum*. Lighting was on a 12 h light:12 h darkness cycle. In the first study, male and female F344 rats were fed rodent chow containing 500 parts per million (ppm) WY-14,643 (WY; ChemSyn Science Laboratories, Lenexa, KS, USA), 8000 ppm gemfibrozil (GEM, Sigma Chemical Co., St Louis, MO, USA) or 20 000 ppm di-*n*-butyl phthalate (DBP, Aldrich Chemical Co., Milwaukee, WI, USA) for 13 weeks. In the second study, male and female F344 rats were given gavage doses of either vehicle (methylcellulose), WY (50 mg/kg body weight), or di-(2-ethylhexyl) phthalate (DEHP; 2000 mg/

kg body weight) in methylcellulose each day for three days. In the third study, male F344 rats were fed rodent chow containing 1.2% DEHP, clofibric acid (CA; 0.5%) or WY (0.1%), for 22 weeks. In the fourth study, male SV129 wild-type, (Taconic, Germantown, NY, USA) and SV129 PPAR α -null mice (Lee *et al.* 1995) 6–8 weeks of age were fed WY (0.1%) or DEHP (0.6%) for three weeks. At the designated time following treatment, animals were deeply anesthetized by pentobarbital injection and killed by exsanguination. The tissues were removed, rinsed with isotonic saline, snap-frozen in liquid nitrogen and stored at -70°C until analysis.

Hepatocyte isolation and cell culture

For preparation of primary hepatocytes, adult male Fischer F344 rats (approximate weight 200–250 g), were obtained from Charles River Breeding Laboratories. The animals were maintained under conditions of constant temperature and humidity and allowed rodent chow and water *ad libitum*. Hepatocytes were isolated by nonrecirculating collagenase perfusion through the portal vein of pentobarbital-anesthetized rats according to the method of Kedderis *et al.* (1988). A thin coat of Matrigel (Becton Dickinson, Bedford, MA, USA) was applied to 100 mm diameter plastic culture dishes and allowed to gel at room temperature. Cells, 2.6×10^6 per 100 mm plate with viability greater than 80% as determined by trypan blue exclusion, were plated in 3 ml modified Waymouth media containing insulin (0.2 $\mu\text{g}/\text{ml}$) as the only hormone. Cultures were maintained in an incubator at 37°C in an atmosphere containing 5% CO_2 . Medium was replaced daily, commencing 24 h after the cells were plated. Cells were cultured for five days before treatment as described (Chen *et al.* 1995). Dimethyl sulfoxide or WY at the indicated concentrations was added, and the cells were incubated for 48 h changing the media after 24 h. Media were aspirated from culture dishes and replaced with 3 ml ice-cold, phosphate-buffered saline, with 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. The cells and Matrigel were transferred to 15 ml capped plastic tubes and then allowed to stand on ice for 45 min to dissolve the Matrigel. Cells were then collected by centrifugation at 800 g for 5 min and lysed. Extracts were used for analysis of protein expression.

Northern blot analysis

Total RNA was isolated by a modification of the guanidinium isothiocyanate method using RNAzol B according to the manufacturer's instructions (Tel-Test, Friendswood, TX, USA). Twenty micrograms denatured total RNA was separated on 1.0% formaldehyde-agarose gels and transferred to Hybond-N nylon membranes in $20 \times$ standard saline citrate buffer. The RNA was fixed by UV cross linking using a Stratalinker UV crosslinker (Stratagene, La

Jolla, CA, USA). The membrane was prehybridized with Hybrisol I (Oncor Inc., Gaithersburg, MD, USA) for 4 h at 42 °C, followed by hybridization overnight. The 1.4 kb EcoRI fragment encoding the rat 17 β -HSD I cDNA and the 1.3 kb EcoRI fragment encoding the rat 17 β -HSD II were generated from the plasmids kindly provided by Dr Hellevi Peltoketo (University of Oulu, Oulu, Finland). The 740 bp SacI-KpnI fragment encoding the mouse 17 β -HSD III was generated from a plasmid kindly provided by Dr Paul J Baker (University of Glasgow Veterinary School, Glasgow, UK). The rat full-length 2.4 kb 17 β -HSD type IV cDNA probe was generated by PCR using M13 forward and M13 reverse primers and pBluescript IISK plasmid encoding 17 β -HSD IV (Corton *et al.* 1996). The β -actin cDNA was obtained from Oncor Inc. β -Actin was used as a loading control since the level does not change after short-term exposure to WY (Goldsworthy *et al.* 1993). The probes for Northern analysis were labeled with [α -³²P]dCTP (Amersham, Cleveland, OH, USA) using the random-primer DNA labeling kit according to the manufacturer's instructions (Stratagene). After washing, the filter was exposed to BioMax X-ray film (Eastman Kodak Co.) at -70 °C with intensifying screen. The size of mRNA was determined by comparing mobility relative to an RNA ladder (GIBCO/BRL, Gaithersburg, MD, USA).

Western blot analysis

Whole-cell protein extracts (100 μ g total protein), made according to Wilcke and Alexson (1994), were denatured and size-separated by 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose (Stratagene) and visualized by Ponceau Red to confirm transfer. The membrane was washed with phosphate-buffered saline-Tween (PBST). The membranes were blocked by incubating in PBST containing 5% non-fat dry milk. Immunoreactivity was detected by incubation with primary antibody followed by horseradish peroxidase conjugated goat-anti-rabbit IgG (Amersham) as secondary antibody. The reaction was detected by enhanced chemiluminescence (SuperSignal, Pierce Chemical Co., Rockford, IL, USA). The sizes of the reactive immunoproteins were estimated by the gel fragment sizer software (DogStar Software, Bloomington, IN, USA) by comparison to the mobility of protein standards (Rainbow marker; Amersham). Autoradiograms for Northern blots and Western blots were densitometrically scanned using Image-1 image analysis system (Universal Imaging Corporation, West Chester, PA, USA) and NIH Image 1.54 software. The 17 β -HSD IV antibodies from two sources (Jerzy Adamski, GSF Research Center, Neuherberg, Germany and Takashi Hashimoto, Shinshu University School of Medicine, Matsumoto, Nagano, Japan) gave the same results. Antibodies obtained from J Adamski were prepared against porcine 17 β -HSD IV but they crossreact with human, rat and mouse

tissues. The anti-acyl-CoA oxidase (ACO) antibody was from Stefan Alexson (Huddinge University Hospital, Huddinge, Sweden).

Results

Alteration of 17 β -HSD mRNA expression after exposure to PPC

The mRNA levels of 17 β -HSD I, 17 β -HSD II, 17 β -HSD III and 17 β -HSD IV were determined by Northern blot analysis in rats treated in the diet for 13 weeks with three PPC (WY, GEM, and DBP). The type I mRNA was detected in uterus as a 1.5 kb mRNA but not in liver, kidney, or testis from control rats. The 17 β -HSD II mRNA (1.6 kb) was expressed to high levels in liver and moderate levels in kidney in control rats. The type III mRNA was detected as a 1.4 kb mRNA in testis but not in liver, kidney or uterus from control rats. The pattern of expression of the 17 β -HSD I, II, and III is consistent with earlier studies (Mustonen *et al.* 1997 and references therein). After PPC treatment the abundance of types I, II, or III was not altered in any of the tissues examined (data not shown). In contrast the 2.8 kb 17 β -HSD IV mRNA levels were increased (~3–4 fold) in rats fed WY, GEM and DBP compared with control rats (Fig. 1A). In the kidneys of rats fed PPC, there was no significant induction of 17 β -HSD IV mRNA (Fig. 1B). Weak constitutive expression of 17 β -HSD IV mRNA could be detected in heart and testis, but no changes in levels were observed after PPC exposure (data not shown). Thus, the increases in 17 β -HSD mRNA levels were specific for the type IV gene.

Alterations in 17 β -HSD IV protein levels after exposure to PPC

In parallel with the analysis of 17 β -HSD mRNA expression, we analyzed the 17 β -HSD IV protein levels after a 13 week exposure to PPC in whole-cell extracts by Western blot analysis from the same tissues used for mRNA expression. In the absence of treatment we could detect only weak expression of the 80 kDa full-length 17 β -HSD IV protein (Fig. 2A). The full-length 17 β -HSD IV protein was strongly induced after treatment by GEM (95-fold) and induced to lesser extents by WY (9-fold) and DBP (4-fold). The preferential induction by GEM under these conditions is in contrast to the almost equal induction of the mRNA by the three PPC. As noted before, posttranscriptional differences in protein translation efficiency or protein stability could be responsible (Corton *et al.* 1996). Immunoreactive proteins of 66 kDa, 56 kDa, 42 kDa, and 32 kDa were also strongly induced by GEM. After WY and DBP treatment, the 66 kDa and 56 kDa proteins were induced but to lesser extents. These changes

Figure 1 Expression of 17 β -HSD IV mRNA after PPC exposure. Northern blot analysis of 17 β -HSD IV mRNA in liver (A, upper) and kidney (B, upper) from control and treated rats. Total RNA was isolated from F344 male rats fed a control diet (Control) or diets which contained 500 ppm WY, 8000 ppm GEM, or 20 000 ppm DBP for 13 weeks. Total RNA was separated on formaldehyde-agarose gels, transferred to nylon membranes and probed with either the full-length rat 17 β -HSD IV cDNA or a fragment of β -actin. The autoradiograms were densitometrically scanned and 17 β -HSD IV expression in liver (A, lower) and kidney (B, lower) was normalized to β -actin expression. The histograms show the mean induction (mean $\pm$ S.D.) from two different samples. * $P < 0.05$ compared with control.

were similar to those observed earlier in liver after PPC treatment (Corton *et al.* 1996) using a different antibody to 17 β -HSD IV.

In parallel with the increases in 17 β -HSD IV protein levels in rat liver after PPC treatment, we observed increases in the expression of ACO protein (Fig. 2A, bottom). Interestingly, the pattern of induction of different forms of the ACO protein was unique to the compound. WY induced expression of two forms of ACO to approximately equal extents: ACO-A, the 72 kDa full-length ACO protein and ACO-B, a 56 kDa form which results from proteolytic processing of ACO-A. After GEM treatment, most of the induced ACO protein was found to be ACO-B with little ACO-A observed. Treatment with DBP resulted in weak induction of ACO-B only.

In control kidneys the full-length 17 β -HSD IV protein plus proteins of 42 kDa and 32 kDa (bands 3 and 4 respectively) were observed. The 42 and 32 kDa proteins were indistinguishable in size to proteins induced after GEM treatment in liver. After exposure to GEM, the 80 kDa protein was induced 9-fold in rat kidneys. WY and DBP also induced the 80 kDa protein albeit to lesser extents (4-fold and 3-fold respectively). Immunoreactive proteins of 66 kDa, 56 kDa, and 32 kDa were also strongly induced by GEM following the pattern observed in the liver of GEM-treated rats. Slight induction of the 56 kDa protein was seen in kidneys after WY or DBP treatment.

In contrast to liver, the expression of the 42 kDa protein did not change after exposure to any PPC.

ACO protein was also differentially induced by PPC in the kidneys. Increases in the levels of ACO-A and ACO-B were detected after WY or GEM treatment (Fig. 2B). Little, if any, increase in ACO was detected after DBP treatment.

In a separate feeding study rats were treated in the diet for 22 weeks with PPC (WY, CA and DEHP), and 17 β -HSD IV expression was analyzed in interscapular brown fat and adrenals. In the brown fat from control rats, expression of full-length 17 β -HSD IV, and variable expression of the 42 kDa fragment were detected (Fig. 2C). After exposure to CA or WY, slight induction of the full-length protein was detected (1.6-fold and 2.5-fold respectively) but was not statistically significant. Weak induction of the 56 kDa and 32 kDa fragments was also observed after WY treatment. WY was the strongest inducer of ACO-A and ACO-B in brown fat, with DEHP and CA treatment resulting in little, if any, induction.

In adrenals from control rats only the 42 kDa fragment was expressed to appreciable levels (Fig. 2D). After PPC exposure no induction of 17 β -HSD IV or any of the immunoreactive fragments was observed. However, we did observe that the expression of full-length protein was induced by CA in liver in the same feeding study (data not shown). Induction of ACO-A was observed in adrenals

Figure 2 Expression of 17 β -HSD IV protein after chronic PPC exposure. Western blot analysis of 17 β -HSD IV protein in liver (A), kidney (B), brown fat (C), and adrenal (D) from control and treated rats. Whole-cell liver or kidney protein extracts were made from the rats used in Fig. 1. Whole-cell brown fat or adrenal protein extracts were made from rats fed DEHP (1.2%), CA (0.5%) or WY (0.1%) in the diet for 22 weeks. Protein extracts of each sample (100 μ g) were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies against 17 β -HSD IV or ACO. In (B), protein extracts from livers of rats treated with 8000 ppm GEM for 13 weeks were run as a control (L). 17 β -HSD IV, position of the full-length 80 kDa 17 β -HSD IV protein; 1–4, positions of immunoreactive proteins with molecular masses of 66, 56, 42 and 32 kDa respectively. The position of ACO forms A and B which have molecular masses of 72 and 52 kDa respectively are indicated. Numbers on the left are the position of molecular mass markers (kDa).

from rats treated with WY. Induction of ACO-B was not observed after any treatment.

To begin to determine how rapidly accumulation of the full length 17 β -HSD IV and fragment changes after exposure, and to determine if sex-specific differences in induction exist, 17 β -HSD IV and ACO protein expression was also examined in the livers of male and female rats given a gavage dose of WY or DEHP each day for three days. As shown in Fig. 3, exposure to WY resulted in increased abundance of the full-length 17 β -HSD IV

(3-fold in males and 2-fold in females). Treatment with DEHP did not appreciably alter the full-length 17 β -HSD IV expression. There was a high level constitutive expression of the 42 and 32 kDa proteins that was not observed in earlier studies. Exposure to either WY or DEHP resulted in increased expression of ACO-B with WY inducing more than DEHP.

Next we determined if the PPC-induced expression could be reconstituted *in vitro* by examining the expression of 17 β -HSD IV in rat primary hepatocytes after a 48-h

Figure 3 Expression of 17 β -HSD IV protein after acute PPC exposure. Western blot analysis of 17 β -HSD IV protein from livers of male and female F344 rats given daily gavage doses of WY (50 mg/kg body weight) or DEHP (200 mg/kg body weight) for 3 days. Whole-cell protein extracts (100 μ g) were separated and analyzed as described in the legend to Fig. 2.

Figure 4 Increased expression of 17 β -HSD IV protein by WY *in vitro*. Rat primary hepatocytes were treated with solvent control (lane 1), and 100 μ M (lane 2), 200 μ M (lane 3) or 400 μ M (lane 4) WY for 48 h. Protein extracts were analyzed by Western blot for expression of 17 β -HSD IV and ACO as described in the legend to Fig. 2.

treatment with different doses of WY. As shown in Fig. 4, expression of the full-length 17 β -HSD IV but not the immunoreactive fragments and ACO-B increased after WY exposure in a dose-dependent manner increasing to maximal levels at 200 μ M WY. At 400 μ M WY the expression of both proteins was detectably decreased. Inhibition of PPC-inducible gene expression at high doses of PPC has been observed before (Rocos & Ledwith 1997 and references therein).

Dependence of 17 β -HSD IV induction by PPC on expression of PPAR α

To determine if PPAR α is important in the induction of 17 β -HSD IV after PPC exposure, we examined mRNA and protein expression in tissues from wild-type mice and mice which lack a functional form of PPAR α (PPAR α -

null mice). Wild-type and PPAR α -null mice were fed a control diet or diets containing 0.1% WY or 0.6% DEHP for 3 weeks. As shown in Fig. 5A, expression of the 2.5 kb 17 β -HSD IV mRNA was increased in the livers of wild-type mice after treatment with WY or DEHP (3-fold and 4-fold respectively). This induction was abolished in PPAR α -null mice treated with either WY or DEHP. In kidneys from wild-type mice, the levels of 17 β -HSD IV mRNA increased after WY and DEHP treatments (2.2-fold and 2.0-fold respectively) (Fig. 5B). The increase was abolished in the PPAR α -null mice. In contrast to the increases in 17 β -HSD IV mRNA levels after treatment in liver and kidney, the expression of 17 β -HSD IV did not change after exposure in testis (Fig. 5C) or heart (data not shown) from wild-type or PPAR α -null mice.

The expression of the 17 β -HSD IV protein was also examined in wild-type and PPAR α -null mouse tissues. In livers of both mouse types, expression of the 17 β -HSD IV 80 kDa protein and the 42 kDa immunoreactive protein was detected in the absence of exposure. In wild-type mice, the 80 kDa 17 β -HSD IV protein was induced after WY or DEHP exposure (7-fold and 4-fold respectively) (Fig. 6A). Immunoreactive proteins of 66 kDa and 56 kDa were also induced by WY or DEHP in wild-type mice. The 42 kDa fragment was not induced by PPC treatment. No induction of the 80 kDa protein or any of the immunoreactive proteins was observed in PPAR α -null mice after WY or DEHP exposure. ACO-A and ACO-B proteins were strongly induced in wild-type but not PPAR α -null mice after WY or DEHP treatment as demonstrated previously for ACO mRNA (Lee *et al.* 1995).

The dependence of 17 β -HSD IV inducibility on PPAR α expression was also examined in whole-cell extracts from kidneys. Both mouse strains constitutively expressed the 42 kDa protein in the absence of exposure (Fig. 6B). Weak expression of the 32 kDa fragment was also detected in both mouse strains. After exposure to WY or DEHP, the 80 kDa full-length protein was induced in kidneys of wild-type mice (9-fold and 7-fold respectively). In the PPAR α -null mice, no induction of the full-length 17 β -HSD IV or fragments was observed after exposure to WY or DEHP. Expression of ACO-A and ACO-B was increased after WY but not DEHP exposure in wild-type mice (Fig. 6B). Although little, if any, increase in ACO-B could be observed in the kidneys of PPAR α -null mice after WY exposure, ACO-B was increased after DEHP treatment. This induction may be mediated by other PPAR isoforms which are expressed in the kidney (Braissant *et al.* 1996).

In testis both wild-type and PPAR α -null mice lacked expression of the full-length protein but strongly expressed the 42 kDa immunoreactive protein and a unique 38 kDa immunoreactive protein (Fig. 6C). No changes in the expression of the full-length 17 β -HSD IV, 17 β -HSD IV immunoreactive proteins or ACO were

Figure 5 Regulation of 17 β -HSD IV mRNA levels after PPC exposure in wild-type and PPAR α -null mice. Northern blot analysis of 17 β -HSD IV mRNA in liver (A, left-hand panel), kidney (B, left-hand panel), and testis (C, left-hand panel) from male SV129 wild-type (PPAR^{+/+}) and SV129 PPAR α -null mice (PPAR^{-/-}) fed a control diet (Control), WY (0.1%) or DEHP (0.6%) for three weeks. Total RNA samples were separated on formaldehyde-agarose gels, transferred to nylon membranes and probed with either the full-length rat 17 β -HSD IV cDNA or a fragment of β -actin. The autoradiograms were densitometrically scanned and mouse 17 β -HSD IV expression in liver, kidney and testis was normalized to β -actin expression. The histograms show the mean induction (mean $\pm$ s.d.) in wild-type mice (middle panels) or PPAR α -null mice (right-hand panels) from two or three different samples. * P <0.05 compared with control. KO, knock out.

observed after PPC exposure in wild-type mice or PPAR α -null mice.

Discussion

The 17 β -hydroxysteroid dehydrogenase type IV was identified as a gene whose mRNA was elevated in the livers of rats after exposure to peroxisome proliferator chemicals (Corton *et al.* 1996). Despite their structural dissimilarity these chemicals elicit a common set of responses in rodents including peroxisome proliferation, increased hepatocyte proliferation and decreased apoptosis, alteration in fatty acid metabolism, and after long-term exposure, hepatocarcinogenesis (reviewed in Lapinskas & Corton 1998). Treatment may also lead to alterations in endocrine function as changes in serum estrogen levels

in both male and female rats have been observed after exposure to some PPC (summarized in Corton *et al.* 1997). In an effort to characterize the tissue-specificity and mechanism of the PPC-responsiveness of HSD family members, we examined expression in different tissues of rats and mice after exposure to diverse PPC. The results led to three main conclusions.

First, the induction of the 17 β -HSD mRNA by PPC was restricted to the type IV gene and only in those tissues which significantly respond to the enzyme inductive effects of PPC. Alteration in 17 β -HSD I, II or III mRNA levels was not observed in a number of tissues important in sex hormone synthesis or degradation. The strongest induction of 17 β -HSD IV was observed in rat and mouse liver, with moderate induction in the kidney. Induction of 17 β -HSD IV in the liver was not limited to one subgroup of PPC but to all PPC tested in this study including

Figure 6 Regulation of 17 β -HSD IV protein levels after PPC exposure in wild-type and PPAR α -null mice. Western blot analysis of 17 β -HSD IV protein levels in whole-cell extracts from livers (A), kidneys (B) and testis (C) of mice described in the legend to Fig. 5. Protein extracts (100 μ g) were separated by 12.5% SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies against 17 β -HSD IV or ACO. In (B) and (C), a liver protein extract from a rat treated with 8000 ppm GEM for 13 weeks was run as a control (L). 17 β -HSD IV, position of the full-length 80 kDa rat 17 β -HSD IV protein; 1–4, positions of immunoreactive proteins with molecular masses of 66, 56, 42 and 32 kDa respectively. Position of ACO forms A and B are indicated. The numbers on the left indicate the position of molecular mass markers (kDa).

hypolipidemic drugs (WY, GEM and CA) and phthalate ester plasticizers (DBP and DEHP). In contrast, another group (Qin *et al.* 1997) found that CA had no effect on 17 β -HSD IV protein levels in liver. Although we could detect constitutive expression of the mRNA and/or protein in many other tissues including testes, heart, and adrenals in the absence of exposure, expression of 17 β -HSD IV could not be induced by PPC in these tissues even after long-term exposure to relatively high doses of PPC. This tissue-restricted pattern of PPC-inducibility of 17 β -HSD IV is identical to the inducibility of other peroxisomal genes/proteins which catalyze β -oxidation of fatty acids such as ACO, the multifunctional protein, and thiolase (Nemali *et al.* 1988). In our studies we detected induction of ACO in all tissues where 17 β -HSD IV was induced, although weak induction of ACO but not 17 β -HSD IV was also detected in adrenals and brown fat. Taken together, these results indicate that inducibility of β -oxidation genes and inducibility of 17 β -HSD IV share a common mechanism.

Secondly, we have shown that the induction of the 17 β -HSD IV mRNA and protein by PPC is mediated by a member of the nuclear receptor superfamily called PPAR α . This receptor has been shown to mediate most, if not all, of the effects of PPC in the liver including enzyme induction, peroxisome proliferation (Lee *et al.* 1995), cell proliferation and carcinogenesis (Peters *et al.* 1997). PPC-induced increases in 17 β -HSD IV levels were completely abolished in both livers and kidneys from mice which lack a functional PPAR α . No induction of 17 β -HSD IV in heart (data not shown) or testis (Fig. 6C) was detected even though PPAR α and other PPAR isoforms which are activated by PPC are expressed in these tissues (Braissant *et al.* 1996). It is possible that PPAR α , the preferential target of PPC action, is not expressed in these tissues to levels high enough to initiate induction or a mechanism is in

place to block the ability of PPAR α to activate 17 β -HSD IV transcription. Our data also demonstrate that 17 β -HSD IV is constitutively regulated in many tissues by a mechanism that does not involve PPAR α but could involve the PPAR δ isoform which is broadly expressed or the PPAR γ isoform which is mainly expressed in adipose tissue including brown fat (Braissant *et al.* 1996). Because PPAR α is also activated by endogenous prostaglandins, 8S-hydroxyecosatetraenoic acid and leukotriene B₄ (summarized in Lapinskas & Corton 1998), 17 β -HSD IV levels could be augmented under physiological conditions in which these eicosanoids are elevated.

Thirdly, we have shown that rat and mouse tissues express a number of proteins with conserved sizes which are immunoreactive with the anti-17 β -HSD IV antibody and most likely result from proteolytic processing of the full-length 80 kDa protein for the following reasons. (1) The fragments (66, 56, 42 and 32 kDa) were detected by two different antibodies raised to 17 β -HSD IV and thus are unlikely to result from recognition of an epitope in an unrelated protein. (2) The abundance of the fragments parallels the abundance of the full-length protein. GEM strongly induces the full-length protein as well as the fragments. WY or DBP only weakly induce the full-length protein and the fragments. (3) Fragments of similar sizes with activities associated with 17 β -HSD IV have been isolated by other groups (Adamski *et al.* 1992, Qin *et al.* 1997). Isolation and characterization of a $\sim$ 32 kDa protein with 2-enoyl-CoA hydratase 2 activity from rat liver led to the cloning of the full-length rat 17 β -HSD IV (Qin *et al.* 1997). Additional fragments of 66 and 46 kDa identified by Western analysis using polyclonal antibodies to the hydratase-2 domain were also identified in rat liver in this study. In pigs, a 32 kDa protein was isolated with dehydrogenase activity (Adamski *et al.* 1992). Our data support a model in which the full-length 80 kDa 17 β -HSD IV is

induced by PPC exposure in responsive tissues, followed by immediate limited proteolysis to defined fragments. In the absence of PPC exposure and consistent with the constitutive expression of 17 β -HSD IV mRNA, constitutive expression of the 42 kDa fragment is found in rat kidneys and adrenals and in mouse livers, kidneys, and testes.

Preliminary evidence indicates that the full-length 17 β -HSD IV from rats is proteolytically processed *in situ* between residues 312–326 (Qin *et al.* 1997) at a site similar to that first observed in the pig enzyme (Leenders *et al.* 1994). Cleavage of the rat protein at this site would result in a 32 kDa N-terminal fragment and a C-terminal fragment with a size almost identical to the 46 kDa fragment observed earlier in liver extracts and isolated peroxisomes (Dieuaide-Noubhani *et al.* 1997b, Qin *et al.* 1997) and very similar in size to the 42 kDa fragment observed in our studies. This simplified proteolytic processing scheme, however, does not explain all of the fragments observed in our study as we also observe 66 and 56 kDa fragments only after PPC treatment in liver and kidney and a constitutively expressed 38 kDa protein in mouse testis. The proteolysis of 17 β -HSD IV in many tissues where there is constitutive expression must be rapid since the 42 kDa fragment, but little, if any, of the full-length form is detected in these tissues. Additional processing pathways important in generation of the other fragments awaits characterization.

Based on an analysis of the functional properties of individually expressed putative domains of 17 β -HSD IV, Leenders *et al.* (1996) concluded that individual functions are encoded by different domains of the protein. The N-terminal 32 kDa fragment (amino acids 1–323) is able to perform the dehydrogenase reaction not only with steroids at the C17 position but also with 3-hydroxyacyl-CoA. The central portion of the protein, amino acids 324–596 catalyzes the 2-enoyl-acyl-CoA hydratase reaction. The C-terminal amino acids 597–737 are homologous to sterol carrier protein 2 and facilitate the transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes *in vitro*. Although the origin of the fragments that have 17 β -HSD IV immunoreactivity identified in our studies and those of others has not been defined, it is likely that tissue-specific expression of the fragments will give a characteristic profile of activities important in the metabolism of fatty acids and steroids.

Porcine (Adamski *et al.* 1992) and human (Adamski *et al.* 1995) 17 β -HSD IV principally catalyze the oxidation of the most active estrogen, estradiol to the less active estrogen, estrone. A physiological consequence of over-expression of 17 β -HSD IV by PPC would be a decrease in circulating estradiol levels in responsive rodent species. Decreases in serum estradiol levels have not been observed in male rats but rather, under some but not all conditions of PPC exposure, an increase in liver aromatase activity has been shown to lead to increased serum estradiol levels

(Biegel *et al.* 1995). On the other hand, treatment of female rats with the PPC, DEHP, results in a dramatic drop of serum estradiol levels (Davis *et al.* 1994a). This suppression of estradiol levels leads to secondarily increased serum follicle-stimulating hormone levels, an absence of luteinizing hormone surges necessary for ovulation and results in polycystic ovaries in rats (Davis *et al.* 1994a). In addition to increased expression of 17 β -HSD IV in liver and kidney, the decrease in serum estradiol levels could also be due to lowered secretion of estradiol from preovulatory granulosa cells (Davis *et al.* 1994b). Mouse granulosa cells have been shown to express the 17 β -HSD IV protein (Normand *et al.* 1995), although the effect of PPC exposure on increased levels of 17 β -HSD IV protein is not known in these cells.

In summary, we show 17 β -HSD IV is regulated by at least two different mechanisms: (1) a PPC-inducible mechanism requiring PPAR α in a limited number of tissues and (2) a PPAR α -independent mechanism in many tissues important for constitutive expression. The over-expression of 17 β -HSD IV by PPC in liver and kidney could lead to altered intracellular and extracellular estrogen levels and consequently, disrupt normal homeostasis of estrogen-dependent functions.

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