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Effect of a peroxisome proliferator on 3β-hydroxysteroid dehydrogenase

Jean S. Wong,[1] Xiaoqin Ye,[1] Christy R. Muhlenkamp, and Sarjeet S. Gill*

Environmental Toxicology Graduate Program, Department of Cell Biology and Neuroscience, University of California, 5429 Boyce Hall, Riverside, CA 92521, USA

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Abstract

To better understand the changes that occur following exposure to peroxisome proliferators, we utilized mRNA differential display and microarray to screen for peroxisome proliferator target genes apart from those involved in lipid metabolism in male C57B6 mice by using the ubiquitous plasticizer, di(2-ethylhexyl)phthalate (DEHP). One noted change was the dose-dependent suppression of the mouse hormone metabolizing 3β-hydroxysteroid dehydrogenase V (HSD3b5), which is specifically expressed in the male mouse liver. Northern analysis showed that HSD3b5 mRNA levels decreased dramatically upon one-day exposure to 2.0% dietary DEHP, and were nearly undetectable by one week of treatment. Food restriction also significantly suppressed HSD3b5 expression; however, in this case the suppression was delayed and to a lesser extent. Another mouse 3β-hydroxysteroid dehydrogenase, HSD3b4, predominantly expressed in kidneys, was also regulated by DEHP and food restriction. The sex-specific gene, HSD3b5, was affected more by DEHP and food restriction than the tissue-specific gene, HSD3b4.

Keywords: 3β-Hydroxysteroid dehydrogenase; HSD3b5; HSD3b4; 3-Ketosteroid reductase; Di(2-ethylhexyl)phthalate (DEHP); Food restriction; mRNA differential display; Northern analysis; RT-PCR; Liver

Peroxisome proliferators encompass a diverse range of chemicals including hypolipidemic drugs, herbicides, and plasticizers, and are characterized by their ability to cause peroxisome proliferation and to induce liver cancer [1]. These proliferators transcriptionally activate many genes involved in lipid metabolism, including fatty acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, and the microsomal CYP4A family. Regulation of these genes by peroxisome proliferators is mediated through peroxisome proliferator-activated receptors (PPARs) [2]. Peroxisome proliferators have also been shown to suppress genes apart from lipid homeostasis, such as GRP58 [3], Bip/GRP78 [4], *cyp2f2* [5], and several steroid-regulated genes, including transthyretin [6], α_{2u}-globulin [7], 17β-hydroxysteroid dehydrogenase IV [8], and 11β-hydroxysteroid dehydrogenase I [9]. In addition, we show here that 3β-hydroxysteroid dehydrogenase V (HSD3b5) is also down-regulated following exposure to a peroxisome proliferator. However, the mechanism of down-regulation of these genes by peroxisome proliferators remains unknown.

HSD3b5 belongs to the 3β-hydroxysteroid dehydrogenase (3β-HSD) family. Payne and coworkers [10–13] identified six murine 3β-HSD isoforms. HSD3b1, HSD3b2, HSD3b3, and HSD3b6 are NAD^+-dependent dehydrogenase/isomerase isoforms, while HSD3b4 and HSD3b5 are NADPH-dependent 3-ketosteroid reductase isoforms [14]. There is 93% amino acid sequence identity between HSD3b4 and HSD3b5; however, these two isoforms have less than 80% amino acid sequence identity to the other four 3β-HSD isoforms [11]. Unlike other members of the 3β-HSD isoforms, HSD3b4 and HSD3b5 do not biosynthesize active steroid hormones, but rather catalyze the inactivation of steroid hormones, such as dihydrotestosterone [10]. HSD3b4 is expressed in kidneys of male and female mice [13] and to a lesser extent in testes [10], while HSD3b5 is expressed only in livers of males, with onset of expression around postnatal day 30 [15].

* Corresponding author. Fax: +1-909-787-3087.
E-mail address: sarjeet.gill@ucr.edu (S.S. Gill).
[1] These authors contributed equally.

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Current literature exists for the effect of the peroxisome proliferators on the expression of several genes involved in steroid metabolism. However, while the effects of development and hormones on regulation of 3β-hydroxysteroid dehydrogenase isoforms are known, there are no data on the effects of environmental chemicals and stress on these enzymes. In this paper, we report the suppression of mouse HSD3b5 and HSD3b4 following the dietary treatment with our model peroxisome proliferator and plasticizer, di(2-ethylhexyl)phthalate (DEHP), and following the food restriction. Additionally, the National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, characterized DEHP as a chemical of "serious concern" for having potential detrimental effects on male infant reproductive development [16]. We extend this concern beyond infancy and to a later stage of development of male reproductivity by providing evidence for the disruption of hormone function in mice during puberty by DEHP.

Materials and methods

Animal treatment. In a dose-response studies, 6–7-week-old male C57B6 mice (Charles River Laboratories, Raleigh, North Carolina) were fed ad libitum with diets containing 0.2%, 0.6%, 1.2%, or 2.0% DEHP (di(2-ethylhexyl)phthalate, Aldrich Chemical, Milwaukee, WI) for 4 weeks. In time-course studies, mice were fed 2.0% dietary DEHP for 1 day, 3 day, 1 week, 2 weeks, 4 weeks, or 10 weeks while control mice were fed with untreated rodent diet. Since previous observations indicated that high dose DEHP-fed mice consume less food, an additional control group of food-restricted mice was also used. Here food restricted mice were given untreated diet at 60–70% of that normally eaten by control mice. Mice were killed by cervical dislocation; livers, kidneys, spleens, and testes were isolated and stored in liquid nitrogen.

Total RNA isolation. Total RNA was isolated using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD), subsequently treated with DNase I (Boehringer Mannheim, Indianapolis, IN), and then stored in FORMAzol (Molecular Research Center, Cincinnati, OH) at −80 °C until further use.

mRNA differential display. Pooled total liver RNA isolated from 1-week DEHP-treated mice was used in mRNA differential display (RNAmap kit, GenHunter, Nashville, TN). Each of the three random primers, 5′-GACCGCTTGT-3′, 5′-AGGTGACCGT-3′, and 5′-GGTA CTCCAC-3′, in combination with each of the four oligo(dT) primers, T_{12}MG, T_{12}MA, T_{12}MT, and T_{12}MC (M = G, A, C), were utilized to screen for genes affected by DEHP exposure. Bands consistently and differentially expressed were excised, eluted into water, and PCR amplified using the same primer pairs used in the mRNA differential display. The products were cloned into pCR2.1 vector (Invitrogen, San Diego, CA) and the determined sequences were analyzed by BLASTN.

Microarray screening. Liver total RNA from three mice from each group was used for microarray screening (GeneChip Core, UC San Diego and Microarray Facility, UC Irvine). Total RNA was reverse-transcribed to cDNA, biotinylated, and hybridized to the murine U74av2 GeneChips (Affymetrix, Santa Clara, CA). The data were analyzed using the Microarray Suite 4.0, MicroDB, and Data Mining Tool 2.0 softwares (Affymetrix). A t test was performed to determine the significance of expression change at $p < 0.05$.

Quantitative RT-PCR. First-strand cDNAs were prepared using 5 μg pooled total RNA (Superscript Preamplification System, GIBCO-BRL). To amplify HSD3b4 and HSD3b5, oligonucleotide sense primer 5′-AGGGCATGTCTGCTGTCATCC-3′ (corresponding to nucleotides 320–340 of HSD3b5 and 329–349 of HSD3b4) and antisense primer 5′-TGGGCAAG TGCTTAGGACTGA-3′ (corresponding to nucleotides 1397–1377 of HSD3B5 and 1406–1386 of HSD3b4) were used. Competimers and primers for 18S (Ambion) were used to co-amplify HSD3b4 or HSD3b5 and 18S. Amplification was performed using the following protocol: denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 2 min.

PCR cycle numbers were chosen so that amplification was in the exponential phase as predetermined by Southern blot analysis. PCR products were capillary transferred overnight to Zeta-probe GT Blotting Membranes (Bio-Rad, Hercules, CA). HSD3b5 cDNA and the 18S probes were labeled with ^{32}P-α-dATP (Amersham) using Prime-it II random primer labeling kit (Stratagene, La Jolla, CA). The membranes were hybridized with both ^{32}P-labeled HSD3b5 and 18S probes in hybridization buffer (0.5 M Na_2HPO_4, pH 7.2, 7% SDS) at 65 °C overnight. Washes were carried out as follows: twice at 65 °C for 60 min with low stringency washing buffer (40 mM Na_2HPO_4, pH 7.2, 5% SDS) and twice at 65 °C for 60 min with high stringency washing buffer (40 mM Na_2HPO_4, pH 7.2, 1% SDS). The bands were quantified using a Phosphorimager (BioRad).

Northern blot analysis. Fifteen micrograms of total RNA was separated by formaldehyde-denaturing gel electrophoresis and subsequently transferred to Zeta-probe GT Blotting Membranes for Northern analysis. Hybridization and washes were performed as described above for RT-PCR, except the blot was probed with HSD3b5 first and then re-probed with 18S as a control.

Statistical analysis. Comparisons between groups were performed using ONEWAY analysis followed by Fisher's pairwise comparisons (Minitab). T test was used to determine differences between means at the level of significance set at $p < 0.01$ or $p < 0.05$.

Results and discussion

One of the main target organs of peroxisome proliferators and specifically DEHP is the liver. Therefore mRNA differential display was employed to identify novel DEHP target genes in the livers of mice fed with 2.0% DEHP diets for 1 week. One of the genes identified to be regulated by DEHP was 3β-hydroxysteroid dehydrogenase V (HSD3b5) (GenBank accession no. L41519/MUSHSD3) (Fig. 1). Screening of the mouse U74av2 genechip using total RNA isolated from three mice from control, food restriction, and 1.0% DEHP treatment also showed HSD3b5 to be down-regulated (Table 1). Further studies with Northern analysis dem-

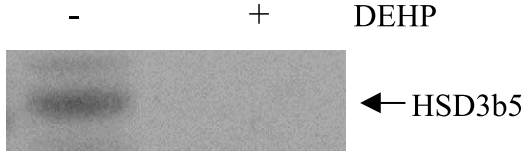

Fig. 1. Identification of HSD3b5 as a novel DEHP target gene by mRNA differential display. Pooled total RNA isolated from 1 week DEHP-treated mouse livers was converted to cDNA. mRNA differential display was performed using RNAmap kit from GenHunter (Nashville, TN). A band corresponding to the HSD3b5 gene as determined by sequencing consistently disappeared after 2.0% dietary DEHP treatment.

Table 1
Induce expression of 3-βHSD5 using DNA microarray

	Control	FR	DEHP	Description
Average signal	27836	2455	349	L41519 *Mus muluscus* 3-ketosteroid reductase (HSD3b5)

Average signal of $n = 3$ for each control/treatment group as determined by hybridization of labeled probe generated from each mouse hybridized to the mouse U74av2 genechip (Affymetrix). Significant difference was determined by t test with $p < 0.05$.

onstrated the down-regulation of HSD3b5 to be dose-dependent (Fig. 2).

Since our dose-response study demonstrated a complete abolishment of expression of HSD3b5 by 4 weeks of treatment with 2.0% DEHP, we wanted to determine the specific time point in which expression was first affected. The time-response study showed significant down-regulation of HSD3b5 by more than 50% within only one day of exposure (Fig. 3). By one week of exposure to DEHP, the levels were nearly undetectable, about 8.5% of control. No detectable levels of HSD3b5 were observed by Northern analysis in the 2, 4, or 10 week exposed mice (data not shown). Mice given 2.0% dietary DEHP consumed about 60–70% of food eaten by control mice. Since food restriction could also regulate stress related genes [17], the expression of HSD3b5 mRNA was also measured in livers of food-restricted mice to confirm that the suppression of HSD3b5 mRNA was due to DEHP rather than the result of decreased food consumption. Food restricted mice were given the same amount of untreated food as that consumed by 2.0% DEHP treated mice. Although food restriction resulted in some decrease in HSD3b5 mRNA, additional and significant decrease in HSD3b5 mRNA levels observed following 2.0% DEHP treatment was the result of exposure to DEHP, not due to a decrease in food intake. It has been demonstrated that typical PPARα-

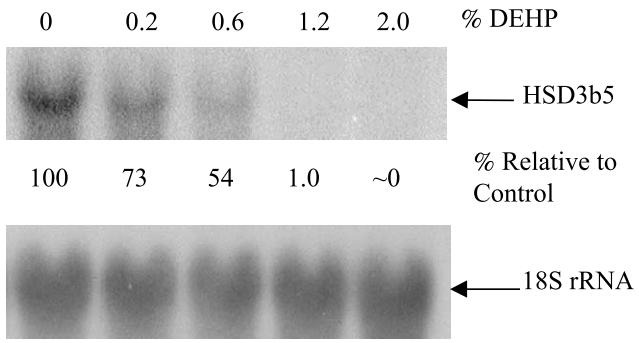

Fig. 2. Dose-dependent differential expression of HSD3b5 in liver following DEHP treatment and food restriction. Each lane of the Northern blot was loaded with 15 μg liver total RNA isolated from mice treated for 4 weeks with DEHP. Hybridization with HSD3b5 probe detected as a single band of ~1.6 kb.

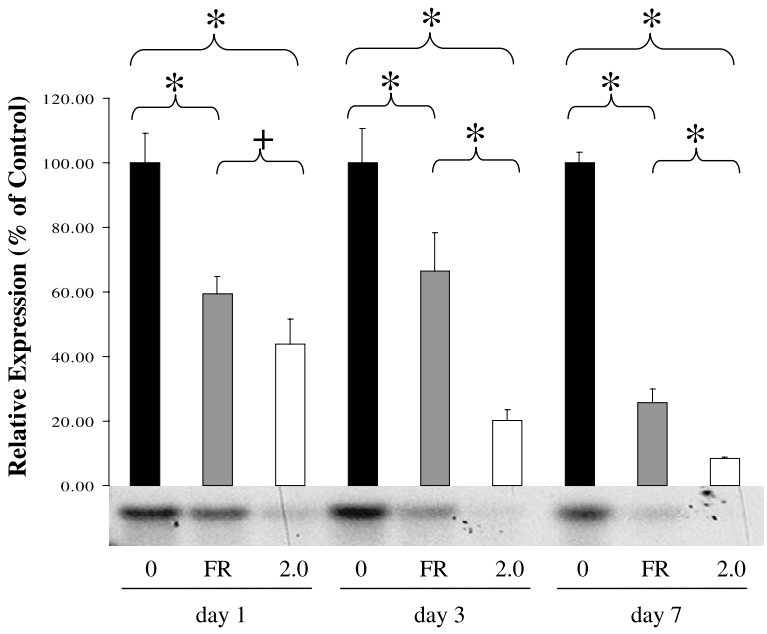

Fig. 3. Northern analysis of total liver RNA isolated from individual mice after 1, 3, and 7 days of treatment with 2.0% DEHP. One representative Northern blot of total RNA from one mouse is presented here. A sample population of $n = 3$ yielded mean ± SD with statistical significance from control, indicated by $*$ ($p < 0.01$) and $+$ ($p < 0.05$). Gene expression in control (0% DEHP) mice was set as 100%. The expression of HSD3b5 was normalized with that of 18S rRNA.

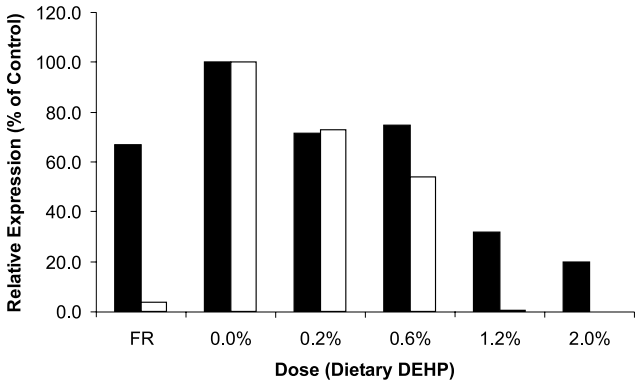

Fig. 4. Comparison of HSD3b5 and HSD3b4 mRNA levels following DEHP treatment and food restriction using quantitative RT-PCR. Quantification of HSD3b4 and HSD3b5 mRNA levels upon 4 weeks of food restriction and 2.0% dietary DEHP treatment in pooled total RNA from kidneys and livers, respectively. The expression of HSD3b4 and HSD3b5 was normalized with 18S rRNA. Gene expression in control mice (0% DEHP) was set as 100%. Open histograms represent HSD3b5 and filled histograms represent HSD3b4.

responsive genes, including CYP4A enzymes, acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase, were also regulated by fasting [18]. A requirement for PPARα in mediating regulation of these genes by fasting and peroxisome proliferators was confirmed by abolished responses in PPARα-deficient mice [18,19], suggesting that PPARα could be the convergent point between fasting and peroxisome proliferation. Since PPARα cross-talks with other nuclear hormone receptors [20], it could be the sensor of diet and hormonal signaling [21].

HSD3b4 is expressed mainly in kidneys and at very low levels in testes but does not co-express with HSD3b5 in the liver [10,13]. HSD3b4 has the highest sequence homology (93%) with HSD3b5 in the murine HSD family [13]. Since only HSD3b4 and HSD3b5 in the 3β-HSD family function as 3-ketosteroid reductases, we also analyzed if HSD3b4 was down-regulated by DEHP and food restriction. Furthermore it was of interest to determine if the 3β-hydroxysteroid dehydrogenase expression was affected by DEHP in the other two target organs, kidneys and testes. Using RT-PCR, we demonstrated that the suppression profile of kidney-specific HSD3b4 was different from that of the liver-specific HSD3b5 (Fig. 3). Compared to more than a 150-fold reduction of HSD3b5 mRNA levels by 4 weeks of 2.0% DEHP treatment as indicated by RT-PCR, HSD3b4 mRNA levels were suppressed by only 5-fold. In addition, food-restriction had a lesser impact on the suppression of HSD3b4 as compared to HSD3b5 (Fig. 4). Using RT-PCR, we were only able to amplify a very faint band of about 1 kb from the testes (data not shown).

HSD3b5, like the mouse major urinary proteins (MUPs), is a sex-specific gene. The expression of both genes is dramatically inhibited by DEHP [22]. Two tissue-specific genes, *cyp2f2* [5] and HSD3b4, are also regulated by DEHP. However, their down-regulation by DEHP is less than that of the sex-specific HSD3b5 and MUP. The different pattern of suppression could indicate that DEHP affects sex-specific genes, such as HSD3b5 and MUP, more than tissue-specific genes, such as HSD3b4 and *cyp2f2*. It is also known that serum testosterone levels are reduced by DEHP treatment [23] and by food restriction [24]. Collectively, these data imply that there is a possible connection between hormone levels and gene regulation by DEHP. There exists a growing body of evidence for phthalates as endocrine disruptors, [25–27] and here we demonstrated yet another gene involved in steroid metabolism, specifically sex hormone inactivation, to be a target of phthalate plasticizer. One possible mechanism of peroxisome proliferators, such as DEHP, to repress transcription of steroid metabolizing genes is the activation of an antagonist to bind PPARα this complex in turn facilitates co-repressor binding while simultaneously disrupting interactions with co-activators [28]. Understanding the mechanism of regulation of peroxisome proliferators and phthalate plasticizers would allow for better risk assessment of its production and use.

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