Simvastatin and Dipentyl Phthalate Lower Ex Vivo Testicular Testosterone Production and Exhibit Additive Effects on Testicular Testosterone and Gene Expression Via Distinct Mechanistic Pathways in the Fetal Rat

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ABSTRACT

Sex differentiation of the male reproductive tract in mammals is driven, in part, by fetal androgen production. *In utero*, some phthalate esters (PEs) alter fetal Leydig cell differentiation, reducing the expression of several genes associated with steroid synthesis/transport, and consequently, lowering fetal androgen and InsI3 hormone levels. Simvastatin (SMV) is a cholesterol-lowering drug that directly inhibits HMG-CoA reductase. SMV may also disrupt steroid biosynthesis, but through a different mode of action (MOA) than the PEs. As cholesterol is a precursor of steroid hormone biosynthesis, we hypothesized that *in utero* exposure to SMV during the critical period of sex differentiation would lower fetal testicular testosterone (T) production without affecting genes involved in cholesterol and androgen synthesis and transport. Secondly, we hypothesized that a mixture of SMV and a PE, which may have different MOAs, would reduce testosterone levels in an additive manner. Pregnant Sprague Dawley rats were dosed orally with SMV, dipentyl phthalate (DPeP), or SMV plus DPeP from gestational days 14–18, and fetuses were evaluated on GD18. On GD18, SMV lowered fetal T production and serum triglycerides, low density lipoprotein, high density lipoprotein, and total cholesterol levels, and downregulated two genes in the fetal testis that were different from those altered by PEs. When SMV and DPeP were administered as a mixture,

fetal T production was significantly reduced in an additive manner, thus demonstrating that a mixture of chemicals can induce additive effects on fetal T production even though they display different MOAs.

Key words: simvastatin; phthalates; steroidogenesis; cholesterol; sexual differentiation

During gonadal and early sex differentiation of the mammalian reproductive system, the fetus contains an undifferentiated gonadal ridge as well as Müllerian and Wolffian structures that serve as precursors for the female and male reproductive tracts, respectively. In the presence of the Y chromosome, or more specifically, the sex determining region of the Y chromosome (SRY), the undifferentiated gonadal ridge develops into testes. Once formed, Sertoli cells in the testes secrete anti-Müllerian hormone (also known as Müllerian inhibitory substance), which promotes the regression of the Müllerian (female) reproductive tract (Jost et al., 1973) and the Leydig cells secrete Insl3 and testosterone. Anti-Müllerian hormone, Insl3, and testosterone are all essential for the morphological differentiation of the male reproductive system (Viger et al., 2005). Conversion of testosterone to the more potent androgen, dihydrotestosterone, in the target tissues is also essential in the development of several male reproductive tissues (Sharpe, 2006).

Androgen signaling in the early fetus is one of the critical pathways in the proper development of the male reproductive tract, and disruption of androgen signaling during the critical period of sex differentiation may produce congenital disorders in humans (Ferlin et al., 2008). Environmental compounds that mimic or antagonize the effects of hormones, including androgen signaling, are endocrine disrupting chemicals (EDCs) that can induce permanent, adverse effects with *in utero* exposure. Among such EDCs are some phthalate esters (PEs). PEs are compounds that have a wide range of uses, mainly as plasticizers for polyvinyl chloride products, but are also found in personal care products, medical devices, and toys (Blount et al., 2000). Gestational exposure to some PEs and their metabolites during the masculinization programming window leads to reproductive tract malformations in androgen- (and Insl3-) dependent fetal tissues that are apparent later in life during adulthood (Barlow and Foster, 2003; Gray et al., 2000; Mylchreest et al., 1999; Parks et al., 2000; Scott et al., 2008; Welsh et al., 2008; Wilson et al., 2004). Although the proximate molecular target for phthalate action in the developing male fetus has not been identified, the mode of action (MOA) involves alterations in fetal Leydig cell differentiation, causing a reduction in the expression of genes associated with steroid synthesis/transport, and consequently, lowered fetal androgen levels during the masculinization programming window. These alterations can be used as quantitative predictors of postnatal phenotypes (Barlow and Foster, 2003; Foster et al., 1983; Howdeshell et al., 2008; Lehmann et al., 2004; McKinnell et al., 2005; Thompson et al., 2004).

In addition to PEs, our laboratory has been interested in other classes of chemicals that disrupt the androgen signaling pathway via diverse modes of toxicity and if they interact in a dose or response additive manner when exposures are combined. The purpose of the present study was to investigate the dose response effects of gestational exposure to simvastatin, a member of the statin family of cholesterol-lowering drugs, on fetal rat testosterone production and other maternal and fetal parameters as well as to begin to describe how a binary mixture of simvastatin and dipentyl phthalate (DPeP) behaves. Simvastatin disrupts the steroid pathway in a manner distinct from the PEs by directly inhibiting 3-hydroxy-3-methylglutaryl coen-

zyme A (HMG-CoA) reductase, the rate determining step of the cholesterol biosynthetic pathway. Our initial objective was to determine if simvastatin disrupted fetal testosterone production following exposure on gestational days (GD) 14–18 during the masculinization programming window. Our hypothesis, as cholesterol is a precursor for steroid hormone biosynthesis, is that simvastatin would reduce fetal testosterone production by reducing the amount of intracellular free cholesterol available for steroidogenesis. Following this, we exposed the dams on GD 14–18 to a mixture of simvastatin and DPeP to determine if the effects were additive.

MATERIALS AND METHODS

Animals. Time-mated Sprague Dawley (SD) rats, approximately 90 days old, were purchased from Harlan Laboratories (Indianapolis, IN). Sperm-positive females were shipped to the U.S. Environmental Protection Agency (EPA) on gestational day 8 (GD8) (the day sperm were identified in the vagina was defined as GD 0). Upon arrival at the EPA, pregnant dams were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding and provided with NIH07 rat chow and filtered (5 µm) tap water *ad libitum*. Water provided to the US EPA is tested every 4 months for a subset of heavy metals, pesticides, and other chemical contaminants and tested monthly for *Pseudomonas*. The study was divided into two parts, a dose response and a binary mixture experiment. This study was conducted in accordance with the National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee. Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care maintained at 20–22°C, 45–55% humidity, and a 12:12 h photo period (light/dark cycle, lights off at 7:00 P.M.).

Doses and administration of chemicals. The first and second experiments were each conducted in two blocks with 15 dams per block. In each block, the 15 dams were ranked by weight and assigned to five groups with 3–4 rats per group in a manner that produced groups with similar weights and variances. Sample sizes were based on power calculations performed previously in similarly conducted studies which demonstrated that two blocks with 3–4 litters per dosage group per block were sufficient for detecting reductions in testosterone production of about 40% or greater and reductions in gene expression (as measured herein).

In the first experiment, time-mated rats were dosed daily by oral gavage with the vehicle (water) control, 15.6, 31.25, 62.5, or 125 mg simvastatin/kg body weight on GD 14–18, a dosing period which covers the critical period of sex differentiation in the developing male rat fetus (Carruthers and Foster, 2005; Wolf et al., 2000). Simvastatin (CAS 79902-63-9; purity: 98% (HPLC); lot no. 11B7D41K) was purchased from AK Scientific (Union City, CA). The initial dosage range for simvastatin was selected based on original US Food & Drug Administration data (<http://www.accessdata.fda.gov/Scripts/cder/DrugsatFDA/index.cfm?fuseaction=Search.Label.ApprovalHistory#apphist>)

and other studies conducted in independent labs to encompass doses that have been reported to cause minimal (if any) adverse effects and those that can cause adverse effects without causing maternal toxicity (Jabbari et al., 2011; Smith et al., 1991; Westwood et al., 2005).

In the second experiment, time-mated dams were dosed with corn oil (vehicle), 62.5 mg/kg/day simvastatin, 50 mg/kg/day dipentyl phthalate, or 62.5 mg/kg/day simvastatin + 50 mg/kg/day DPeP. These dosage levels were selected based on data from the first experiment (simvastatin dose response) and a previous DPeP study (Hannas et al., 2011). These doses were expected to partially but not entirely reduce testosterone production individually, enabling us to determine if the mixture of the two act in a cumulative or antagonistic manner. DPeP (CAS 131-18-0, lot no. 1431420) and corn oil (CAS 8001-30B7) were purchased from Sigma-Aldrich Corp (St Louis, MO). Corn oil was used as the vehicle in these blocks because DPeP is water insoluble.

Necropsies. Dams were euthanized by decapitation on GD18, and maternal trunk blood was collected into a serum separator vacutainer tube and stored at 4°C during the necropsy. At the end of the necropsy, tubes were centrifuged at 3000 rpm for 10 min. Serum was dispensed in 2 ml vials and stored at -80°C until clinical chemical analyses were performed. Maternal adrenals, livers, and ovaries were excised, trimmed, and weighed. One adrenal, a 2–3 mm³ piece of liver (mid lobe), and one ovary per dam were each transferred to individual microcentrifuge tubes with 80 µl TRI reagent (Sigma, St Louis, MO) for immediate homogenization with an electric handheld homogenizer for later mRNA analyses. Another section of the liver was fixed in Bouin's solution for 72 h, transferred to 70% ethanol, and submitted for histopathological evaluation. All necropsies began 1 h following administration of the final maternal dose and were conducted within a 3 h time frame between 8:00 and 11:00 A.M. EST to avoid any potential confounding effects on fetal growth or time of day on the fetal endpoints.

Trunk blood was collected in heparinized capillary tubes from each fetus. Once the fetuses were sexed, heparinized blood was transferred into a 0.65 ml siliconized microcentrifuge tube. Blood from males and females was pooled separately for each litter. One testis from the first three males in each litter was used for *ex vivo* testosterone production. Fetal adrenals and the remaining testes were each pooled by litter and immediately homogenized in TRI reagent on ice using a Kontes pestle homogenizer for mRNA analyses. Fetal livers were placed in individual tubes of TRI reagent and finely snipped with sterile scissors followed by homogenization with a handheld homogenizer. All tissue homogenates were stored at -80°C until RNA was extracted.

Serum/plasma lipid and progesterone analysis. Clinical chemical analysis of serum (dams) or plasma (fetuses) concentrations of total cholesterol, triglycerides, high density lipoproteins (HDLs), low density lipoproteins (LDLs), alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen, creatinine, total protein, total bile acids (TBA), total bilirubin (TBil), direct bilirubin, and albumin were performed using an Olympus AU400e chemistry analyzer (Beckman Coulter, Inc., Irving, TX) using reagents obtained from the manufacturer (cholesterol and triglycerides); reagents for the HDL, LDL, and TBA analysis were obtained from Diazyme Laboratories (Poway, CA). Progesterone concentrations in the maternal serum were measured by radioimmunoassay (RIA) according to the manufacturer's instructions (Coat-A-Count Kits; Siemens

Corp., Los Angeles, CA). The limit of detection was 0.02 ng/ml for progesterone. Radioactivity was measured using a Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Waltham, MA).

Ex vivo fetal testicular T production. One testis from the first three males was placed into a single well on a 24-well plate containing 500 µl M-199 media with reduced methionine (Hazelton Biologicals, Inc., St Lenexa, KS) and incubated for 3 h at 37°C in humidified ambient air. After incubation, the media were collected and stored at -80°C until used for testosterone RIA as outlined in Hannas et al. (2011).

Maternal and fetal organ gene expression analysis. Preparation of RNA for gene expression analysis is outlined in Wilson et al. (2004). Briefly, RNA was extracted from the maternal or fetal tissue homogenate and cleaned to eliminate any potential genomic DNA contamination. RNA concentration and purity were determined with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE), followed by an additional genomic DNA elimination and then complementary DNA (cDNA) synthesis. For each individual sample, 300 ng of cDNA was added to a single reaction, to be used across a 96-well array plate. The template cDNA was then added to RT2 SYBR Green qPCR Master Mix (SABioscience, Fredrick, MD), and 25 µl was added to each well of the plate. Two gene array plates were used to analyze the samples. A 96-well gene array plate (SABioscience) was custom designed to contain 89 individual target genes, three housekeeping genes (beta-actin, beta-glucuronidase, and lactate dehydrogenase), an interassay control, a genomic DNA control, a reverse transcription control, and a positive PCR control. To verify interassay consistency in C_T cycling, aliquots of fetal testicular cDNA from males of a corn oil or water (control)-treated dam were added to a well containing primers from the housekeeping gene beta-2 microglobulin on all plates. For both gene array plates, data were analyzed using the comparative C_T method with raw data normalized to the housekeeping genes. PCR reaction was run on an iCycler iQ Real-Time Detection System (BioRad, Hercules, CA) using the cycling parameters described previously (Hannas et al., 2012). The purity of the product was verified by melting curve analysis. Lipoprotein transport and cholesterol metabolism were examined using Rat Lipoprotein Signaling and Cholesterol Metabolism RT² PCR array (SABioscience, Catalog no. PARN-080A) according to manufacturer's instructions.

Data analysis and statistics. Data analysis for maternal body/organ weights and progesterone was performed using one- (first experiment) or two-way (second experiment) analysis of variance (ANOVA) through the general linear model procedure in the Statistical Analysis System software (SAS, SAS Institute, Cary, NC). Organ weight data were analyzed with and without body weight as a covariate in the regression model to determine if this altered the interpretation of the results. Post hoc t-tests were performed with the LSMEANS procedure between dose groups when the overall dose effect was significant as determined by ANOVA. Individual maternal serum values and pooled (by gender) litter mean plasma values were also analyzed by ANOVA for lipid concentrations and liver biomarkers.

Fetal testosterone production and gene expression data were analyzed using litter mean values. Data were log₁₀-transformed (correcting for heterogeneity of variance) and analyzed by ANOVA. Testosterone values from the RIA are in ng/ml. These values were converted to ng/testis/3 h by correcting for the incubation volume (500 µl) and dilution of the sample prior

(if any) to running the RIA. Percent of control (PC) testosterone production values were generated for each testis as follows: $PC = 100 \times (\text{the individual testis T Prod value}) / (\text{mean T prod value of the concurrent control group})$. ED50 and slope parameters for the dose response data were determined using a four parameter logistic regression analysis with GraphPad 5.1 software (GraphPad Software, Inc., La Jolla, CA).

Gene expression data were converted to fold (F) versus the control values by the equation $F = 2^{-\Delta\Delta C_T}$. Gene expression for fetal organs was analyzed using litter mean values and individually for maternal organs. Data were analyzed using SAS 9.1 Software available at the USEPA, and fold changes for each gene were considered statistically significant if the F and t-test values for the overall ANOVA model and post hoc comparison were $p < 0.01$ ($0.05 > p > 0.01$ values were considered “equivocal” changes). Messenger RNA samples with mean C_T values > 33 were considered too low to be reliably detected.

RESULTS

Experiment 1. Simvastatin Dose Response

Litter effects. Simvastatin induced 100% fetal mortality in the high dose (125 mg/kg/day) group, whereas fetal viability, litter sizes, and fetal liver weights were not affected at lower simvastatin dose levels (see Supplementary table 1). Simvastatin at 62.5 mg/kg/day significantly downregulated the expression of *Cyp46a1* in the fetal testes and *Akr1d1* in the fetal livers compared with control, but there were no other significant changes in the expression of genes involved in the cholesterol biosynthesis or sex differentiation pathways in fetal adrenals, livers, and testes out of the 183 genes assessed (Figs. 1A and 1B). Simvastatin treatment at 15.6, 31.25, and 62.5 mg/kg/day induced significant decreases in fetal HDLs, triglycerides, and total cholesterol in the circulating plasma; whereas significant reductions in LDLs only occurred in the 62.5 mg/kg dose group (Fig. 2). The sexes did not differ significantly from one another.

Ex vivo testicular testosterone production. Dose-response studies were performed to assess the relationship between *in utero* exposure to simvastatin and fetal *ex vivo* testicular testosterone production. Simvastatin reduced testosterone production in a dose-related manner, with the reduction being statistically significant in the 62.5 mg/kg dose group ($p < 0.01$ vs. control) (Fig. 3). The ED50 and slope values were 133.0 mg/kg/day and -1.509 , respectively.

Maternal effects. Overt toxicity was seen in dams in the high dose (125 mg/kg/day) group, as indicated by reduced body weights and lethargy (Table 1). As soon as this was detected, dosing was terminated so some dams in this group were dosed for only 4 days. Although simvastatin caused a decrease in total maternal weight gain at 62.5 mg/kg/day, the lower treatment groups did not show any evidence of overt toxicity. Liver weight displayed a slight nonmonotonic dose response, being significantly increased by simvastatin treatment at 31.25 and 62.5 mg/kg/day but reduced at 125 mg/kg/day (Table 1) when analyzed with maternal body weight as a covariate.

Liver histology was unaffected at 31.25 and 62.5 mg/kg/day in spite of increases in liver weight, whereas histopathological lesions characterized by increased numbers of portal biliary duct profiles and/or irregular rows or islands of biliary epithelial cells that often entrapped islands of hepatocytes were seen in the livers of dams exposed to 125 mg/kg/day (see Supplementary fig. 1). Furthermore, serum concentrations of ALT, AST, total bile

acids, and direct and TBil were significantly elevated, but only in the highest dose group (Supplementary table 2).

In addition to the minimal effect on the liver of the dam, simvastatin doses below 125 mg/kg/day did not alter maternal ovary or adrenal weights (Table 1), circulating lipids (Fig. 4), or progesterone levels (Fig. 5) as compared with controls. Of the maternal endpoints collected, maternal liver weight appeared to be the most sensitive indicator of simvastatin-induced maternal effects. *Tm7sf2* (upregulated), *Apoa4* (downregulated), and *Cyp39a1* (downregulated) were altered in the maternal liver as a result of the 62.5 mg/kg/day simvastatin exposure at the $p < 0.01$ level. In addition, the expression of several genes was equivocal but may be downregulated in a dose-dependent manner, although these genes expression changes at 62.5 mg/kg/day did not affect liver function (see Supplementary table 2). The expression of several genes involved in cholesterol metabolism, transport, and synthesis were altered in the maternal liver in response to 125 mg/kg/day simvastatin (Fig. 6). For example, the downregulation of genes such as *Abca1* and *Apoa2* and the upregulation of genes including *Mvk* and *Cyp51* promote elevated cholesterol levels in the dam and are consistent with the elevated cholesterol concentrations measured in maternal serum at the high dose.

Simvastatin/Dipentyl Phthalate (DPeP) Binary Mixture

Litter effects and maternal body weight. The impact of 62.5 mg/kg/day simvastatin alone on maternal weight gain was similar to that observed in the dose-response study (Table 2), whereas a 5-day exposure to 50 mg/kg/day DPeP did not affect maternal weight gain. The simvastatin/DPeP mixture caused a decrease in maternal body weight gain that was similar in magnitude to the decrease that was achieved by simvastatin exposure alone. There were no significant differences among fetal survival in any of the treatment groups compared with control.

Ex vivo testicular testosterone production. Simvastatin or DPeP alone decreased testosterone production to a similar extent (44% of control and 37% of control, respectively, $p < 0.0001$). The mixture of simvastatin and DPeP reduced testosterone production to 19% of control values, which was a 50% greater reduction than simvastatin or DPeP alone ($p < 0.0001$ vs. control; $p < 0.0001$ vs. 62.5 mg simvastatin/kg/day; $p < 0.0001$ vs. 50 mg DPeP/kg/day) (Fig. 7, top).

Gene expression analysis in the fetal testes. The effect of simvastatin, DPeP, and the mixture of simvastatin and DPeP on gene expression in the fetal testis following 5-day *in utero* exposure was assessed using two different PCR arrays containing 89 and 84 target genes that are involved in cholesterol biosynthesis or sex differentiation, respectively. DPeP significantly downregulated several genes involved in cholesterol transport and synthesis, sex differentiation, and steroidogenesis as reported previously (Hannas et al., 2012). These genes were *Cyp11a1*, *Nr0b1*, *Star*, *Cyp11b2*, *Hsd3b*, *Cyp17a1*, *Scarb1*, *Insl3*, *Dhcr7*, *Cyp11b1*, and *Inha* (Fig. 7, bottom). The simvastatin/DPeP mixture induced reductions in the expression levels of 13 genes in the fetal testes, and these were identical to the two genes downregulated by simvastatin and the 11 genes downregulated by DPeP. In the mixture group, there were no synergistic or antagonistic interactions between DPeP and simvastatin on any of the 13 affected genes (Fig. 8).

(A)

(B)

				Fetal Testes		
Group	Gene	Refseq #	Cont C _T	SIM 15.6	SIM 32.25	SIM 62.5
1	Wt1	NM_031534	23.8			
1	Nr5a1	XM_001054966	23.9			
1	Lhx9	NM_181367	26.4			
1	Gata4	NM_144730	25.2			
1	Emx2	XM_574698	27.1			
1	Lhx1	NM_145880	30.5			
2	Cbx2	XM_221185	27.3			
2	Dmrt1	NM_053706	26.2			
2	Dmrt2	XM_219927	31.7			
2	Sry	NM_012772	31.3			
2	Sox9	XM_343981	25.6			
2	Zfpn2	XM_235253	26.7			
2	Nr0b1	NM_053317	26.6			
2	Sox8	XM_220283	28.6			
2/4	Amhr2	NM_030998	27.1			
2	Rhox5	NM_022175	27.8			
2	Ptgds2	NM_031644	29.2			
5	Star	NM_031558	22.8			
5	Cyp11a1	NM_017286	21.9			
5	Cyp11b2	NM_012538	31.9			
5	Hsd3b	NM_001042619	24.3			
5	Cyp17a1	NM_012753	21.6			
5	Hsd17b3	NM_054007	25.2			
5	Lhcgr	NM_012978	25.9			
5	Scarb1	NM_031541	22.3			
5	Mapk3	NM_017347	25.4			
4	Insl3	NM_053680	21.7			
5	Dhcr7	NM_022389	24.8			
5	Tspo	NM_012515	26.5			
5	Cyp11b1	NM_012537	28.2			
3	Dhh	XM_343327	27.7			
3	Pdgfa	NM_012801	28.3			
3	Fgf9	NM_012952	28.1			
3	Fgf8	NM_133286	29.4			
3	Ptch1	XM_345570	26.6			
3	Pdgfra	XM_214030	23.3			
3	Fog1	XR_007127	33.5			
3	Smo	NM_012807	26.5			
3	Tgfb1	NM_021578	25.6			
4	Rhox10	NM_001037581	27.2			
2	Hoxa2	NM_012581	32.4			
2	Wnt7a	XM_342723	30.9			
10	Ntf3	NM_031073	29.5			
10	Ntrk3	NM_019248	31.9			
11	Inhbb	XM_344130	23.8			
11	Inha	NM_012590	22.0			
11	Acvr2b	NM_031554	27.7			
11	Inhba	NM_017128	25.5			

				Fetal Testes		
Group	Gene	Refseq #	Cont C _T	SIM 15.6	SIM 32.25	SIM 62.5
8	Sfrp1	XM_224987	23.8			
8	Sfrp2	XM_227314	28.7			
8	Sfrp4	NM_053544	30.4			
8	Sfrp5	XM_219887	32.5			
6	Pcaf	NM_001024252	27.1			
7	Axin1	NM_024405	26.5			
7	Axin2	NM_024355	26.8			
7	Dixdc1	NM_001037654	28.0			
7	Dvl1	NM_031820	27.2			
7	Dvl2	XM_239254	27.4			
7	Dvl3	XM_221304	27.1			
7	Tle1	XM_342851	25.5			
7	Tle2	NM_001039013	26.7			
7	Dkk3	NM_138519	29.6			
7	Dkk1	XM_219804	32.3			
7	IGD156304	XR_008686	31.9			
6	Sra1	NM_183329	25.2			
6	Ar	NM_012502	27.8			
12	Esr1	NM_012689	27.9			
12	Esr2	NM_012754	30.4			
12	Vdr	NM_017058	31.5			
12	Nr4a2	NM_019328	29.1			
12	Nr1d1	NM_145775	27.9			
12	Nr3c2	NM_013131	29.8			
12	Nr3c1	NM_012576	25.9			
9	Pparg	NM_013124	35.2			
9	Ppard	NM_013141	27.5			
9	Ppara	NM_013196	28.0			
9	Rxra	NM_012805	25.6			
9	Rxrb	NM_206849	26.7			
9	Rxrg	NM_031765	31.9			
9	Rara	NM_031528	26.3			
9	Rarb	XM_223843	29.3			
9	Rarg	XM_217064	27.2			
9	Acox1	NM_017340	26.1			
9	Cyp4a1	NM_175837	36.0			
9	Fabp1	NM_012556	34.1			
9	Apoa1	NM_012738	31.8			
14	Adh1	NM_019286	31.3			
14	Aldh1a1	NM_022407	22.7			
13	Pou5f1	NM_001009178	27.7			
15	Actb	NM_031144	19.0			
16	B2m	NM_012512	23.2			
15	Gusb	NM_017015	25.7			
15	Ldha	NM_017025	23.2			
16	RGDC	U26919	38.3			
16	RTC	SA_00104	23.5			
16	PPC	SA_00103	19.3			

Legend		
1 Sex determination	9	PPAR/RXR signaling
2 Sex differentiation	10	neurotrophins
3 Testis cell differentiation/signaling	11	inhibins/activins
4 Gubernaculum dev't/insl3-related	12	Other nuclear receptors
5 Steroidogenesis	13	cell differentiation marker
6 AR/coregulator proteins	14	metabolism
7 Frizzled signaling pathway	15	Housekeeping genes
8 Wnt signaling pathway	16	RT-PCR controls

Down-regulated (p<0.01)
Down-regulated (p<0.05)
Up-regulated (p<0.05)
Up-regulated (p<0.01)
No change
Undetected (Cont C _T >33)

FIG. 1. (A) Gene expression changes in the cholesterol pathway in fetal testes, adrenals, and livers on GD 18 following 5-day *in utero* exposure to simvastatin. Control threshold (C_T) refers to the mean (±SEM) of control litter values for comparison of relative expression. Control C_T values ≥ 33 were considered nondetectable and represented by black boxes. Red and yellow boxes indicate downregulation with $p < 0.01$ and $p < 0.05$, respectively, and green and blue boxes represent upregulation with $p < 0.01$ and $p < 0.05$, respectively. (B) Gene expression changes in fetal testes on GD 18 following 5-day *in utero* exposure to simvastatin. Group number corresponds to listed groups below heat map. Control threshold (C_T) refers to the mean (±SEM) of control litter values for comparison of relative expression. Control C_T values ≥ 33 were considered nondetectable and represented by black boxes. Red and yellow boxes indicate downregulation with $p < 0.01$ and $p < 0.05$, respectively, and green and blue boxes represent upregulation with $p < 0.01$ and $p < 0.05$, respectively.

FIG. 2. LDL (A), HDL (B), triglycerides (C), and total cholesterol (D) concentrations in plasma from pooled (by litter) female (black bars) and male (white bars) fetuses after 5-day in utero exposure to vehicle, 15.6, 31.25, 62.5, or 125 mg/kg-maternal body weight/day simvastatin on GD 14–18. Data represent means $\pm$ SEM. For dam: $n = 5$ for control and 15.25 groups; $n = 7$ for 31.25 and 62.5 groups. * $p < 0.05 > 0.01$, ** $p < 0.001$ versus controls (male and female); asterisk(s) between bars: effect level for both sexes; asterisk(s) over black bar: female effect level; asterisk(s) over white bar: male effect level.

TABLE 1. Maternal Weight Gain, Organ Weight, and Fetal Survival Following Oral Administration of Increasing Doses of Simvastatin (SMV) from GD 14–18

	Control	SMV 15.6 mg/kg	SMV 31.25 mg/kg	SMV 62.5 mg/kg	SMV 125 mg/kg
Numbers of dams	5	5	7	7	3 [‡]
Final weight ^a	340.5 $\pm$ 10.38	315.58 $\pm$ 6.47	341.49 $\pm$ 8.79	325.19 $\pm$ 7.49	240 $\pm$ 8.54**
Change in weight (g) ^b	45.2 $\pm$ 5.34	40.1 $\pm$ 4.34	43.6 $\pm$ 3.37	28.6 $\pm$ 5.86[†]	-41.0 $\pm$ 7.22**
Ovary weight (mg) ^a	68.08 $\pm$ 8.61	71.28 $\pm$ 8.91	67.08 $\pm$ 5.54	66.86 $\pm$ 4.06	57.27 $\pm$ 1.70
Adrenal weight (mg) ^a	32.5 $\pm$ 1.66	31.86 $\pm$ 1.02	32.77 $\pm$ 0.47	34.61 $\pm$ 1.23	42.87 $\pm$ 2.15***
Liver weight (g) ^a	13.65 $\pm$ 0.44	12.72 $\pm$ 0.41	14.83 $\pm$ 0.38[†]	14.83 $\pm$ 0.44*	10.52 $\pm$ 0.29*
No. of live fetuses	10.4 $\pm$ 2.01	10.8 $\pm$ 1.5	12.71 $\pm$ 0.92	10.86 $\pm$ 1.65	0 $\pm$ 0**
Fetal survival (%) ^c	98.6 $\pm$ 1.4	96.7 $\pm$ 3.3	99.0 $\pm$ 1.0	88.4 $\pm$ 9.3	0 $\pm$ 0**

Note. Data are means $\pm$ SEM.

^aFinal weight, Ovary weight, Adrenal weight, Liver weight = maternal body weight or organ weight at GD 18.

^bChange in weight = maternal body weight GD 18 – maternal body weight GD 14.

^cFetal survival = (number of live fetuses/total fetuses) $\times$ 100.

[†] $p < 0.05$ versus control, * $p < 0.01$ versus control, ** $p < 0.001$ versus control, *** $p < 0.0001$ versus control. Shaded and bold values differ significantly from control.

[‡]Overt maternal toxicity in SMV 125 group; experiment was not repeated at this dose resulting in only three animals in this group.

TABLE 2. Maternal Weight Gain and Fetal Survival Following Gestational Exposure from Days 14–18 to Simvastatin (SMV) and/or Dipentyl Phthalate (DPeP)

	Control	SMV 62.5 mg/kg/d	DPeP 50 mg/kg/d	SMV 62.5 plus DPeP 50 mg/kg/d
Numbers of litters	8	6	8	8
Final weight ^a	334.7 ± 8.23	301.4 ± 8.99***	329.3 ± 7.68	290.5 ± 4.84***
Change in weight (g) ^b	51.7 ± 4.33	13.0 ± 5.4***	52.2 ± 3.45	13.1 ± 2.74***
No. of live fetuses	12.6 ± 1.64	9.4 ± 2.18	12.6 ± 0.96	12.1 ± 0.85
Fetal survival (%) ^c	100.0 ± 0.00	86.5 ± 11.7	100.0 ± 0.00	99.0 ± 0.96

Note. Data are means ± SEM.

^aFinal weight = maternal body weight or organ weight at GD 18.

^bChange in weight = maternal body weight GD 18 – maternal body weight GD 14.

^cFetal survival = number of live fetuses/total fetuses.

****p* < 0.0001 versus control.

n = number of pregnant dams.

Shaded and bold values differ significantly from control.

FIG. 3. Fetal testicular ex vivo testosterone production following 3-h incubation from SD rats exposed *in utero* to simvastatin during sex differentiation on GD 14–18. The data represent testosterone production as PC per nanogram testis, and each data point represents the mean ± SEM of the litter means. **p* < 0.01 versus control.

DISCUSSION

In the current study, we evaluated the dose-related effects of a 5-day simvastatin exposure on fetal testosterone production and gene expression. Dose-dependent reductions in fetal testosterone production were observed in response to simvastatin, which attained statistical significance at the 62.5 mg/kg/day dose (*p* < 0.011). Simvastatin also significantly decreased fetal HDLs, triglycerides, and total cholesterol at every dose level and LDLs at 62.5 mg/kg/day without causing overt fetal or maternal toxicity or affecting maternal lipids at the same dose. In addition, the second objective of this study was to examine the cumulative effects of a simple binary mixture of simvastatin and DPeP. Examination of the mRNA alterations in fetal testes genes clearly shows that simvastatin and DPeP are disrupting androgen production through different modes of action

but when combined still produce an additive reduction in testosterone production.

Cholesterol is essential for normal differentiation and growth of many organs during fetal development, including the reproductive system. Fetal cholesterol can be imported from the maternal circulation in the form of LDL or HDL, it can be obtained from the cell membrane, or it can be synthesized *de novo* by the fetus from acetate (Scott et al., 2009). Once cholesterol is available, the biosynthesis of testosterone from cholesterol can occur in the fetal Leydig cells within the testes (Payne and Youngblood, 1995). Simvastatin is highly lipophilic and can cross the placenta, and it is likely that simvastatin exposure during the critical period of sex differentiation resulted in inhibition of fetal *de novo* cholesterol synthesis and possibly cholesterol import to the testes in the fetus. Although statins reduce cholesterol primarily through inhibition of HMG-CoA reductase, they also reduce circulating LDL cholesterol by increasing the expression of LDL receptors in hepatocytes (Kazmin et al., 2007). Gene expression data from the fetal testes showed that simvastatin consistently altered *Srx17* (encodes an LDL receptor) and *Acaa2* (encodes an acetyl-Coenzyme A acyltransferase), suggesting that the mechanism of action does not involve altering the expression of enzymes that facilitate the reactions in the cholesterol biosynthetic pathway at the transcriptional level. Although we did not observe any significant changes in gene expression in fetal livers, the decrease in circulating fetal lipids suggests that this alteration does not occur at the level of the mRNA, but rather simvastatin inhibits enzyme activity rather than mRNA and enzyme protein levels.

All steroid hormones are derived from cholesterol; therefore, we hypothesized that inhibition of cholesterol synthesis by simvastatin could lower steroid hormone levels. If steroidogenesis is impaired during the masculinization programming window, it could result in reproductive tract malformations in the male. While statins are contraindicated in pregnancy in the United States (category X—not for use in pregnancy), there is still a risk for inadvertent exposure. Women who are on statin drugs that become unexpectedly pregnant may continue to take statins for weeks before pregnancy is detected. Because the early program-

FIG. 4. Maternal serum lipid panel. LDL (A), HDL (B), triglycerides (C), and total cholesterol (D) serum concentrations in pregnant SD rats receiving 0 (control), 15.6, 31.25, 62.5, or 125 mg/kg-body weight/day simvastatin by gavage on GD 14–18. Data represent means $\pm$ SEM ($n = 5$ for control and 15.6 groups; $n = 7$ for 31.25 and 62.5 groups, and $n = 3$ in 125 group). ** $p < 0.001$, *** $p < 0.0001$ versus controls. SMV = simvastatin.

FIG. 5. Maternal serum progesterone concentrations from SD rats given vehicle, 15.6, 31.25, 62.5, or 125 mg/kg-body weight/day simvastatin by gavage from GD 14–18. Each bar represents the mean $\pm$ SEM ($n = 5$ for control and 15.6 groups; $n = 7$ for 31.25 and 62.5 groups, and $n = 3$ in 125 group). ** $p < 0.001$ versus control.

ming window in humans is likely to be 8–14 weeks of gestation (Welsh et al., 2008), fetal exposure to statins during this window is possible. Further, statin drugs and other pharmaceuticals are found in some source waters (Benotti et al., 2009), and if the population of statin users continues to increase, statin levels in the water supply may also increase. Taken together, because the potential for exposure to statins during pregnancy exists, the re-

productive and developmental effects of inadvertent exposure to these drugs during pregnancy should be evaluated.

The available data for inadvertent human exposure to statins during early pregnancy are limited (Hosokawa et al., 2003). Although conclusive evidence that statins cause congenital abnormalities to a greater extent than what is found in the general population has not been reported (Alberts et al., 1980; Manson et al., 1996), it is thought that only a small proportion of adverse outcomes due to statin exposure is reported, and these reports are likely to be biased toward more severe outcomes (Edison and Muenke, 2004; Staffa et al., 2002). In addition, there are case reports documenting central nervous system and limb anomalies after first trimester statin exposure (Edison and Muenke, 2004). Citing this case report, the Australian Drug Evaluation Committee changed the pregnancy classification of the statin drugs from category C (assigned to drugs that have caused or may be suspected of causing harmful effects on the human fetus or neonate without causing malformations and may be reversible) to category D (assigned to drugs that have caused, are suspected to cause, or may be expected to cause, an increase incidence of human fetal malformations or irreversible damage).

A previous study with rats demonstrated that developmental toxicity that resulted from atorvastatin exposure only occurred at maternally toxic doses, 300 mg/kg/day from GD 6–15 (Dostal et al., 1994). Fetuses were examined for external, visceral, and skeletal malformations and variations, and there were no malformations or variations observed at doses ≤ 100 mg/kg/day. However, because the duration of dosing for that study did not

Down-regulated (p<0.01)
Down-regulated (p<0.05)
Up-regulated (p<0.05)
Up-regulated (p<0.01)
No change
Undetected (Cont C ₇ >33)

FIG. 6. Gene expression changes in the cholesterol pathway in maternal livers on GD 18 following 5-day oral administration of simvastatin. Control threshold (C_T) refers to the mean ($\pm$ SEM) of control values for comparison of relative expression. Control C_T values ≥ 33 were considered nondetectable and represented by black boxes. Red and yellow boxes indicate downregulation with $p < 0.01$ and $p < 0.05$, respectively, and green and blue boxes represent upregulation with $p < 0.01$ and $p < 0.05$, respectively.

FIG. 7. (Top) Fetal testicular ex vivo testosterone production following 3-h incubation from SD rats exposed in utero to simvastatin and/or dipentyl phthalate during sex differentiation on GD 14–18. The data represent testosterone production as PC per nanogram testis, and each data point represents the mean $\pm$ SEM of three pooled litter values. *** $p < 0.0001$ versus control, SMV/DPeP; # $p < 0.0001$ versus control, SMV 62.5, DPeP 50. (Bottom) Genes downregulated in the fetal testes by simvastatin (SMV), dipentyl phthalate (DPeP), or both (SMV+DPeP). Equal sign indicates no significant enhancement of SMV or DPeP gene expression in the mixture group. * $p < 0.01$: altered by SMV or DPeP versus control.

cover the entire critical period of sex differentiation and the fetuses were examined on GD 20, reproductive alterations, if present, would be difficult if not impossible to detect.

A one generation reproduction study examined the effects of *in utero* exposure to atorvastatin from gestation day 7 through lactation day 20 on reproduction and development and found no significant treatment-related effects on F1 fertility (Henck *et al.*, 1998), however, this study assessed a limited number of reproductive endpoints in the prenatally exposed offspring. If simvastatin induced a low incidence of reproductive malformations in androgen-dependent tissues, it is possible that such alterations would be undetected unless the reproductive system of the offspring was examined more thoroughly (Blystone *et al.*, 2010).

Although we did not find any publications on the effects of statins on fetal rat Leydig cell androgen synthesis *in vitro*, such studies have been conducted with Leydig cells from adult mice and rats. *In vitro* studies using murine Leydig cells found no reductions in testosterone production after a 12-h incubation with lovastatin (Hou *et al.*, 1990), and they concluded that the murine Leydig cell stores enough lipids to sustain androgen production

FIG. 8. MOA for simvastatin and DPeP action on fetal testicular testosterone production. (A) Simvastatin acts to inhibit enzyme action (specifically HMG-CoA) in the cholesterol biosynthetic pathway resulting in the lowering of cholesterol. As a precursor for steroidogenesis, the amount of cholesterol available for steroidogenesis is reduced resulting in a decrease in testosterone production. (B) DPeP alters fetal Leydig cell differentiation, resulting in the downregulation of genes involved in steroid synthesis and transport, which also leads to decreased testosterone production. (C) Co-administration of simvastatin and DPeP, though acting through distinct pathways, lower testosterone production to a greater extent than each compound individually.

over this incubation period. In contrast to mice, rat and human Leydig cells have relatively low lipid stores, and when adult rat Leydig cells were cultured for 18 h with statins, including simvastatin, testosterone production in cells treated with luteinizing hormone was reduced by about 50% of control (Klinefelter *et al.*, 2014). Although the latter study does support our argument that cholesterol inhibition by statin drugs directly inhibits androgen synthesis in the fetal Leydig cell, conclusions about treatment-induced effects on steroidogenesis seen with adult Leydig cells cannot always be extrapolated to fetal Leydig cells. While fetal and adult Leydig cells are similar in many ways, they also are distinct cell populations that can display a number of significant functional and morphological differences.

In the current study, we observed that simvastatin significantly reduced fetal ex vivo testosterone production, similar in magnitude to the reductions seen by Klinefelter *et al.* (2014) *in vitro* with adult Leydig cells. We are in the process of determining the biological relevance of these statistically significant re-

ductions in fetal testosterone production in a study to evaluate the postnatal reproductive consequences. We are currently conducting fetal and postnatal simvastatin dose-response studies in Charles River Sprague Dawley rats to determine the biological relevance of *in utero* exposure to simvastatin on reproductive development in F1 rats through adulthood and to determine if a 30–50% reduction in fetal *ex vivo* testosterone production induces functional or morphological reproductive alterations in male rat offspring exposed to simvastatin *in utero*.

In addition to effects on testosterone production, we also examined simvastatin-induced changes in gene expression in several fetal tissues. Genes that are known to be affected by PE exposure during the critical period of sex differentiation resulting in reductions in testicular testosterone production were not similarly affected by simvastatin. This supports our prediction that simvastatin and PEs reduce testosterone production in the developing fetus by different modes of action.

The effects of simvastatin on the pregnant dam differed drastically from those on the fetus. At dose levels below 125 mg/kg/day, simvastatin reduced total weight gain in the dam and slightly elevated maternal liver weights, but had little effect on any other maternal endpoints investigated in this study. For example, although simvastatin significantly reduced circulating lipids in the fetus at all dosage levels, there were no corresponding effects on maternal lipids. Serum lipids generally increase steadily throughout normal pregnancy, with triglycerides increasing 300–400% and total cholesterol increasing 25–90% compared with levels found in the non-pregnant state (Manson *et al.*, 1996). Although simvastatin lowers LDLs and total cholesterol in rodents (Elahi *et al.*, 2008) and humans (Blumenthal, 2000), a 5-day exposure to simvastatin during pregnancy, during which rats normally display relatively high serum levels of cholesterol, is not sufficient to cause any significant changes in maternal lipid levels.

There was unexpected maternal toxicity observed in the highest dose group (125 mg/kg/day), which included elevated cholesterol and progesterone levels, lesions and gene expression changes in the liver, and 100% fetal loss. Maternal livers examined via histopathology displayed hepatocyte degeneration, hepatocyte necrosis, hepatocyte atypia, and biliary epithelial hyperplasia. Similar histological changes in the rat have been reported in male rats after administration of high doses of simvastatin (180 mg/kg/day) and lovastatin (1600 mg/kg/day) (MacDonald *et al.*, 1988; MacDonald and Halleck, 2004); however, these changes were observed after 13 and 14 weeks of dosing, respectively. Some morphologic changes also occurred during “short-term” high dose administration of simvastatin (100 mg/kg/day) (MacDonald and Halleck, 2004), but even this observation occurred after a 14-day dosing period. Therefore, it was surprising to observe such significant lesions in our high dose group after only a 5-day exposure. It is possible that some of the physiological changes of pregnancy (e.g., cardiovascular and renal adaptations) may alter the toxicokinetics of this drug and exacerbate these effects.

In addition to the lesions, the high dose group had elevated total cholesterol in the serum. Previous studies using ML-263B (compactin), another HMG-CoA reductase inhibitor, showed that high dose administration of this drug elevated hepatic levels of HMG-CoA reductase activity in rats (Endo *et al.*, 1979). Later immunohistochemical studies demonstrated that short-term, high dose administration of simvastatin or lovastatin increased the percentage of HMG-CoA reductase-positive hepatocytes, suggesting significant induction of this enzyme (MacDonald and Halleck, 2004; Singer *et al.*, 1984). The induction of HMG-CoA re-

ductase in response to high dose administration of HMG-CoA reductase inhibitors may contribute to the elevated serum cholesterol observed in the high dose simvastatin group.

The dams in the high dose group (125 mg/kg/day) also had elevated circulating progesterone. Our hypothesis for this study was that inhibition of HMG-CoA reductase by simvastatin would lower cholesterol which would reduce the amount of substrate available for steroid hormone biosynthesis. However, in the high dose group, both cholesterol and progesterone were significantly elevated. One explanation for the increase in progesterone is an inference derived from the hypothesis: if reducing the amount of cholesterol available for steroidogenesis caused a decrease in the steroid hormone production, then elevated cholesterol may increase the amount of steroid hormone production. Another explanation, and one we favor, is that progesterone levels increased because the failing liver was unable to metabolize the hormone, resulting in accumulation in the circulation. The gross observations of pale color (compared with lower treatment and control groups), significantly smaller weight, evidence of hepatocyte necrosis, downregulation of several genes involved in steroid metabolism, as well as elevated liver biomarkers in the maternal serum confirm that the livers had been damaged and that liver function was also impaired, thereby supporting the latter explanation.

The results of the simvastatin dose-response study support that inhibition of the cholesterol pathway in the fetal rat affects testicular steroidogenesis, resulting in reductions in testosterone production. Because the mode by which this occurs differs from that of PEs, which involves alterations in fetal Leydig cell differentiation and reduction of several genes associated with steroid synthesis/transport, we next wanted to determine how co-administration of two compounds with different modes of action would affect testosterone production. We conducted a simple binary mixture study with simvastatin and DPeP to determine the effects of a 5-day *in utero* exposure on fetal testosterone production and gene expression. As expected, simvastatin or DPeP alone reduced fetal testosterone production to a similar extent. However, co-administration of the compounds resulted in an additive effect, reducing fetal testosterone production to levels below that achieved by either compound alone.

In summary, these studies demonstrate that *in utero* exposure to simvastatin lowers fetal testosterone production and alters the fetal lipid profile of both males and females at dosage levels that had no effect on the maternal lipid profile. In addition, we have shown that a mixture of chemicals with different modes of action can induce additive effects on a common adverse outcome in the fetal rat during sex differentiation, however future studies will need to be conducted in order to delineate whether the additive nature of this mixture followed response and/or dose addition theory.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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