Hormonal and Chemical Regulation of Paraoxonases in Mice

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ABSTRACT

In humans and rodents, paraoxonase (PON/Pon) 1 expression and activity in livers and serum are higher in females than in males, and some drugs increase paraoxonase's expression. However, the underlining mechanisms of gender-divergent expression and chemical regulation of Pon1 remain largely unknown. The present study determined the regulatory mechanisms contributing to gender-divergent and chemically altered Pon expression in mouse livers. Pon1 mRNA was much more abundant in the livers of mice than other tissues, with higher levels in female livers than male livers at mRNA and protein levels. Pon2 mRNA was ubiquitously expressed in mouse tissues, but minimally in mouse liver. Pon3 mRNA was most abundant in mouse lung and liver and less abundant in other tissues. Pon1 mRNA was lowest in fetal liver, markedly increased at parturition, and remained relatively constant thereafter. Pon2 and Pon3 mRNA are highly expressed in fetal liver and decreased after birth. Male-pattern growth hormone (GH) administration in hypophysectomized and *lit/lit* mice decreased Pon1 expression. Sex hormones and female-pattern GH administration had no effect on Pon1 expression, indicating the importance of male-pattern GH in regulating Pon1. Aryl hydrocarbon receptor, pregnane X receptor, and NF-E2-related factor activators had no effect on Pon1 mRNA. A constitutive androstane receptor (CAR) activator decreased Pon1 expression in wild-type but not CAR-null mice. In conclusion, Pon1 mRNA was most abundant in adult mouse livers, whereas Pon2 and Pon3 mRNAs were most abundant in fetal mouse livers. Female-predominant Pon1 expression in mouse livers is caused by the inhibitory effects of male-pattern GH secretion, and CAR activation decreases Pon1 expression.

Introduction

Oxidation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) is a critical risk factor in the development of atherosclerosis and other cardiovascular diseases. Paraoxonases (PONs/Pons) are a family of proteins that protect LDL and HDL from oxidation, thus preventing the development of atherosclerosis and other cardiovascular diseases (Aviram and Rosenblat, 2005).

Human PON1 was first reported to hydrolyze organophosphates, such as paraoxon (Mazur, 1946). PON1 was originally classified as an A-esterase because of its paraoxonase and arylesterase activities (Sorenson et al., 1995). In addition, PON1 has lactonase activity and can metabolize a number of drugs and prodrugs such as dihydrocoumarin and homocysteine thiolactone (Draganov and La Du, 2004). In contrast, PON2 and PON3 lack paraoxonase or arylesterase activities, but have lactonase activity capable of hydrolyzing aromatic and long-chain aliphatic lactones, such as dihydrocoumarin and 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid lactone (Draganov et al., 2000; Ng et al., 2001).

PON1 and PON3 are synthesized primarily in liver and secreted into plasma, where they are associated with HDL (Mackness and Walker, 1983, 1988; Draganov et al., 2000). PON2 is not present in blood, but is expressed widely in a number of tissues, including liver, lung, brain, and heart (Ng et al., 2001).

In addition to its ability to hydrolyze paraoxon, PON1 attenuates the production and accumulation of lipoperoxides

ABBREVIATIONS: LDL, low-density lipoprotein; HDL, high-density lipoprotein; AhR, aryl hydrocarbon receptor; bDNA, branched DNA; BHA, butylated hydroxyanisole; BL, blocker; BNF, β-naphthoflavone; CAR, constitutive androstane receptor; CE, capture extender; CLFB, clofibric acid; CPFB, ciprofibrate; DAS, diallyl sulfide; DEHP, diethylhexylphthalate; DEX, dexamethasone; DHT, 5α-dihydroxytestosterone; E2, 17β-estradiol; ETHOXYQ, ethoxyquin; GH, growth hormone; GHRH, GH-releasing hormone; FPGH, female-pattern GH; MPGH, male-pattern GH; GNX, gonadectomized; HX, hypophysectomized; LE, label extender; Nrf2, NF-E2 related factor; OPZ, oltipraz; PB, phenobarbital; PCB126, polychlorinated biphenyl 126; PCN, pregnenolone 16α-carbonitrile; PON/Pon, paraoxonase; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; RLU, relative light units; SPR, spironolactone; STAT5b, signal transducer and activator of transcription 5b; TBS-T, Tris-buffered saline containing 0.1% Tween 20; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP, 1,4-*bis*[2-(3,5-dichloropyridyloxy)] benzene.

in LDL (Mackness et al., 1991, 1993) and protects phospholipids from oxidation in HDL (Costa et al., 2003). Similar to PON1, both PON2 and PON3 have antioxidant function (Draganov and La Du, 2004; Costa et al., 2005).

Serum Pon1 activity in mice and rats is 14 to 26% higher in females than males (Wehner et al., 1987; Costa et al., 2005; Thomàs-Moyà et al., 2006, 2007). Human serum PON1 activity is also higher in women than men (Mueller et al., 1983). Pon1 mRNA in livers of female mice is 40% higher than in males (bin Ali et al., 2003). Gonadectomy results in an increase in Pon1 mRNA expression in male, but not female mice (bin Ali et al., 2003), suggesting that endogenous androgens, but not endogenous estrogens, regulate Pon1 expression.

Dietary polyphenols (Gouédard et al., 2004) and aspirin (Jaichander et al., 2008) increase PON1 gene expression through the activation of the aryl hydrocarbon receptor (AhR). In addition, alcohol, wine consumption, resveratrol, pitavastatin, and probucol increase Pon1/PON1 expression and activity in rodents and humans (Rao et al., 2003; Ota et al., 2005; Hong et al., 2006; Rajdl et al., 2007; Curtin et al., 2008). In contrast, dietary taurocholate decreased Pon1 expression in mice through the activation of the farnesoid X receptor-Fgf15-Fgfr4 pathway (Gutierrez et al., 2006). Proinflammatory cytokines, such as interleukin-1β and tumor necrosis factor α, decreased Pon1 expression in HepG2 cells (Kumon et al., 2002).

In addition to sex hormones, growth hormone (GH) plays important roles in gender-divergent gene expression in livers (Waxman and O'Connor, 2006). Except for the AhR signaling pathway, the characterization of chemical regulation of Pon1 remains largely unknown. Therefore, the current study was performed to determine the tissue distribution, ontogeny, and hormonal and chemical regulation of Pon1, Pon2, and Pon3 in mice.

Materials and Methods

Materials

Micro-O-protect was purchased from Roche Diagnostics (Indianapolis, IN). Formaldehyde, 4-morpholinepropanesulfonic acid, sodium citrate, and $NaHCO_3$ were purchased from Fischer Chemicals (Fairlawn, NJ). Chloroform, agarose, and ethidium bromide were purchased from AMRESCO (Solon, OH). Rat growth hormone was obtained from Dr. Albert F. Parlow at the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Disease (Torrance, CA). Pellets for subcutaneous release of the hormones used in this study, including 5α-dihydroxytestosterone (DHT), 17β-estradiol (E2), GH, and placebo, were purchased from Innovative Research of America, Inc. (Sarasota, FL). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was a gift from Dr. Karl Rozman (University of Kansas Medical Center, Kansas City, KS). Oltipraz (OPZ) was a gift from Dr. Stephen Safe (Texas A&M University, College Station, TX). Polychlorinated biphenyl 126 (PCB126) was obtained from AccuStandard (New Haven, CT). All other chemicals, unless otherwise indicated, were purchased from Sigma (St. Louis, MO).

Anti-human PON1-3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), β-actin antibody (Abcam Inc., Cambridge, MA), and goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody (Sigma) were commercially available. According to the manufacturer, anti-human PON1-3 antibody recognizes mouse Pon1, Pon2, and to a lesser extent PON3 protein. Because Pon1, but not Pon2, is generally highly expressed in mouse liver, this antibody will detect mainly Pon1 in mouse liver.

Animals and Treatment

Eight-week-old adult male and female C57BL/6 mice (n = 5/gender) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed according to the Association for Assessment and Accreditation of Laboratory Animal Care International.

Tissue Distribution Study. Twelve tissues [liver, kidney, lung, stomach, duodenum, jejunum, ileum, colon, heart, brain, and gonad (testes in males and ovaries in females)] were collected from adult (approximately 8 weeks of age) male and female C57BL/6 mice (n = 5/gender). Placenta was removed from pregnant mice on gestation day 17. The intestine was longitudinally dissected, rinsed in saline, and divided into three equal-length sections (referred to as duodenum, jejunum, and ileum) before being snap-frozen in liquid nitrogen.

Ontogenic Study. C57BL/6 mice were bred in the animal facilities at the University of Kansas Medical Center. Livers from male and female C57BL/6 mice were collected at −2, 0, 5, 10, 15, 22, 30, 40, and 45 days of age (n = 5/gender/age), snap-frozen in liquid nitrogen, and stored at −80°C.

Treatment of Mice with Microsomal Enzyme Inducers. Adult (approximately 8 weeks of age) male C57BL/6 mice (n = 5/treatment) were separated into groups. Groups of five mice were administered one of the following chemicals once daily for 4 days: AhR ligands, TCDD (40 μg/kg i.p. in corn oil), β-naphthoflavone (BNF; 200 mg/kg i.p. in corn oil), and PCB126 (300 μg/kg p.o. in corn oil); constitutive androstane receptor (CAR) activators, phenobarbital (PB; 100 mg/kg i.p. in saline), 1,4-*bis*[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP; 3 mg/kg i.p. in corn oil), and diallyl sulfide (DAS; 200 mg/kg i.p. in corn oil); pregnane X receptor (PXR) ligands, pregnenolone-16α-carbonitrile (PCN; 200 mg/kg i.p. in corn oil), spironolactone (SPR; 200 mg/kg i.p. in corn oil), and dexamethasone (DEX; 75 mg/kg i.p. in corn oil); peroxisome proliferator-activated receptor α (PPARα) ligands, clofibric acid (CLFB; 500 mg/kg i.p. in saline), ciprofibrate (CPFB; 40 mg/kg i.p. in saline), and diethylhexylphthalate (DEHP; 1000 mg/kg p.o. in corn oil); NF-E2-related factor (Nrf2) activators, butylated hydroxyanisole (BHA; 350 mg/kg i.p. in corn oil), ethoxyquin (ETHOXYQ; 250 mg/kg p.o. in corn oil), and OPZ (150 mg/kg p.o. in corn oil). Four different vehicle control groups (corn oil intraperitoneally, corn oil orally, saline intraperitoneally, and saline orally) were used. No statistical difference between these control groups was observed, thus these groups were averaged together as a single vehicle control group. All injections were administered in a volume of 10 ml/kg. Livers were removed on day 5, snap-frozen in liquid nitrogen, and stored at −80°C.

Hormone Replacement Treatment in Gonadectomized, Hypophysectomized, and *lit/lit* Mice. Both gonadectomized (GNX) mice and hypophysectomized (HX) mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). *lit/lit* mice (GH-releasing hormone receptor mutant heterozygous mice, C57BL/6J-Ghrhr^*lit*) were purchased from The Jackson Laboratory. The doses and routes of treatment of sex hormones and growth hormone in these mouse models have been reported previously (Cheng et al., 2006). After treatment, livers were removed for total RNA isolation.

TCPOBOP Treatment of WT and CAR-Null Mice. Breeding pairs of CAR-null mice in the C57BL/6 background were obtained from Dr. Ivan Rusyn (University of North Carolina, Chapel Hill, NC) and engineered by Tularik Inc. (South San Francisco, CA) as described previously (Ueda et al., 2002). CAR-null and C57BL/6 mice (n = 5) were administered TCPOBOP (3 mg/kg i.p. in corn oil) or the vehicle corn oil once daily for 4 days. Livers were removed on day 5, snap-frozen in liquid nitrogen, and stored at −80°C.

Total RNA Isolation

Total RNA was isolated by using RNA Bee reagent (Tel-Test Inc., Friendswood, TX) according to the manufacturer's protocol. RNA

pellets were resuspended in diethyl pyrocarbonate-treated deionized water. Total RNA concentrations were quantified spectrophotometrically at 260 nm.

Development of Specific Oligonucleotide Probe Sets for Branched DNA Analysis

Gene sequences of Pon1, Pon2, and Pon3 were accessed from GenBank. The strategy of multiple oligonucleotide probe sets design has been described previously (Hartley and Klaassen, 2000). Probe sets for each mouse paraoxonase [including capture extenders (CEs), label extenders (LEs), and blockers (BLs)] are shown in Table 1. Probe sets were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA).

bDNA Assay

Reagents required for RNA analysis (i.e., lysis buffer, amplifier/label probe dilution buffer, and substrate solution) were supplied in the Quantigene bDNA signal amplification kit (Panomics Inc., Fremont, CA). Each paraoxonase mRNA was analyzed according to the method of Hartley and Klaassen (2000). Data are presented as relative light units (RLUs) per 8 µg of total RNA.

Liver Protein Preparation

Liver (50–100 mg) was minced in 1 ml of ice-cold homogenizing buffer (0.25 M sucrose and 10 mM Tris-HCl at pH 7.5, containing 25 µg/ml leupeptin, 50 µg/ml aprotinin, 40 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml pepstatin, and 50 µg/ml antipain). The minced tissue was poured into a Dounce homogenizer (Kontes Glass, Vineland, NJ) and homogenized on ice for 10 strokes. The homogenate was centrifuged at 10,000g for 15 min at 4°C. The resulting supernatants were collected for Western blots. Protein concentration of each sample was determined with a Bradford protein assay kit (Sigma).

Western Blots

Liver protein samples mixed with sample loading buffer (75 µg protein/lane) were loaded after heating onto a 12.5% SDS-polyacrylamide gel. After electrophoresis, proteins in the gel were electrotransferred to a nitrocellulose membrane for 4.5 h at 30 V at room temperature. Membranes were blocked for 5 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Blots were then incubated overnight with anti-human PON1-3 (H-300) antibody at 4°C. β-Actin antibody was used as a loading control. After thorough washing (three 20-min washes with excess TBS-T), blots were incubated with goat anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:5000 dilution with 5% nonfat milk in TBS-T) for 1 h. Blots were washed again. Immunoreactive bands were detected with an enhanced chemical luminescence kit (Thermo Fisher Scientific, Waltham, MA). Pon1 and β-actin protein bands were visualized by exposure to Fuji (Tokyo, Japan) Medical X-Ray film. The intensity of the protein band on the films was quantified with Gel-Pro 3.1 image analysis software (Media Cybernetics, Inc., Bethesda, MD).

Statistical Analysis

Data were expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance, followed by Duncan's post hoc test using Statistica software (StatSoft, Tulsa, OK). Data of gender difference between male and female mouse tissue were analyzed by Student's t test. Statistical significance was considered at $p < 0.05$.

Results

Tissue Distribution of Mouse Paraoxonases. mRNA expression of mouse Pon1, Pon2, and Pon3 was quantified in 13 major tissues (Fig. 1). Expression of Pon1 mRNA (Fig. 1) was highest in mouse liver, detectable in mouse lung, and
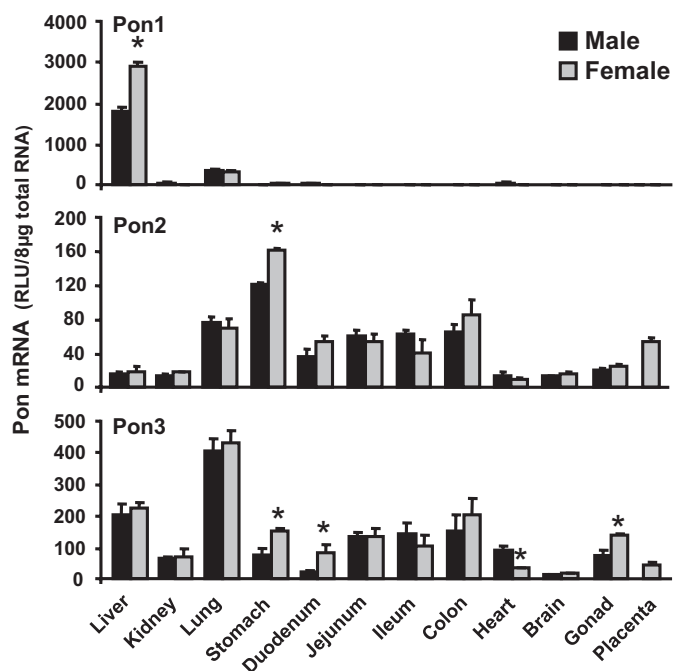
TABLE 1

Oligonucleotide probes generated for analysis of mouse paraoxonase mRNA expression by bDNA signal amplification assay

Function refers to the type of bDNA oligonucleotide probe represented by each sequence. GenBank accession numbers for each transcript are given with the gene name.

Function	Probe Sequence	Function	Probe Sequence
Pon1 (NM_011134)		**Pon3 (NM_173006)**	
CE	gagggtgagtgctagcagccttcTTTTTctcttggaaagaaagt	CE	tggtctagtgagactgtgattccgTTTTTctcttggaaagaaagt
CE	cggaaagcatttaatcttgtttgatTTTTTctcttggaaagaaagt	CE	ttcccagctgaatgacctttcaTTTTTctcttggaaagaaagt
CE	tgagggttaaatgaagatatatccaaTTTTTctcttggaaagaaagt	CE	cctggaggatcctcagggttataTTTTTctcttggaaagaaagt
LE	gcagcaacactggtcagtcctgTTTTTaggcataggaccgtgtct	CE	ccaaatataaagacgttttagaaagatctTTTTTctcttggaaagaaagt
LE	gccatggcctgagacagatgTTTTTaggcataggaccgtgtct	LE	tgttacatcagctacatagacaaacttcTTTTTaggcataggaccgtgtct
LE	ctcggattccttTaactaaattacagttaTTTTTaggcataggaccgtgtct	LE	ctggagttaaatcccaattatcatgTTTTTaggcataggaccgtgtct
LE	cttactgggatcgaaactttTtTattTTTTTaggcataggaccgtgtct	LE	caacggtcaagttatccactaaggTTTTTaggcataggaccgtgtct
LE	tgtatttcctataaattctaactctgacacTTTTTaggcataggaccgtgtct	LE	aaatatctcccgtggctggatTTTTTaggcataggaccgtgtct
LE	ggacgaggagtctggatggtttTTTTTaggcataggaccgtgtct	LE	tcctggatgcgtagtacttctgacTTTTTaggcataggaccgtgtct
BL	gacaggctgaggatggacaagt	LE	agaggtcacagtacagagctttgtgTTTTTaggcataggaccgtgtct
BL	agtgccaacaccagtcccac	LE	ttagcatacaaattacaattttactttttgTTTTTaggcataggaccgtgtct
BL	aggaagaccgatggttcttgtaa	LE	tgataattgtaaataattacagttccccTTTTTaggcataggaccgtgtct
BL	ggaagttctactggcgttacttca	BL	ttggcagaactgaatccttgg
BL	atctctaagtcttcagcacccgt	BL	ttttttcattatgtgaatgttcttagc
BL	aaagaaagttagtccattaggcaga	BL	gggtggcagcctgcca
BL	ccaggatactttagcccagtgct	BL	gatcaacagcttcatggggtta
BL	gtccatcagaagtatttttccagg	BL	ccctgggcttatctgacaaagag
BL	tgctggctccttcttcttgttcaa	BL	ttgttcgcatacagtgtgctca
BL	ttcatctgtgaatgtactaatccca	BL	gccctgaagcacagagccg
BL	accaccagtaggtacacagtgttatc	BL	atacacagaagccacggtgct
Pon2 (NM_183308)		BL	aaatatagtacctatgagcattctcttatg
CE	gcctggagaccgaggcgTTTTTctcttggaaagaaagt	CE	tggtctagtgagactgtgattccgTTTTTctcttggaaagaaagt
CE	ccctctccagaggatttcccTTTTTctcttggaaagaaagt	CE	ttcccagctgaatgacctttcaTTTTTctcttggaaagaaagt
CE	aagagccccttacatagagcttTTTTTctcttggaaagaaagt	CE	cctggaggatcctcagggttataTTTTTctcttggaaagaaagt
LE	aagcccctctgtgcaaactgTTTTTaggcataggaccgtgtct	CE	ccaaatataaagacgttttagaaagatctTTTTTctcttggaaagaaagt
LE	cagacctgtttaccacactcccTTTTTaggcataggaccgtgtct	LE	tgttacatcagctacatagacaaacttcTTTTTaggcataggaccgtgtct
LE	acactcctaacttgtaaatcagtaaacaTTTTTaggcataggaccgtgtct	LE	ctggagttaaatcccaattatcatgTTTTTaggcataggaccgtgtct
LE	ccctaggagtcacttctagcctcaTTTTTaggcataggaccgtgtct	LE	caacggtcaagttatccactaaggTTTTTaggcataggaccgtgtct
LE	tgattcctgtccaaccgaatgTTTTTaggcataggaccgtgtct	LE	aaatatctcccgtggctggatTTTTTaggcataggaccgtgtct
LE	cggttgtgttttatttttattttttccaataTTTTTaggcataggaccgtgtct	LE	tcctggatgcgtagtacttctgacTTTTTaggcataggaccgtgtct
BL	ggaggcacggagggggg		
BL	gcttcagtggactcttacgtggt		
BL	aagcccagcaggcgca		
BL	aagtgttatctatcagtagtcctgcac		
BL	aagccggatgtagaaaacatcc		
BL	cccggattttgattagtcttgaat		
BL	ttgggaattaaacacaaacaactc		
BL	tcatgagaaattaacatggccat		

Fig. 1. Tissue distribution of Pon1 (top), Pon2 (middle), and Pon3 (bottom) mRNA in mice. Total RNA from both male and female C57BL/6 mouse tissues ($n = 5$/gender) was analyzed by bDNA assay for expression of each paraoxonase mRNA. Data are presented as mean ± S.E.M. *, statistical differences between male and female mice ($p < 0.05$).

Fig. 2. Ontogenic expression of mouse Pon1 (top), Pon2 (middle), and Pon3 (bottom) mRNA in mouse liver. Total RNA from C57BL/6 mice at each age ($n = 5$/gender) was analyzed by bDNA assay. Data are presented as mean ± S.E.M. *, statistical differences between male and female mice ($p < 0.05$).

minimal in other tissues. A gender difference in Pon1 mRNA expression was observed in mouse liver with higher levels in females. Expression of Pon2 mRNA in mice was highest in stomach, followed by colon, lung, placenta, and small intestine (Fig. 1). Pon2 mRNA expression in mouse stomach was higher in females than males. Pon3 mRNA (Fig. 1) was ubiquitously expressed in mouse tissues, with the highest in lung, less in liver, ovary, and gastrointestinal tract (approximately 40–60% of that in lung), and least in kidney and brain (less than 20% of that in the lung). A gender difference of Pon3 expression was noted in mouse stomach, duodenum, and gonad, with higher mRNA in females. In contrast, Pon3 mRNA expression in mouse heart was higher in males than females.

Ontogenic Expression of Paraoxonases in Male and Female Mouse Liver. The neonatal and postnatal mRNA expression patterns of the three mouse paraoxonases in male and female livers are shown in Fig. 2. Pon1 is highly expressed in adult mouse liver (Fig. 1). Expression of Pon1 in mice was low 2 days before birth, but increased markedly at parturition to its highest level. After birth Pon1 mRNA decreased to half of that at birth. By 15 days of age Pon1 mRNA returned to the high levels seen at birth. After 30 days of age Pon1 mRNA decreased in male mice, but was maintained in female mice, leading to the gender differences in expression, with higher levels observed in female mice. Pon2 mRNA was low in the livers of adult mice (Fig. 1). The ontogeny study showed that Pon2 mRNA was expressed at high levels in mouse livers at birth and 5 days of age, but decreased rapidly to its lowest level and remained low at most time intervals thereafter (Fig. 2). It is noteworthy that at 40 days of age there was an unexpected peak in the mRNA expression of Pon2. The developmental pattern of Pon3 was somewhat similar to that of Pon2 (Fig. 2). Pon3 mRNA was highly
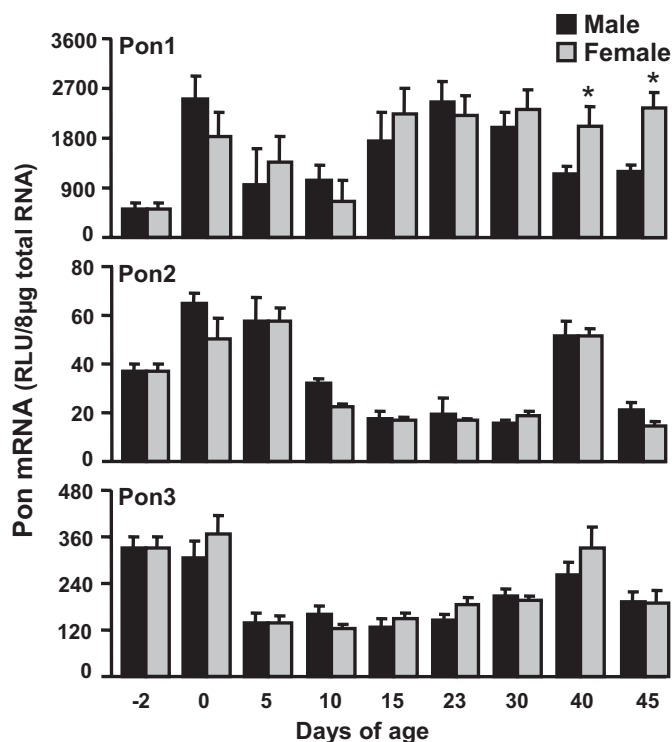
expressed before and at birth. After birth Pon3 mRNA expression decreased by approximately 60% by 5 days of age and remained at relatively low levels thereafter. Similar to Pon2, at 40 days of age there was a peak in the mRNA of Pon3.

Protein Levels of Pon1 in Adult Male and Female Mouse Livers. To determine whether gender differences in Pon1 mRNA expression result in a gender difference at the protein level, Pon1 protein levels were evaluated in the livers of adult male and female mice (Fig. 3). Similar to mRNA, Pon1 protein was 170% higher in female than male mouse livers.

Regulation of Gender Differences in Liver Pon1 by Sex Hormones and Growth Hormone. Pon1 mRNA and protein are higher in adult female than male mouse livers (Figs. 1–3). Gender differences in gene expression may be the result of regulation by sex hormones and/or gender-dimorphic GH secretion patterns. Therefore, the effects of sex hormones and growth hormone replacement on Pon1 mRNA were determined in gonadectomized, hypophysectomized, and *lit/lit* mice.

As shown in Fig. 4, castration of naive mice increased Pon1 mRNA, whereas ovariectomy of naive mice did not alter Pon1 mRNA expression, thus leading to a disappearance of the gender difference in Pon1 mRNA levels. In gonadectomized mice, both estrogen (E2) and androgen (DHT) replacement decreased Pon1 mRNA in both male and female mice.

The removal of pituitary glands, as indicated by the hypophysectomized mice (Fig. 4), increased Pon1 mRNA in male, but not female, mice, also resulting in the loss of the gender-divergent Pon1 mRNA pattern. In hypophysectomized mice, administration of male-pattern GH (MPGH) and
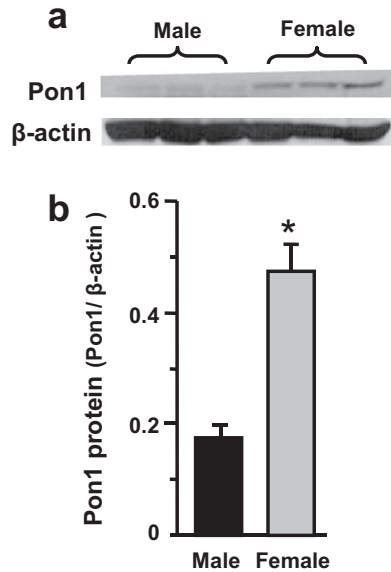
Fig. 3. Protein levels of Pon1 in adult male and female mouse livers. Total protein was isolated from adult C57BL/6 mouse livers. Protein samples ($n = 3$/gender) were analyzed by Western blotting. a, protein levels of Pon1 and β-actin in mouse liver homogenates were analyzed by Western blotting. b, protein levels of Pon1 and β-actin in mouse liver homogenates are expressed as a ratio of Pon1 to β-actin protein levels per 75 μg of total liver protein. Data are presented as mean ± S.E.M. ∗, statistical differences between male and female mice ($p < 0.05$).
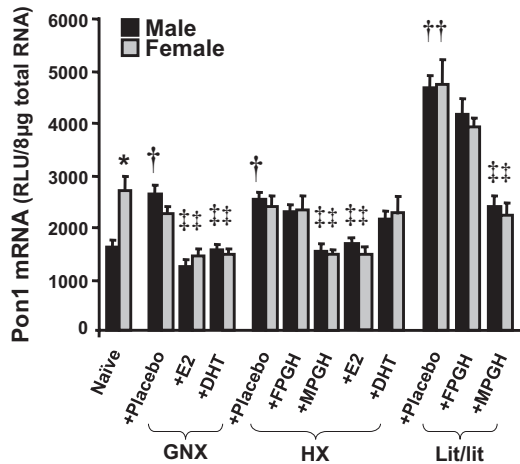

Fig. 4. Effects of GH and sex hormones on the gender differences in Pon1 mRNA expression in mouse liver from naive, GNX, HX, and *lit/lit* mice. Total liver RNA was isolated and analyzed by bDNA assay for Pon1 mRNA expression. The data are presented as mean RLU ± S.E.M. ($n = 6$–7). The study was separated into groups: GNX/HX/Lit + Plac (placebo administered to gonadectomized, hypophysectomized, or *lit/lit* mice); HX/Lit + MPGH (rat GH twice daily administered by intraperitoneal injection to hypophysectomized or *lit/lit* mice mimicking male-pattern GH secretion); HX/Lit + FPGH (continuous infusion of GH to hypophysectomized or *lit/lit* mice via subcutaneously implanted 21-day-release, 1-mg rat GH pellet mimicking female-pattern GH secretion); GNX/HX + DHT (DHT administered to gonadectomized or hypophysectomized mice); and GNX/HX + E2 (E2 administered to gonadectomized or hypophysectomized mice). ∗, statistical differences ($p < 0.05$) between male and female mice. †, statistical differences ($p < 0.05$) between naive mice and the same gender, placebo-treated gonadectomized, hypophysectomized, or *lit/lit* mice. ‡, statistical differences ($p < 0.05$) between placebo-treated gonadectomized, hypophysectomized, or *lit/lit* mice and the same gender, gonadectomized, hypophysectomized, or *lit/lit* mice after hormone replacement treatment.

estrogen (E2) decreased Pon1 mRNA in both male and female mice. In contrast, administration of female-pattern GH (FPGH) and androgen (DHT) did not alter Pon1 mRNA.

Loss of function of GH, as shown in *lit/lit* mice, in which the GH-releasing hormone receptor gene was mutated, caused a very high constitutive expression of Pon1 mRNA. Similar to that in hypophysectomized mice, male-pattern GH administration decreased Pon1 mRNA in both male and female *lit/lit* mice.

Regulation of Pon1, Pon2, and Pon3 by Prototypical Microsomal Enzyme Inducers. Drugs and other xenobiotics can alter target gene expression through the activation of nuclear receptors and other transcription factors such as AhR, CAR, PXR, PPARα, and Nrf2 (Klaassen and Aleksunes, 2010). To address the effects of distinct transcription factor pathways on the hepatic Pon mRNA expression, regulation of Pon1, Pon2, and Pon3 by well studied prototypical microsomal enzyme inducers was determined. As shown in Fig. 5, the AhR ligand PCB126 and all three CAR activators (PB, TCPOBOP, and DAS) decreased Pon1, Pon2, and Pon3 mRNA. Two PPARα activators (CLFB and CPFB) also decreased Pon1 and Pon2 mRNA.

Regulation of Pon1, Pon2, and Pon3 by TCPOBOP in CAR-Null and WT Mouse Livers. CAR-null mice were used to further determine whether down-regulation of Pon expression by TCPOBOP, the prototypical CAR activator, depends on CAR activation. As depicted in Fig. 6, TCPOBOP decreased Pon1, Pon2, and Pon3 mRNA in wild-type mice.
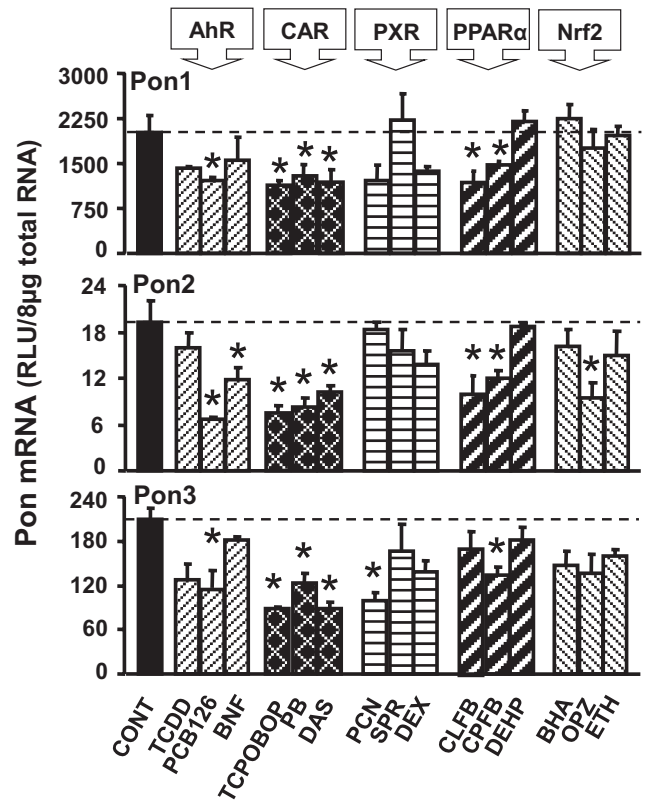

Fig. 5. Expression of Pon1 (top), Pon2 (middle), and Pon3 (bottom) mRNA in C57BL/6 mouse liver after administration of prototypical drug-metabolizing enzyme inducers: TCDD, BNF, PCB126, PB, TCPOBOP, DAS, PCN, SPR, DEX, CLFB, CPFB, DEHP, BHA, ETHOXYQ, and OPZ. Total RNA from five chemically treated male livers was analyzed by bDNA assay. All data are expressed as mean ± S.E.M. for five animals in each group, except for controls (CONT), which were combined from the four individual controls after it was determined that they were not statistically different. ∗, statistical differences between treated and control mice ($p < 0.05$).
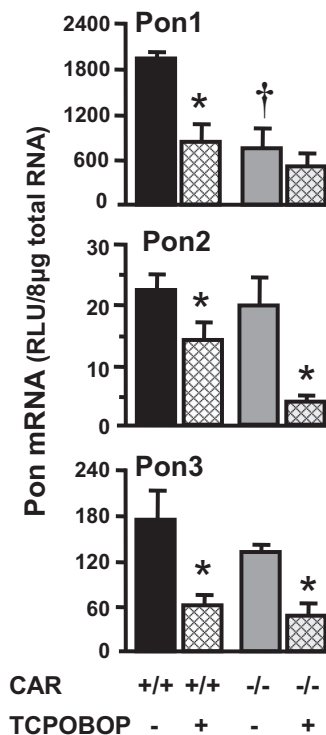
Pon mRNA (RLU/8μg total RNA)

CAR	+/+ +/+	-/-	-/-
TCPOBOP	- +	-	+

Fig. 6. Effects of TCPOBOP on Pon1 (top), Pon2 (middle), and Pon3 (bottom) mRNA expression in wild-type and CAR-null mice. The black/gray bars depict the mRNA of each Pon after vehicle treatment, and the striated bars depict the mRNA of each Pon after TCPOBOP treatment. Adult C57BL/6 or CAR-null male mice ($n = 5$) were intraperitoneally administered TCPOBOP (3 mg/kg in corn oil) or the vehicle corn oil once daily for 4 days. Total RNA from untreated or treated mouse livers was analyzed by bDNA assay. All data are expressed as mean ± S.E.M. of five mice for each treatment. ∗, statistical differences between TCPOBOP-treated and control mice ($p < 0.05$). †, statistical differences ($p < 0.05$) between the vehicle-treated wild-type male mice and vehicle-treated CAR-null male mice.

Deficiency of CAR functional protein, as confirmed in the CAR-null mice, decreased basal mRNA of Pon1, but not Pon2 or Pon3. In CAR-null mice, TCPOBOP administration did not decrease Pon1 mRNA expression, but decreased Pon2 and Pon3 mRNA. Therefore, down-regulation of Pon1 by TCPOBOP is CAR-dependent, but regulation of Pon2 and Pon3 seems to be regulated by other mechanisms.

Discussion

The present study systematically investigated the constitutive and chemical regulation of mouse Pons. Consistent with previous reports, Pon1 and Pon3 are highly expressed in mouse liver, and Pon2 and Pon3 are expressed in the gastrointestinal tract (Mackness and Walker, 1983, 1988; Draganov et al., 2000; Ng et al., 2001). In addition, the present study indicates that Pon1, Pon2, and Pon3 all are expressed in mouse lung (Fig. 1), indicating paraxonases may play detoxification functions or other physiological roles in the lung.

In mice and rats, serum and liver Pon1 enzyme activity is very low at birth and increases until 21 days of age, with a parallel increase in liver mRNA (Li et al., 1997; Moser et al., 1998). In humans, serum PON1 activity is minimal before and at birth, but gradually increases after birth, reaching a plateau between 6 and 15 months of age (Augustinsson and

Barr, 1963; Ecobichon and Stephens, 1973; Mueller et al., 1983; Cole et al., 2003). In the present study in mice, Pon1 mRNA expression in mouse liver was very low in the fetus, but rapidly increased at birth, reaching adult levels at 15 days of age (Fig. 2). In contrast, Pon2 and Pon3 both were expressed in the livers of mouse fetuses, but decreased after birth (Fig. 2). Because Pon2 and Pon3 do not have paraxonase activity (Draganov et al., 2000; Ng et al., 2001), low Pon1 level during early age contributes mainly to the increased susceptibility of young animals to the toxicity of some organophosphorus insecticides (Li et al., 1997; Moser et al., 1998; Karanth and Pope, 2000). Moreover, a recent study demonstrated that low expression of Pon3 in newborn mice resulted in increased rates of early fetal and neonatal death (Kempster et al., 2012).

Gender-divergent gene expression in liver usually is determined by sex hormones and/or growth hormone (Waxman and O'Connor, 2006). Gender-divergent growth hormone secretion, especially male-pattern growth hormone secretion, often contributes to sex-dependent mouse liver gene expression. In the present study, the lower Pon1 expression in male mouse livers was largely caused by the inhibitory effect of male-pattern growth hormone secretion (Fig. 4). Growth hormone produces the sex-dependent gene expression in liver via activation of signal transducer and activator of transcription 5b (STAT5b) (Clodfelter et al., 2006; Waxman and O'Connor, 2006). In silico analysis of the mouse Pon1 promoter sequence indicated that there is a putative STAT5b response element (5′-TGTCTTGGTTCTTTGAAAACGTACG-3′; consensus sequence of STAT5b response element underlined) located between 4881 and 4889 base pairs upstream of the transcription start site. Therefore, the male-pattern growth hormone decrease in mouse Pon1 gene expression probably is mediated via the STAT5b signaling pathway.

One interesting finding is that GH represses Pon1 expression and GH deficiency induces Pon1 in mice. Both Pons and growth hormone can prevent atherosclerosis and other cardiovascular diseases. Pons protect LDL and HDL from oxidation, thus preventing the development of atherosclerosis and other cardiovascular diseases (Aviram and Rosenblat, 2005). Adult patients with growth hormone deficiency are predisposed to premature atherosclerosis and other cardiovascular diseases (Twickler et al., 2000; Elhadd et al., 2001). Growth hormone replacement in GH-deficient adult patients improves their LDL-cholesterol abnormalities (Twickler et al., 2000; Abdul Shakoor and Shalet, 2003). Therefore, growth hormone also prevents atherosclerosis and other cardiovascular diseases. However, in the present study, we showed that male-pattern (intermittent injection) GH administration decreases Pon1 expression. Loss of GH function, as shown in the livers of *lit/lit* mice, remarkably increases Pon1 constitutive expression in both male and female phenotypes (Fig. 4). Thus, GH represses the expression of Pon1. Our explanation is that the actions of Pons and growth hormone are compensatory. During GH deficiency, Pon1 is induced further to prevent the body from developing atherosclerosis and other cardiovascular diseases.

In addition to growth hormone, DHT decreases Pon1 mRNA expression in gonadectomized mice (Fig. 4) (bin Ali et al., 2003), but not in hypophysectomized mice (Fig. 4), indicating that androgens indirectly decrease Pon1 mRNA in the livers of mice. Sex hormones have the capability of modifying

gender-specific GH secretion and thus might influence gene expression in the liver (Legraverend et al., 1992; Painson et al., 1992; Cheng et al., 2006). Therefore, androgens probably regulate female-predominant Pon1 expression by enhancing male-pattern growth hormone secretion.

Administration of exogenous E2 decreased Pon1 mRNA in the livers of both gonadectomized and hypophysectomized mice (Fig. 3). However, in postmenopausal women estrogen replacement therapy increases serum PON1 (Sutherland et al., 2001; Kumru et al., 2005; Topçuoglu et al., 2005; Fenkci et al., 2006). In addition, menopause is an estrogen-deficient status, in which decreases in serum PON1 levels and activity are observed (Kumru et al., 2005; Topçuoglu et al., 2005). Therefore, the effect of estrogens on Pon1/PON1 expression is not consistent between mice and humans.

A major function of Pon1 is to prevent LDL oxidation; however, the effect of estrogens on LDL oxidation is controversial. In rats, 17β-estradiol treatment increases the peroxidation of LDL (Butterworth et al., 1998; Chiang et al., 2004; Eybl et al., 2004), but in humans estradiol at physiological concentrations is unlikely to act as an antioxidant and even as a pro-oxidant in women with high estradiol levels who have increased myeloperoxidase protein in their plasma (Santanam et al., 1998). However, there is evidence that estrogens protect against oxidation of LDL in humans (Arteaga et al., 1998; Brunelli et al., 2000; Ruiz-Sanz et al., 2001; Rontu et al., 2004). Therefore, it seems that estrogens can function to either decrease or increase Pon1/PON1 expression and activity and depends on the dosage of estrogens and species studied.

Ovariectomy depletes endogenous estrogens, but does not alter hepatic Pon1 mRNA in female mice (Fig. 4) (bin Ali et al., 2003), indicating that endogenous estrogens do not contribute to altering Pon1 expression. Therefore, exogenous E2 decreases hepatic Pon1 mRNA independent of its estrogenic activity. In addition to activating estrogen receptors, estrogens including E2, activate CAR (Mendelsohn and Karas, 1999; Kawamoto et al., 2000; Kretschmer and Baldwin, 2005; Hernandez et al., 2007). Administration of CAR activators, such as TCPOBOP, DAS, and PB, decrease Pon1 mRNA expression (Fig. 5). By using CAR-null mice, it was determined that TCPOBOP decreases Pon1 mRNA in a CAR-dependent manner (Fig. 6). Therefore, CAR activation decreases Pon1 expression, suggesting that exogenous E2 may decrease hepatic Pon1 mRNA in mice through CAR activation. In the present study, we also showed that TCPOBOP decreased Pon2 and Pon3 expression independently of CAR activation (Fig. 6). Pon2 is least expressed in the liver of mice, thus down-regulation of Pon2 in mouse liver is essentially not meaningful. However, we do not know how TCPOBOP, phenobarbital, and diallyl sulfide decrease Pon3 expression. This merits further investigation.

Previous reports have shown that dietary polyphenols (Gouédard et al., 2004) and aspirin (Jaichander et al., 2008) increase PON1 gene expression through activation of the AhR. However, in the present studies, AhR activators did not increase Pon1 mRNA. In contrast, the AhR activator PCB126 decreased Pon1 mRNA (Fig. 5).

Taken together, the current study provides important insight into the tissue-specific, gender-divergent, and ontogenic expression, as well as chemical alteration, of mouse paraoxonases. Female-predominant Pon1 mRNA expression

in mouse liver is caused primarily by the inhibitory effect of male-pattern growth hormone secretion, whereas the activation of CAR decreases Pon1 expression. The present study provides useful information that helps to better predict the disposition of exogenous and endogenous compounds and suggests the alteration of Pon1 might affect the etiology and pathophysiology of atherosclerosis and other cardiovascular diseases.

Authorship Contributions

Participated in research design: Cheng and Klaassen.
Conducted experiments: Cheng.
Performed data analysis: Cheng.
Wrote or contributed to the writing of the manuscript: Cheng and Klaassen.

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