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Oral Benzo[*a*]pyrene in *Cyp1* Knockout Mouse Lines: CYP1A1 Important in Detoxication, CYP1B1 Metabolism Required for Immune Damage Independent of Total-Body Burden and Clearance Rate

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ABSTRACT

CYP1A1 and CYP1B1 metabolically activate many polycyclic aromatic hydrocarbons (PAHs), including benzo[*a*]pyrene, to reactive intermediates associated with toxicity, mutagenesis, and carcinogenesis. Paradoxically, however, *Cyp1a1*$^{-/-}$ knockout mice are more sensitive to oral benzo[*a*]pyrene exposure, compared with wild-type *Cyp1a1*$^{+/+}$ mice (*Mol Pharmacol* **65:**1225, 2004). To further investigate the mechanism for this enhanced sensitivity, *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1b1*$^{-/-}$ single-knockout, *Cyp1a1/1b1*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ double-knockout, and *Cyp1*$^{+/+}$ wild-type mice were analyzed. After administration of oral benzo[*a*]pyrene (125 mg/kg/day) for 18 days, *Cyp1a1*$^{-/-}$ mice showed marked wasting, immunosuppression, and bone marrow hypocellularity, whereas the other five genotypes did not. After 5 days of feeding, steady-state blood levels of benzo[*a*]pyrene were ~25 and ~75 times higher in *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice, respectively, than in wild-type mice. Benzo[*a*]pyrene-DNA adduct levels were highest in liver, spleen, and marrow of *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice. Many lines of convergent data obtained with oral benzo[*a*]pyrene dosing suggest that: 1) inducible CYP1A1, probably in both intestine and liver, is most important in detoxication; 2) CYP1B1 in spleen and marrow is responsible for metabolic activation of benzo[*a*]pyrene, which results in immune damage in the absence of CYP1A1; 3) both thymus atrophy and hepatocyte hypertrophy are independent of CYP1B1 metabolism but rather may reflect long-term activation of the aryl hydrocarbon receptor; and 4) the magnitude of immune damage in *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice is independent of plasma benzo[*a*]pyrene and total-body burden and clearance. Thus, a balance between tissue-specific expression of the CYP1A1 and CYP1B1 enzymes governs sensitivity of benzo[*a*]pyrene toxicity and, possibly, carcinogenicity.

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed environmental toxicants that are largely the byproducts of combustion processes—such as cigarette smoke, charcoal-grilled food, creosote railroad ties, and coke ovens in the petroleum industry. The most thoroughly studied prototype of this class of chemicals is benzo[*a*]pyrene (Pelkonen and Nebert, 1982; Conney et al., 1994; Nebert et al., 2004). In numerous mammalian studies, benzo[*a*]pyrene has been shown to cause cytotoxic, teratogenic, genotoxic, mutagenic, and carcinogenic effects in various tissues and cell types (Nebert, 1989; Ellard et al., 1991). Benzo[*a*]pyrene is implicated as a causative agent in lung carcinogenesis (Alexandrov et al., 2002; Pfeifer et al., 2002) and atherosclerosis (Miller and Ramos, 2001) in cigarette smokers.

The etiology of toxicity and cancer caused by benzo[*a*]pyrene and other PAHs is complex. PAHs induce numer-

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; AHR, aromatic hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCR, polymerase chain reaction; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AUC, area under the curve; RT, reverse transcription; DMBA, 7,12-dimethylbenzo[a]anthracene.

ous enzymes involved in activation and detoxication of PAHs via the aromatic hydrocarbon receptor (AHR). PAHs are typically metabolically activated by phase I (oxidation-reduction) enzymes to reactive intermediates that bind covalently to nucleic acids and proteins; however, PAHs are detoxified by both phase I and phase II (conjugation) enzymes. PAHs affect the expression of many other genes by way of both AHR-dependent and AHR-independent mechanisms (Nebert, 1989, 2000b, 2004; Ryu et al., 1996; Puga et al., 2000; Miller and Ramos, 2001).

Expression of CYP1A1 is constitutively nil but is markedly induced in a large number of tissues after induction by PAHs (Nebert et al., 2004). In contrast, substantial levels of basal CYP1A2 activity occur in mammalian liver. The human and rodent *CYP1A2* gene is inducible by PAHs in liver, gastrointestinal tract, pancreas, brain, and lung (Sesardic et al., 1990; Farin and Omiecinski, 1993; Foster et al., 1993; Dey et al., 1999; Tatemichi et al., 1999; Wei et al., 2001). Although after induction CYP1A2 exists in rodent lung, there remains some question as to whether human CYP1A2 protein is expressed at any significant level in lung (Shimada et al., 1996) or other extrahepatic tissues. CYP1B1 has a high basal activity in such tissues as adrenal cortex, ovary, testis, uterus, prostate and mammary gland, gastrointestinal tract, and immune cells (Walker et al., 1995; Guengerich et al., 2003; Galvan et al., 2005). Constitutive CYP1B1 is extremely low in liver and gastrointestinal tract but is detectable after PAH treatment (Buesen et al., 2002; Zhang et al., 2003).

To examine the role of CYP1A1 in the intact animal receiving daily oral benzo[*a*]pyrene, we compared the *Cyp1a1*$^{-/-}$ knockout with *Cyp1*$^{+/+}$ wild-type mice. Based on many studies in vitro and in cell culture showing that CYP1A1 metabolically activates benzo[*a*]pyrene, we had expected the *Cyp1a1*$^{-/-}$ mouse to be more protected than *Cyp1*$^{+/+}$ wild-type mice; surprisingly, however, damage to the immune system and benzo[*a*]pyrene-DNA adduct formation in liver, small intestine, spleen, and bone marrow were much higher in the knockout than wild-type mice (Uno et al., 2004a). Moreover, total body clearance of benzo[*a*]pyrene was greatly impeded in the *Cyp1a1*$^{-/-}$ mouse. This led us to conclude that oral benzo[*a*]pyrene-induced CYP1A1 in the intestine and/or liver was more important in detoxication than metabolic activation in the intact animal. The contributions of CYP1A2 and CYP1B1 to oral benzo[*a*]pyrene-mediated disease have not been thoroughly evaluated. To address this issue, *Cyp1*$^{+/+}$ wild-type mice were compared with the *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1b1*$^{-/-}$ single-knockout mice, and the *Cyp1a1/1b1*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ double-knockout mice, after daily exposure to oral benzo[*a*]pyrene.

Materials and Methods

Chemicals. Benzo[*a*]pyrene was purchased from Sigma (St. Louis, MO), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from Accustandard, Inc. (New Haven, CT). All other chemicals and reagents were bought from Sigma-Aldrich Chemical Company (St. Louis, MO) as the highest available grades.

Animals. Generation of the *Cyp1a1*$^{-/-}$ (Dalton et al., 2000), *Cyp1a2*$^{-/-}$ (Liang et al., 1996), and *Cyp1b1*$^{-/-}$ (Buters et al., 1999) knockout mouse lines have been described. Subsequent genetic crosses have generated the *Cyp1a1/1b1*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ double-knockout lines. It is not possible for such simple genetic crosses to provide the *Cyp1a1/1a2*$^{-/-}$ double-knockout, because these two genes are located only 13.3 kilobases apart on mouse chromosome 9. All five genotypes were backcrossed into the C57BL/6J background for eight generations; this ensures that the knockout genotype resides in a genetic background that is >99.8% C57BL/6J (Nebert et al., 2000a). Age-matched C57BL/6J *Cyp1*$^{+/+}$ wild-type mice, purchased from The Jackson Laboratory (Bar Harbor, ME), were therefore used as the comparative controls. All experiments with these six genotypes were begun at 6 ± 1 weeks of age. All animal experiments were approved by, and conducted in accordance with, the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee.

Treatment. Preliminary experiments comparing *Cyp1*$^{+/+}$ wild-type with *Cyp1a1*$^{-/-}$ knockout mice showed no substantial differences between male and female mice; thus, one sex (male) was chosen for all subsequent studies. For the pharmacokinetics studies, benzo[*a*]pyrene (15 mg/kg) was given by gavage. In all other experiments, benzo[*a*]pyrene was given in corn oil-soaked food. Rodent chow (Harlan Teklad, Madison, WI) was soaked at least 24 h in benzo[*a*]pyrene-containing corn oil (10, 1.0, or 0.1 mg/ml). By knowing the weight of the food ingested daily by a 20-g mouse and by using [^{3}H]benzo[*a*]pyrene in several experiments (Robinson et al., 1975), the daily oral benzo[*a*]pyrene doses had been estimated to be approximately 125, 12.5, and 1.25 mg/kg/day, respectively. To start day 1 of the experiment, the mice (after having been fasted overnight) were presented with the benzo[*a*]pyrene-laced food; control mice received food soaked in corn oil alone. The mice eagerly ate the corn oil-soaked chow. Oral benzo[*a*]pyrene experiments in this study were run for 5 days or 18 days. Each mouse was weighed daily. All tissues were removed between 9:00 a.m. and 10:00 a.m. to exclude any circadian rhythm effects. Wet weights of liver, thymus, and spleen were recorded.

Total RNA Preparation. Total RNA was isolated (four tissues; $n = 3$ mice per group) using the RNAgents Total RNA Isolation System (Promega, Madison, WI).

Reverse Transcription. Total RNA (2 μg) was added to a reaction containing 3.8 μM oligo(dT)$_{20}$ and 0.77 mM dNTP to a final volume of 13 μl. Reactions were incubated at 65°C for 5 min, then at 4°C for 2 min. To the reaction mixture was added 7 μl of a solution containing 14 mM dithiothreitol, 40 units of RNaseOUT Recombinant RNase inhibitor (Invitrogen, Carlsbad, CA), and 200 units of SuperScript III (Invitrogen). Reactions were incubated at 50°C for 50 min, followed by 75°C for 10 min (to inactivate the reverse transcriptase). Distilled water (80 μl) was added to the isolated cDNA, and this was then stored at −80°C.

Quantification of CYP1 mRNA Copy Numbers. The mRNAs for each of the three CYP1 family members were quantified by fitting RT-PCR data to a curve generated from copy RNAs (cRNAs) for each CYP1 member. In brief, templates for cRNA synthesis were generated by performing PCR on cDNA constructs for each CYP1 family member, cloned into pcDNA3.1(+) (Invitrogen). PCR was performed, using a forward primer containing the T7 promoter sequence and a reverse primer that had an oligo(dT) after the stop codon. The amplified product was purified by electrophoresis. In vitro transcription was performed using T7 RiboMAX Express Large-Scale RNA Production System (Promega). After DNase1 treatment, the cRNAs were quantified spectrophotometrically. cRNAs were used to generate a standard curve in real time PCR reactions from which mRNA copy number from RT-RNA measurements (see below) could be extrapolated.

Real-Time Quantitative PCR. Primers used were: *Cyp1a1*: forward, CCTCATGTACCTGGTAACCA; reverse, AAGGATGAATGCCGGAAGGT; *Cyp1a2*: forward, AAGACAATGGCGGTCTCATC; reverse, GACGGTCAGAAAGCCGTGGT; *Cyp1b1*: forward, ACATCCCCAAGAATACGGTC; reverse, TAGACAGTTCCTCACCGATG; *Gapdh*: forward, TGCACCACCAACTGCTTAG; reverse, GATGCAGGGATGATGTTC. Real-time PCR was performed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems), using

SYBR Premix EX *Taq* (TAKARA BIO Inc., Shiga, Japan). Individual CYP1 family member mRNA abundance was determined by using the standard-curve method (from 10^1 to 10^8 copies/μl), as described previously by K. Livak (PE-ABI Sequence Detector User Bulletin 2) (Winer et al., 1999). Each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Microsomal Protein Immunoblots. Microsomes (S9 fraction) from liver, small intestine (2 cm extending distally from the pylorus), spleen, and bone marrow were prepared as described previously (Uno et al., 2001). Protein concentrations were determined using the Bio-Rad protein reagent (Bio-Rad, Hercules, CA). Microsomal proteins were separated on 0.1% SDS/10% polyacrylamide mini-gels. Separated proteins were transferred to nitrocellulose and visualized with Ponceau S to verify equivalent loading across lanes. Western blot analysis was performed using goat polyclonal anti-human CYP1A1/1A2 and polyclonal anti-human CYP1B1 (both from BD Gentest, Woburn, MA). Alkaline phosphatase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and the alkaline phosphatase conjugate substrate kit (Bio-Rad) were used for visualization; exposure times ranged from 5 to 15 min.

Plasma Enzymes. Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, in mouse plasma previously frozen at −20°C, were determined using kits purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Percent hematocrit and percent methemoglobin were also determined, as described previously (Uno et al., 2004a).

Histology. Blood samples were air-dried on glass slides. Bone marrow was obtained at sacrifice by dissecting the femurs free of muscle, removing the proximal and distal epiphyses, and affixing a tiny polyethylene tube to one end. The marrow was gently blown onto a glass slide, and a second slide was used to squash the droplet of marrow onto the slide. These slides were then air-dried. All slides were stained with Wright-Giemsa stain (University Hospital Bone Marrow Lab).

Differential counts of the peripheral blood and marrow were performed. The myeloid line includes promyelocytes, myelocytes, promegakaryocytes, megakaryocytes, neutrophils, eosinophils, basophils, and monocytes. The lymphoid line includes large, medium, and small lymphocytes and plasma cells. The erythroid line includes basophilic erythroblasts, polychromatophilic erythroblasts, orthochromatic erythroblasts, and red cells. Percentages of different cell types were calculated, usually based on 1000 cells tabulated, and the differentials were always counted on a minimum of 100 lymphocytes per animal.

Pharmacokinetic Studies. A benzo[*a*]pyrene dose of 15 mg/kg body weight (in 150 μl) was given by gavage to *Cyp1*$^{+/+}$ wild-type mice, each of the three single-knockout lines, and each of the two double-knockout lines. Sequential blood samples were drawn from the saphenous vein of the same mouse by a procedure described previously (Hem et al., 1998). There were marked differences in clearance time noted between the *Cyp1a1*$^{-/-}$ or *Cyp1a1/1b1*$^{-/-}$ versus the four other genotypes in preliminary experiments. Consequently, blood was drawn from the same *Cyp1a1*$^{-/-}$ or *Cyp1a1/1b1*$^{-/-}$ mouse at 0.5, 1.0, 1.5, 2, 3, 4, 6, 9, 12, and 15 h; blood was drawn from the same *Cyp1*$^{+/+}$, *Cyp1a2*$^{-/-}$, *Cyp1b1*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mouse at 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, and 6 h. Each time with each mouse, at least 20 μl of blood was collected so that we could analyze 15 μl of whole blood. Before benzo[*a*]pyrene administration by gavage, some mice were treated 48 h beforehand with TCDD (10 μg/kg body weight i.p.) in corn oil vehicle. The principal pharmacokinetic parameters elimination half-life ($t_{1/2}$), total body clearance, and area under the curve (AUC) were determined with the help of Sander Vinks (Cincinnati Children's Hospital, Cincinnati, OH), using his KinFit software program (MW\Pharm, version 3.50; MediWare, Groningen, The Netherlands). In addition to benzo[a]pyrene by gavage, we attempted intravenous benzo[*a*]pyrene pharmacokinetics but found this to be too difficult technically; the veins of the *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice seemed to collapse far more easily than veins of the other four genotypes.

Detection of Benzo[*a*]pyrene in Blood. Benzo[*a*]pyrene levels in blood were quantified by modifying the methods described previously (Garcia-Falcon et al., 1996; Kim et al., 2000). Whole blood (15 μl) was extracted three times with ethyl acetate/acetone mixture [2:1 (v/v)]. The organic extracts were pooled and dried under argon, and the residue was resuspended in 250 μl of acetonitrile. An aliquot (100 μl) aliquot was injected onto a Nova-Pak C_{18} reverse-phase column (4 μm, 150 × 3.9 mm i.d.; Waters Associates, Boston, MA). High-performance liquid chromatography analysis was conducted on a Waters model 600 solvent controller, equipped with a fluorescence detector (F-2000; Hitachi, Yokohama, Japan). Isocratic separation was performed using an acetonitrile/water [85:15 (v/v)] mobile phase at a flow rate of 1 ml/min. Excitation and emission wavelengths were 294 and 404 nm, respectively. Benzo[*a*]pyrene concentrations in blood were calculated by comparing the peak amplitude of samples with those of control blood spiked with different known concentrations of benzo[*a*]pyrene; the calibration curve for benzo[*a*]pyrene showed excellent linearity (correlation coefficient, $r > 0.998$). Four major benzo[*a*]pyrene metabolites were found to run far ahead of benzo[*a*]pyrene on the column and thus did not interfere. The detection limit (defined as 3 times the signal-to-noise ratio) was 0.05 mg/ml, and the limit of benzo[*a*]pyrene quantification was determined to be 0.2 ng/ml. The intraday and interday precision of repeated analyses ($n = 4$) gave us coefficients of variation of ≤12%.

Biohazard Precaution. Benzo[*a*]pyrene and TCDD are very toxic and likely human carcinogens. All personnel were instructed in safe handling procedures. Lab coats, gloves, and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit or by independent contractors. Benzo[*a*]pyrene- and TCDD-pretreated mice were housed separately, and their carcasses were treated as contaminated biological materials.

Measurement of Benzo[*a*]pyrene-DNA Adducts. Benzo[*a*]pyrene-DNA adducts were determined by ^{32}P-postlabeling as described previously (Uno et al., 2004a). DNA was extracted from liver, small intestine, spleen, or bone marrow using the Wizard genome DNA isolation kit (Promega) and hydrolyzed (0.5–1.3 μg DNA/sample) to 3′-phosphodeoxynucleotides by digestion with micrococcal endonuclease and spleen phosphodiesterase (both from Worthington Biochemical Co., Lakewood, NJ). Nuclease P1 treatment (Calbiochem, San Diego, CA) was carried out to enrich the adducts. After *n*-butanol extraction to remove most of the nonadducted 3′-phosphodeoxynucleotides, the 3′-phosphodeoxynucleosides were labeled at the 5′ positions with [^{32}P]ATP (PerkinElmer Life and Analytical Sciences, Boston MA) and T4 polynucleotide kinase (U.S. Biochemical Corp., Cleveland, OH). Two-dimensional thin-layer chromatography on polyethylenimine cellulose sheets were used to resolve the ^{32}P-labeled DNA adducts (EM Scientific, Gibbstown, NJ). Visualization and analyses were performed by scintillation counting (1900 CA; PerkinElmer Life and Analytical Sciences) and autoradiography, respectively. Adduct levels in each DNA sample were calculated from the background-corrected adduct counts and the specific activity of the radiolabeled-ATP and expressed as relative adduct labeling, which is defined as the minimal estimate of the number of nucleotides containing adducts, per 10^9 nucleotides, in the sample. This value is based on the amount of DNA in each sample and on the specific radioactivity of the [^{32}P]ATP used in the assay. Triplicates of each DNA sample were analyzed independently.

Statistical Analysis. For quantitative histology, means and standard-errors-of-the-means were generated with the General Linear Model of the SAS. For adducts and all other measurements, assays were performed in duplicate or triplicate, and the average values were considered as one independent determination. Statistical differences between group-mean values were assessed by analysis of variance and/or Student's pairwise *t* tests. Data were normally

distributed and are presented as means ± S.E.M. A P value of <0.05 was regarded as statistically significant.

Results

Decision on the Benzo[*a*]pyrene Dose. Previous studies from this laboratory had determined a regimen for oral benzo[*a*]pyrene administration. Benzo[*a*]pyrene is delivered in daily long-term doses by offering mice the food pellets soaked in corn oil in which benzo[*a*]pyrene had been dissolved (Robinson et al., 1975). Studies using this regimen demonstrated that C57BL/6 mice, consuming 125 mg/kg/day of benzo[*a*]pyrene, remained healthy for at least 1 year with no apparent toxic effects. For this reason, we compared three doses (125 versus 12.5 versus 1.25 mg/kg/day) of oral benzo-[*a*]pyrene in *Cyp1*$^{+/+}$ wild-type and *Cyp1a1*$^{-/-}$ knockout mice, both genotypes of which have a C57BL/6J genetic background (Uno et al., 2004a). The difference between genotypes was very dramatic: oral benzo[*a*]pyrene at 125 mg/kg/day was lethal to all *Cyp1a1*$^{-/-}$ mice within 30 days, whereas all *Cyp1*$^{+/+}$ mice showed no signs of toxicity for 1 year. *Cyp1a1*$^{-/-}$ mice displayed increases in damage to immune cells, and in benzo[*a*]pyrene-DNA adduct formation, detectable in some tissues—even at the 1.25 mg/kg/day regimen of oral benzo[*a*]pyrene for 18 days.

On the 125 mg/kg/day routine, food consumption during the first 10 days is not significantly different between *Cyp1*$^{+/+}$ and *Cyp1a1*$^{-/-}$ mice; beyond 10 days, this starts to become significantly ($P < 0.05$) different, with *Cyp1a1*$^{-/-}$ mice consuming less than wild-type mice. No differences in food consumption between the genotypes are seen over 30 days on the 12.5 or 1.25 mg/kg/day regimens (Uno et al., 2004a). Consequently, in the present study we chose 5 days or 18 days of oral benzo[*a*]pyrene for all experiments. We found no differences in the eating habits of any of the six genotypes—except beyond 10 days for *Cyp1a1*$^{-/-}$ mice, and to a lesser extent for *Cyp1a1/1b1*$^{-/-}$ mice, on the 125 mg/kg/day dosing regimen (details described under *Wasting and Immunotoxicity Caused by Oral Benzo[a]pyrene*).

CYP1 mRNA Levels after Exposure to Oral Benzo-[*a*]pyrene. The RT-PCR data (Fig. 1) include estimations of mRNA copy numbers for the three CYP1 mRNAs in the four tissues from the six genotypes. With no benzo[*a*]pyrene exposure (Fig. 1, last columns), wild-type mice showed negligible constitutive CYP1A1 mRNA in all four tissues examined by RT-PCR; *Cyp1*$^{+/+}$ mice showed detectable constitutive CYP1A2 mRNA in all four tissues that was lowest in small intestine. Wild-type mice also showed measurable constitutive CYP1B1 mRNA in all four tissues that was lowest in liver. Compared with no benzo[*b*]pyrene, oral benzo[*a*]pyrene for 5 days in *Cyp1*$^{+/+}$ wild-type mice induced CYP1A1 mRNA to high levels in liver, small intestine, spleen, and bone marrow (Fig. 1; compare first and last columns). In these same wild-type mice, accumulation of CYP1A2 mRNA was highest in liver and small intestine and slight in spleen, but there was no significant induction in marrow. In these same wild-type mice, induced CYP1B1 mRNA was highest in spleen and minutely increased in bone marrow, liver, and small intestine.

As expected, genetic ablation of the mouse *Cyp1a1*, *Cyp1a2*, or *Cyp1b1* gene resulted in no detectable CYP1A1, CYP1A2, or CYP1B1 mRNA, respectively, in the four tissues examined (Fig. 1). Also, as expected, the *Cyp1a1/1b1*$^{-/-}$ double-knockout showed the complete absence of the CYP1A1 and CYP1B1 mRNA, and the *Cyp1a2/1b1*$^{-/-}$ double-knockout exhibited no detectable CYP1A2 or CYP1B1 mRNA.

Compared with wild-type mice exposed to oral benzo-

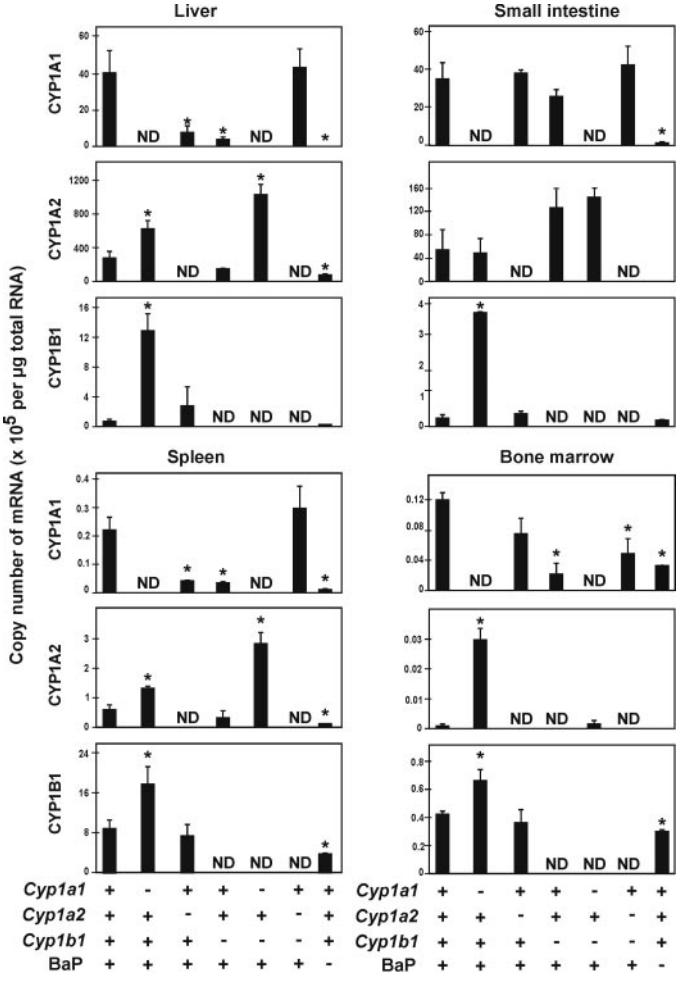

Fig. 1. Estimated copy numbers for the CYP1A1, CYP1A2, and CYP1B1 mRNA transcripts in the six different genotypes in this study, as evaluated by RT-PCR (means ± S.E.). Tissues were removed from each mouse ($n = 3$ per group) after 5 days of oral benzo[*a*]pyrene (125/mg/kg daily) or oil-soaked food without benzo[*a*]pyrene. From total RNA extracted from mouse liver, small intestine, spleen, and bone marrow, mRNA levels are expressed as -fold differences relative to *Cyp1*$^{+/+}$ wild-type mice—after normalization to the housekeeping gene's GAPDH mRNA. + and − (bottom) denote presence or absence, respectively, of the *Cyp1a1*, *Cyp1a2*, or *Cyp1b1* genes in the wild-type, three single-knockout, and two double-knockout lines, as well as treatment with benzo[*a*]pyrene or corn oil alone in the food; the far right column shows wild-type animals not exposed to oral benzo[*a*]pyrene. *, significant difference relative to oral benzo-[*a*]pyrene-treated *Cyp1*$^{+/+}$ mice ($P < 0.05$). ND, not detectable by RT-PCR. Thus, in benzo[*a*]pyrene-treated wild-type liver, maximally induced CYP1A2 mRNA levels are 5 to 10 times higher than maximally induced CYP1A1, which in turn are ~20 times greater than maximally induced CYP1B1. In benzo[*a*]pyrene-treated wild-type small intestine, maximally induced CYP1A1 mRNA levels are not significantly different from maximally induced CYP1A2, which in turn are >100 times higher than maximally induced CYP1B1. In benzo[*a*]pyrene-treated wild-type spleen, maximally induced CYP1B1 mRNA levels are ~50 times higher than maximally induced CYP1A1, which in turn are approximately three times lower than maximally induced CYP1A2. In benzo[*a*]pyrene-treated wild-type bone marrow, maximally induced CYP1B1 mRNA levels are 3 to 4 times higher than maximally induced CYP1A1, which in turn are >100 times greater than maximally induced CYP1A2.

[*a*]pyrene for 5 days, absence of the *Cyp1a1* gene resulted in large increases in CYP1B1 mRNA levels in all four tissues examined, and increased CYP1A2 mRNA in liver, spleen, and bone marrow (Fig. 1). Compared with benzo[*a*]pyrene-treated wild-type mice, absence of the *Cyp1a2* gene led to significant decreases in CYP1A1 mRNA in liver, spleen, and marrow and did not decrease CYP1B1 mRNA in any of the four tissues. Absence of the *Cyp1b1* gene resulted in both large decreases in CYP1A1 mRNA and lowered CYP1A2 mRNA in all four tissues examined. Absence of both the *Cyp1a1* and *Cyp1b1* genes caused increased CYP1A2 mRNA in liver, small intestine, and especially spleen. Absence of both the *Cyp1a2* and *Cyp1b1* genes led to increased CYP1A1 mRNA in spleen and no significant changes in CYP1A1 mRNA levels in liver, small intestine, or bone marrow.

Changes in the levels of a CYP1 mRNA in mice having the genetic absence of one or more other *Cyp1* genes in some cases reflect dramatic differences in benzo[*a*]pyrene pharmacokinetics (see *Benzo[a]pyrene Clearance in the Six Genotypes*). In other cases, these mRNA level changes may also reflect compensatory changes in gene regulation that we do not understand. It should be noted that some modest, seemingly compensatory increases in the basal level of some CYP1 mRNAs have been detected when their family members have been ablated; whether these increases indicate that these genes are somehow primed for more robust benzo[*a*]pyrene-meditated induction is not known.

CYP1 Protein Levels after Exposure to Oral Benzo[*a*]pyrene. Compared with no benzo[*a*]pyrene exposure, oral benzo[*a*]pyrene for 5 days in *Cyp1*$^{+/+}$ wild-type mice induced the CYP1A1 and CYP1A2 proteins in the same tissues (Fig. 2) comparable with what was seen with mRNA increases determined by RT-PCR (Fig. 1). The same increases or decreases in CYP1 proteins in the three oral benzo[*a*]pyrene-treated single-knockout and two double-knockout lines—seen in the Western immunoblots of Fig. 2—very closely paralleled that of the CYP1 mRNA levels; however, the levels of mRNA determined by RT-PCR seemed to be more dramatic and also more quantitative than the Western blot data.

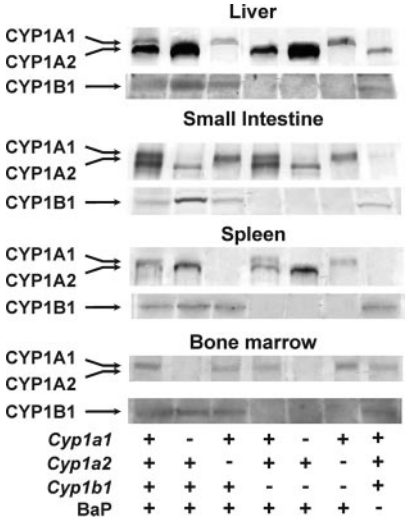

Fig. 2. Western immunoblot analysis of microsomes from liver, small intestine, spleen, and bone marrow—from the same six genotypes (n = 3 mice, combined), and same oral benzo[*a*]pyrene regimen, as in Fig. 1. For the CYP1A1/1A2 blots, the lanes of liver and small intestine were loaded with 4 μg of protein, and lanes of spleen and bone marrow were loaded with 10 μg of protein. For the CYP1B1 blot, the lanes were loaded with 60 μg of microsomal (S9) protein per lane. + and − (bottom) denote presence or absence, respectively, of the *Cyp1a1*, *Cyp1a2*, or *Cyp1b1* genes in the wild-type, three single-knockout, and two double-knockout mouse lines.

Discrepant mRNA-to-protein levels are apparent. For example, liver CYP1B1 mRNA is borderline detectable in *Cyp1*$^{+/+}$ and *Cyp1a2*$^{-/-}$ mice and very prominent in *Cyp1a1*$^{-/-}$ mice, yet CYP1B1 protein levels are slightly greater in *Cyp1a1*$^{-/-}$ and *Cyp1a2*$^{-/-}$ than in wild-type mice; in contrast, the intestinal, spleen, and bone marrow CYP1B1 proteins correlate extremely well with CYP1B1 mRNA measurements by RT-PCR.

Benzo[*a*]pyrene Clearance in the Six Genotypes. Previously (Uno et al., 2004a), we had evaluated the accumulation of benzo[*a*]pyrene in blood after a gavage dose of benzo[*a*]pyrene (15 mg/kg)—chosen because it roughly approximates the amount of benzo[*a*]pyrene consumed as a result of a single feeding of benzo[*a*]pyrene at 125 mg/kg/day. When benzo[*a*]pyrene in whole blood was compared, we found that *Cyp1a1*$^{-/-}$ knockout mice show a 4-fold greater AUC, a 4-fold slower clearance rate, and a 2-fold longer half-life than in *Cyp1*$^{+/+}$ wild-type mice. Although these differences in benzo[*a*]pyrene clearance are quite dramatic, they probably do not reflect that in mice consuming benzo[*a*]pyrene daily for 5 or 18 days; in mice not previously induced, CYP1A1 levels are extremely low, approaching undetectable, but become induced after at least 6 or 12 h of oral benzo[*a*]pyrene. Thus, to study also the effect of induced CYP1A1 on circulating benzo[*a*]pyrene, we pretreated mice 48 h beforehand with TCDD (10 μg/kg), which is known to induce *Cyp1* gene expression maximally without overt toxicity (Nebert, 1989). After TCDD pretreatment and compared with *Cyp1*$^{+/+}$ wild-type mice, the *Cyp1a1*$^{-/-}$ AUC is 7-fold greater, the clearance rate is 7-fold slower, and the half-life is 4-fold longer (Uno et al., 2004a).

The present study was thus extended to the additional knockout mouse lines (Table 1). Without or with TCDD pretreatment, the primary determinant of benzo[*a*]pyrene clearance was CYP1A1, although—in the absence of prior CYP1A1 induction—CYP1B1 plays a modest role. Thus, compared with *Cyp1*$^{+/+}$ mice, benzo[*a*]pyrene clearance is greatly affected only in *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice. In addition, without or with TCDD pretreatment, *Cyp1a1/1b1*$^{-/-}$ mice—having total-body complement of CYP1A2 but no CYP1A1 or CYP1B1—showed substantially greater AUC values, slower benzo[*a*]pyrene clearance rates, and longer half-lives than those of *Cyp1a1*$^{-/-}$ mice (Fig. 3).

Perhaps the most revealing data were the steady-state blood concentrations of benzo[*a*]pyrene after 5 days of feeding (Table 1). Compared with *Cyp1*$^{+/+}$ wild-type mice, the *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice showed ~25- and ~75-fold higher amounts of blood benzo[*a*]pyrene, respectively. Hence, whereas the total-body absence of CYP1A1 dramatically impairs benzo[*a*]pyrene clearance and elevates blood levels of benzo[*a*]pyrene in oral benzo[*a*]pyrene-treated mice, the combined absence of both CYP1A1 and CYP1B1 produces a striking increase in blood levels of benzo[*a*]pyrene over that seen in *Cyp1a1*$^{-/-}$ mice. For the remainder of this article, one will note that the parameters in *Cyp1*$^{+/+}$, *Cyp1a2*$^{-/-}$, *Cyp1b1*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mice are not substantially different after administration of oral benzo[*a*]pyrene; from this point forward, therefore, we will focus principally on the

comparison of *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice with *Cyp1*$^{+/+}$ wild-type animals.

Wasting and Immunotoxicity Caused by Oral Benzo[*a*]pyrene. At 125 mg/kg/day, normal increases in body weight over the 18 days (Fig. 4, top) were most severely lacking in *Cyp1a1*$^{-/-}$ and then in *Cyp1a1/1b1*$^{-/-}$ mice; curiously, *Cyp1a2*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ mice actually gained weight significantly better than wild-type mice. At 12.5 and 1.25 mg/kg/day for 18 days, body weight increases in *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, *Cyp1b1*$^{-/-}$, and *Cyp1a1/1b1*$^{-/-}$ mice were significantly less than that in *Cyp1*$^{+/+}$ wild-type, whereas *Cyp1a2/1b1*$^{-/-}$ weight gains were not different from those of wild-type mice.

At 125 mg/kg/day for 18 days, spleen weights compared with wild-type (Fig. 4, second from top) were most decreased in *Cyp1a1*$^{-/-}$ and then in *Cyp1a1/1b1*$^{-/-}$ mice—with no significant drop in the other three genotypes. At 12.5 mg/kg/day for 18 days, spleen weights compared with wild-type were significantly decreased in the *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1a1/1b1*$^{-/-}$ mice. No significant differences occurred at the 1.25 mg/kg/day dose.

At 125 mg/kg/day for 18 days, thymus weights compared with wild-type (Fig. 4, third from top) were equally strikingly decreased in *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice—with significant increases in thymus weights in *Cyp1a2*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ mice. At 12.5 mg/kg/day for 18 days, spleen weights compared with wild-type were significantly decreased in the *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1a1/1b1*$^{-/-}$ mice. At 1.25 mg/kg/day for 18 days, significant decreases in thymus weights were still significant especially in *Cyp1a1/1b1*$^{-/-}$ mice.

At 125 mg/kg/day and at 12.5 for 18 days, liver weights compared with wild-type (Fig. 4, bottom) were increased in *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice—with significant decreases in *Cyp1a2/1b1*$^{-/-}$ mice. At 1.25 mg/kg/day for 18 days, compared with wild-type liver weights, decreases in liver weights were found in *Cyp1b1*$^{-/-}$, *Cyp1a1/1b1*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mice. It is noteworthy that the effects of oral benzo[*a*]pyrene on total body weight and spleen weight in *Cyp1a1/1b1*$^{-/-}$ mice are intermediate between those in *Cyp1a1*$^{-/-}$ and wild-type mice; in contrast, the effects of oral benzo[*a*]pyrene on liver weight and thymus weight in *Cyp1a1/1b1*$^{-/-}$ and *Cyp1a1*$^{-/-}$ are equally strikingly different from *Cyp1*$^{+/+}$ wild-type mice

Effects of Oral Benzo[*a*]pyrene on Plasma AST and ALT Levels and Peripheral Blood Cells. At 125 mg/kg/day for 18 days (Fig. 5, top two), plasma AST and ALT levels were markedly elevated in *Cyp1a1*$^{-/-}$ mice and less elevated (but ALT is significant) in *Cyp1a1/1b1*$^{-/-}$ mice. At 12.5 and 1.25 mg/kg/day for 18 days, the six genotypes showed no significant differences in plasma AST and ALT levels.

At oral benzo[*a*]pyrene 125 mg/kg/day and compared with *Cyp1*$^{+/+}$ wild-type, the hematocrit (Fig. 5, third from top) was significantly depressed in *Cyp1a1*$^{-/-}$ mice—and, to a lesser extent, *Cyp1a1/1b1*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ mice. At benzo[*a*]pyrene 12.5 mg/kg/day, *Cyp1a1*$^{-/-}$ mice also showed a significantly lower hematocrit. At benzo[*a*]pyrene 1.25 mg/kg/day, no effects were seen in any of the six genotypes.

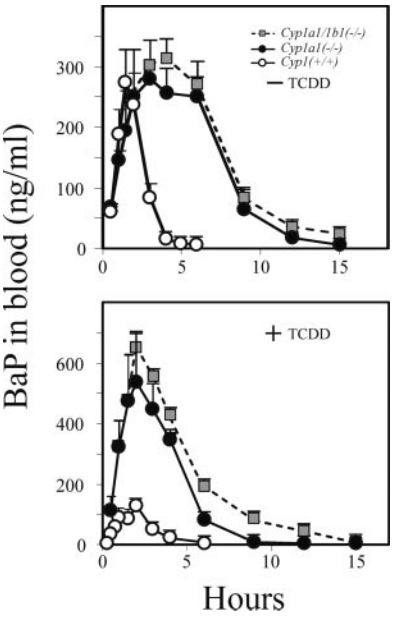

Fig. 3. Comparison of benzo[*a*]pyrene clearance between *Cyp1*$^{+/+}$ wild-type (○), *Cyp1a1*$^{-/-}$ (●), and *Cyp1a1/1b1*$^{-/-}$ (▨) mice, after stomach gavage of benzo[*a*]pyrene in corn oil at 15 mg/kg: top, without TCDD pretreatment; bottom, with TCDD pretreatment (10 μg/kg body weight; in corn oil vehicle 48 h before benzo[*a*]pyrene). n = 6 to 8 animals/group. Values in this figure are given as means ± S.D. To avoid clutter, the clearance curves for *Cyp1b1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mice are not shown but were not significantly different ($P > 0.05$) from that of *Cyp1*$^{+/+}$ wild-type mice.

TABLE 1

Comparison of benzo[*a*]pyrene pharmacokinetic parameters in *Cyp1*$^{+/+}$ wild-type mice, the three single-knockout mouse lines, and the two double-knockout mouse lines

Individual mice (n = 3) were repeatedly tested at 9 or 10 time points for each of the six genotypes, after stomach gavage of benzo[*a*]pyrene (15 mg/kg in corn oil). TCDD pretreatment denotes 10 μg/kg body weight (in corn oil vehicle) 48 h before benzo[*a*]pyrene administration by gavage. *Cyp1b1*$^{-/-}$, *Cyp1a2*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mice were not significantly different from *Cyp1*$^{+/+}$ wild-type mice. All parameters of *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice were significantly different from *Cyp1*$^{+/-}$ wild-type animals at $P < 0.01$ and $P < 0.005$, respectively. Values are given as means ± S.D.

	No TCDD Pretreatment			TCDD Pretreatment			Benzo[*a*]pyrene 125 mg/kg/day × 5 days[a]
	AUC	Clearance	Half-Life	AUC	Clearance	Half-Life	
	min × g/ml	*ml/min/kg*	*min*	*min × g/ml*	*ml/min/kg*	*min*	*ng/ml*
Cyp1$^{+/+}$	32 ± 9.1	490 ± 134	31 ± 11	16 ± 5.0	984 ± 310	16 ± 3.2	2.2 ± 1.6
Cyp1a1$^{-/-}$	121 ± 8	123 ± 10	61 ± 13	119 ± 34	135 ± 40	63 ± 10	56 ± 5.7
Cyp1a2$^{-/-}$	37 ± 11	418 ± 94	36 ± 6.2	26 ± 4.9	629 ± 59	18 ± 3.4	1.5 ± 0.51
Cyp1b1$^{-/-}$	53 ± 13	327 ± 92	46 ± 2.9	11 ± 4.5	979 ± 280	14 ± 4.1	5.6 ± 9.1
Cyp1a1/1b1$^{-/-}$	140 ± 78	106 ± 75	70 ± 41	151 ± 80	115 ± 50	77 ± 49	165 ± 46
Cyp1a2/1b1$^{-/-}$	35 ± 5.2	452 ± 76	48 ± 12	17 ± 7.2	999 ± 270	13 ± 7.1	4.0 ± 3.3

[a] Whole-blood benzo[*a*]pyrene concentrations, after 5 days of benzo[*a*]pyrene feeding at the 125 mg/kg/day dose. Individual mice (n = 3) were tested between 10 and 11 AM each morning on days 3, 4, and 5; these values were similar on all 3 days and are averaged together, thus confirming that by days 3 to 5, the benzo[*a*]pyrene had already reached steady-state levels in the blood.

Hemoglobin levels (not shown) paralleled the hematocrit values.

An enhanced methemoglobin level is a sign of oxidative stress in the intact animal. At oral benzo[*a*]pyrene 125 mg/kg/day (Fig. 5, bottom), methemoglobin levels compared with *Cyp1*$^{+/+}$ wild-type were strikingly increased in *Cyp1a1*$^{-/-}$ mice and also significantly increased in *Cyp1a1/1b1*$^{-/-}$ mice. At oral benzo[*a*]pyrene 12.5 mg/kg/day, methemoglobin levels remained abnormally high and statistically significant in *Cyp1a1*$^{-/-}$ compared with wild-type; no significant differences from wild-type were seen in the other genotypes. At the 1.25 mg/kg/day dose, no differences in any of the six groups were detectable. Hence, elevated plasma AST and ALT levels, decreased hematocrit, and increased methemoglobin concentrations in *Cyp1a1/1b1*$^{-/-}$ were intermediate between those in *Cyp1a1*$^{-/-}$ and in *Cyp1*$^{+/+}$ mice (Fig. 5).

At oral benzo[*a*]pyrene 125 mg/kg/day and compared with *Cyp1*$^{+/+}$ wild-type mice (Fig. 6, top two), the *Cyp1a1*$^{-/-}$ knockout showed a striking decrease in percentage of lymphocytes, combined with a significant increase in percentage of neutrophils. At oral benzo[*a*]pyrene 12.5 mg/kg/day compared with *Cyp1*$^{+/+}$ wild-type mice, *Cyp1a1*$^{-/-}$ and *Cyp1b1*$^{-/-}$ mice showed significant increases in percentage of neutrophils and decreases in percentage of lymphocytes; at oral benzo[*a*]pyrene 1.25 mg/kg/day compared with *Cyp1*$^{+/+}$ wild-type mice, *Cyp1b1*$^{-/-}$ mice continued to show significant increases in percentage of neutrophils and decreases in

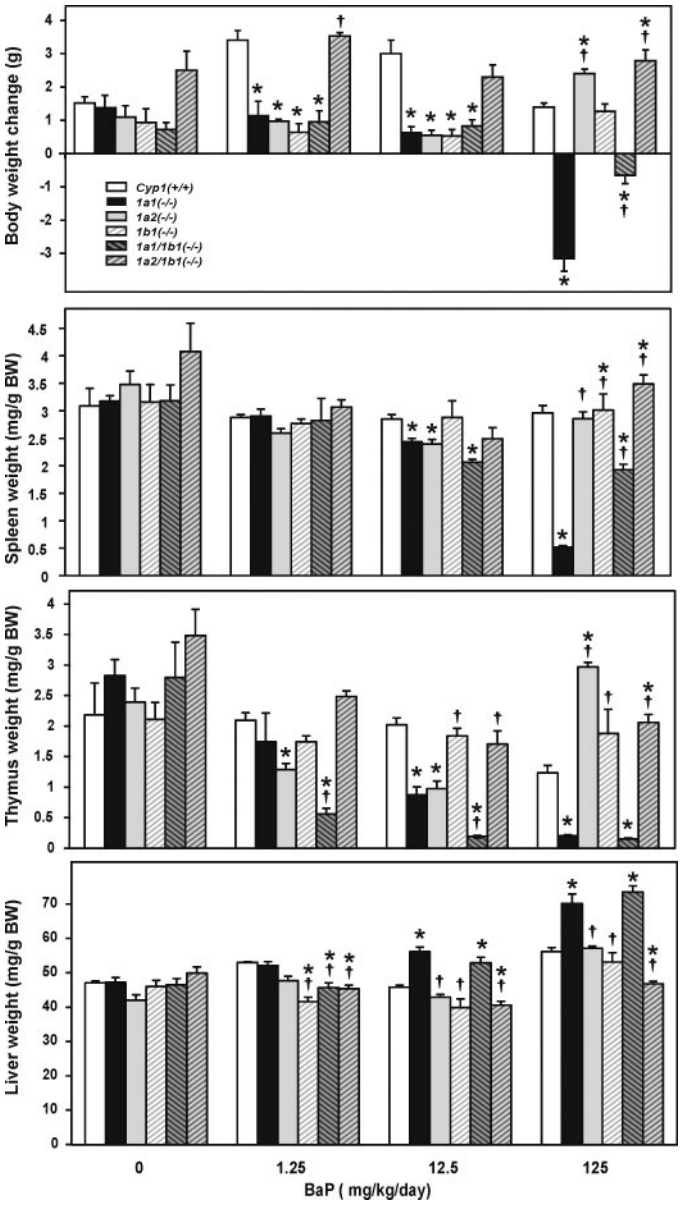

Fig. 4. Comparison of the effects of oral benzo[*a*]pyrene (125, 12.5, or 1.25 mg/kg/daily) for 18 days in the six genotypes under study: percentage change in body weight (top) and spleen (second from top), thymus (third from top), and liver (bottom) wet weights per kilogram of body weight. Order of the bars, from left to right, is consistent throughout the entire article: *Cyp1*$^{+/+}$, *Cyp1a1*$^{-/-}$, *Cyp1a2*$^{-/-}$, *Cyp1b1*$^{-/-}$, *Cyp1a1/1b1*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ mice. $n = 6$ animals/group. Values are given as means ± S.E.M. *, significant difference ($P < 0.05$) relative to the *Cyp1*$^{+/+}$ wild-type. †, significant difference ($P < 0.05$) relative to *Cyp1a1*$^{-/-}$ knockout mice.

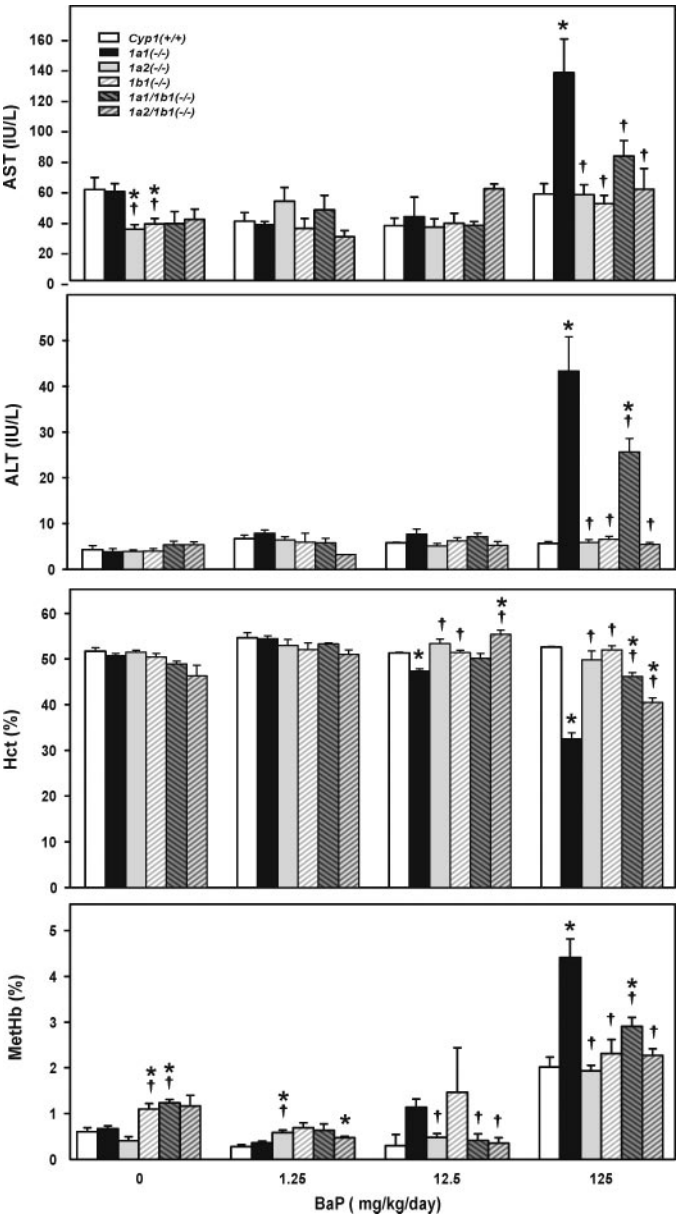

Fig. 5. Comparison of the effects of oral benzo[*a*]pyrene (125 mg/kg/daily) for 18 days in the six genotypes under study: plasma AST and ALT activities (top two rows) and hematocrit and percent methemoglobinemia (bottom two rows). Order of the bars, from left to right, and statistical comparisons, are the same as those in Fig. 4. $n = 6$ animals/group.

percentage of lymphocytes. Doses of oral benzo[*a*]pyrene 10 or 100 times lower than the 125 mg/kg/day thus seem to uncover some interesting subtle differences in the peripheral lymphocyte/neutrophils ratios not seen at the highest benzo[*a*]pyrene dose; this same finding is true of benzo[*a*]pyrene-DNA adducts (described under *Benzo[a]pyrene-DNA Adducts*).

Effects of Oral Benzo[*a*]pyrene on Bone Marrow. Compared with wild-type mice treated with oral benzo[*a*]pyrene 125 mg/kg/day for 18 days (Fig. 6, bottom), *Cyp1a1*$^{-/-}$ showed an overall loss of cellularity in the marrow. Megakaryocyte density (semiquantitative 1+ to 4+) was also significantly ($P = 0.003$) decreased in *Cyp1a1*$^{-/-}$ mice but not in the other five genotypes; in quantitative differential counts of marrow ($n = 12$), *Cyp1a1*$^{-/-}$ showed a significant ($P = 0.035$) decrease of eosinophils, compared with that in wild-type and the other four genotypes. Megakaryocytes and eosinophils are two components of the myeloid line.

Histology of the bone marrow (Fig. 7) showed dramatic hypocellularity in oral benzo[*a*]pyrene-treated *Cyp1a1*$^{-/-}$, compared with *Cyp1*$^{+/+}$ wild-type mice (Fig. 7, top two). Benzo[*a*]pyrene-treated *Cyp1a1*$^{-/-}$ showed decreases in the myeloid and erythroid cell lines, hypercondensation of the nuclei, an increase in Howell-Jolly bodies, and a slight increase in number of micronuclei. The other four benzo[*a*]pyrene-treated genotypes—including the *Cyp1b1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice—displayed no significant decreases in cellularity. In mice receiving the 12.5 or 1.25 mg/kg/day benzo[*a*]pyrene dose for 18 days, no significant changes in cellularity were found in any of the genotypes (data not shown).

Benzo[*a*]pyrene-DNA Adducts. The relevance of benzo[*a*]pyrene-DNA adducts associated with benzo[*a*]pyrene-induced CYP1A1-mediated tissue damage, mutagenesis, teratogenesis, and cancer is well established (Pelkonen and Nebert, 1982; Nebert, 1989; Conney et al., 1994; Knize et al., 1999; Miller and Ramos, 2001). At the oral benzo[*a*]pyrene 125 mg/kg/day dose for 18 days (Fig. 8, top) and compared with *Cyp1*$^{+/+}$ wild-type mice, *Cyp1a1*$^{-/-}$ mice showed slightly more benzo[*a*]pyrene-DNA adducts in liver and very significantly higher adduct levels in small intestine, spleen

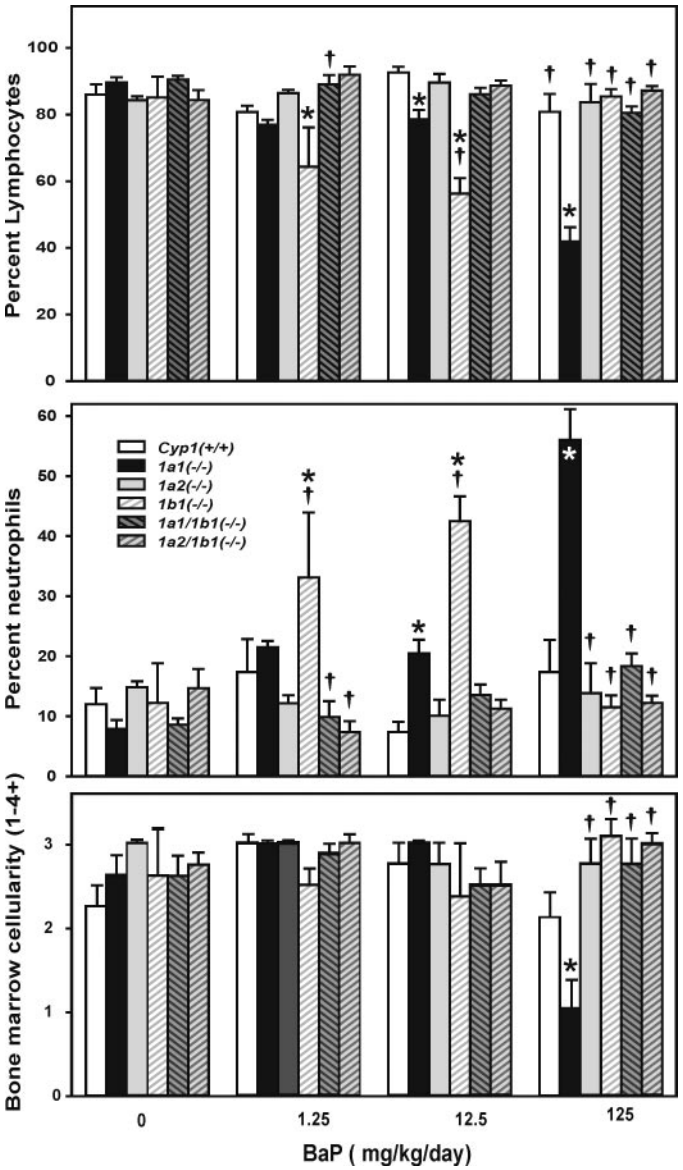

Fig. 6. Comparison of the effects of oral benzo[*a*]pyrene (125 mg/kg/daily) for 18 days in the six genotypes under study: peripheral blood lymphocytes and neutrophils (top two rows) and bone marrow relative cellularity (bottom row). Order of the bars, from left to right, and statistical comparisons, are the same as that in Fig. 4. $n = 6$ animals/group.

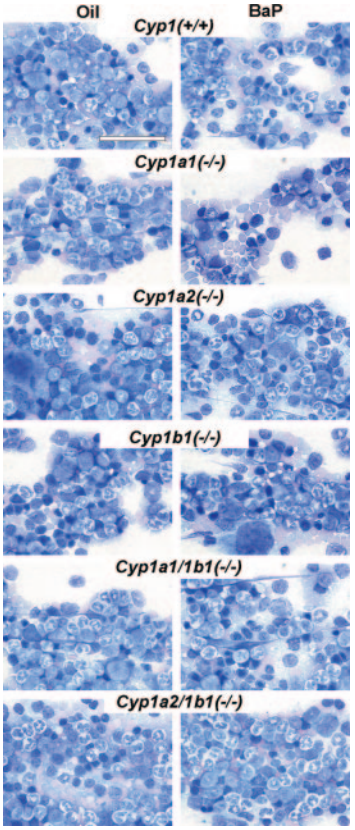

Fig. 7. Histology of bone marrow, comparing control with the effects of oral benzo[*a*]pyrene on the six genotypes in the present study. The benzo[*a*]pyrene-treated *Cyp1a1*$^{-/-}$ knockout shows striking hypocellularity compared with the other five benzo[*a*]pyrene-treated genotypes and all six control genotypes. Consistent with hypocellularity seen in *Cyp1a1*$^{-/-}$ mice, the myeloid and erythroid lines demonstrated increased nuclear hypercondensation, and there were an abnormally high number of Howell-Jolly bodies. Oral benzo[*a*]pyrene treatment was 125 mg/kg/day for 18 days. Scale bar, 20 μm.

and bone marrow. Both *Cyp1a2*$^{-/-}$ and *Cyp1b1*$^{-/-}$ mice showed significantly more adducts in small intestine but fewer in bone marrow. *Cyp1a1/1b1*$^{-/-}$ showed significantly fewer adducts in small intestine. The relative levels of benzo[*a*]pyrene-DNA adducts—comparing *Cyp1*$^{+/+}$ with *Cyp1a1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice, especially in spleen and bone marrow—parallel quite closely the relative magnitude of immunotoxicity described above in the three genotypes.

At the oral benzo[*a*]pyrene 12.5 mg/kg/day dose for 18 days (Fig. 8, *bottom*) and compared with *Cyp1*$^{+/+}$ mice, *Cyp1a1*$^{-/-}$ mice showed significantly more benzo[*a*]pyrene-DNA adducts in liver, spleen, and bone marrow but not small intestine. *Cyp1a1/1b1*$^{-/-}$ showed significantly higher adducts in liver, spleen, and marrow and fewer adducts in small intestine.

Benzo[*a*]pyrene-DNA Adduct Patterns. We found more or less unique patterns and levels of benzo[*a*]pyrene-DNA adducts among liver, small intestine, spleen, and bone marrow from all six of the genotypes (Fig. 9). In liver, there were at least nine distinct spots (putative benzo[*a*]pyrene-DNA adducts) in *Cyp1a1*$^{-/-}$ mice, compared with only five in the *Cyp1*$^{+/+}$ wild-type mouse; the patterns seen in the *Cyp1a2*$^{-/-}$ and *Cyp1a2/1b1*$^{-/-}$ were similar, with at least eight distinct spots. The *Cyp1b1*$^{-/-}$ pattern was different, but most similar to the wild-type pattern. The *Cyp1a1/1b1*$^{-/-}$ pattern was similar to the *Cyp1a1*$^{-/-}$ pattern but showed perhaps three additional spots. In small intestine, there were fewer spots in *Cyp1a1*$^{-/-}$ than in *Cyp1*$^{+/+}$ mice; the *Cyp1a1/1b1*$^{-/-}$ pattern had by far the fewest number of spots.

In spleen, the *Cyp1a1*$^{-/-}$ pattern (with at least eight spots) was similar to that seen in liver and intestine, whereas the *Cyp1*$^{+/+}$ pattern was very different from that seen in liver or intestine, showing only three spots. The *Cyp1a1/1b1*$^{-/-}$ pattern was similar to those of *Cyp1a2*$^{-/-}$, *Cyp1b1*$^{-/-}$, and *Cyp1a2/1b1*$^{-/-}$ but also showed several unique spots. In bone marrow, the *Cyp1a1*$^{-/-}$ pattern showed at least six spots, whereas the wild-type pattern showed only three faint spots. The *Cyp1a1/1b1*$^{-/-}$ pattern was more similar to the *Cyp1*$^{+/+}$ pattern but with slightly greater intensity of the spots. Intriguingly, the relative intensity of "the most prominent spot" (in the lower left quadrant of spleen and marrow; Fig. 9) seems to be proportionate to the relative degree of immunotoxicity: *Cyp1a1*$^{-/-}$ ⋙ *Cyp1a1/1b1*$^{-/-}$ > *Cyp1*$^{+/+}$ = *Cyp1a2*$^{-/-}$ = *Cyp1b1*$^{-/-}$ = *Cyp1a2/1b1*$^{-/-}$ treated with oral benzo[*a*]pyrene for 18 days; this spot is also seen in liver and small intestine but showed a similar intensity among the six genotypes.

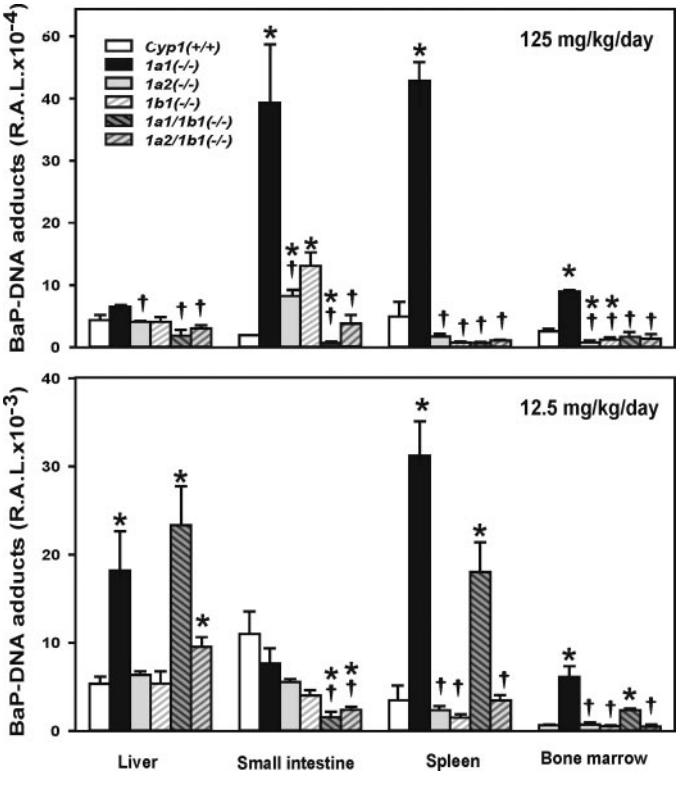

Fig. 8. Comparison of benzo[*a*]pyrene-DNA adduct formation in liver, small intestine, spleen, and bone marrow from the six genotypes under study after 18 days of oral benzo[*a*]pyrene at two doses: 125 mg/kg/day daily (top) and 12.5 mg/kg/day daily (bottom). Order of the bars, from left to right, is the same as that in Fig. 4. n = 4 animals/group. R.A.L., relative adduct levels, as detailed under *Materials and Methods*. Note that the labeling on the ordinate differs by 1 order of magnitude between the top and bottom panels.

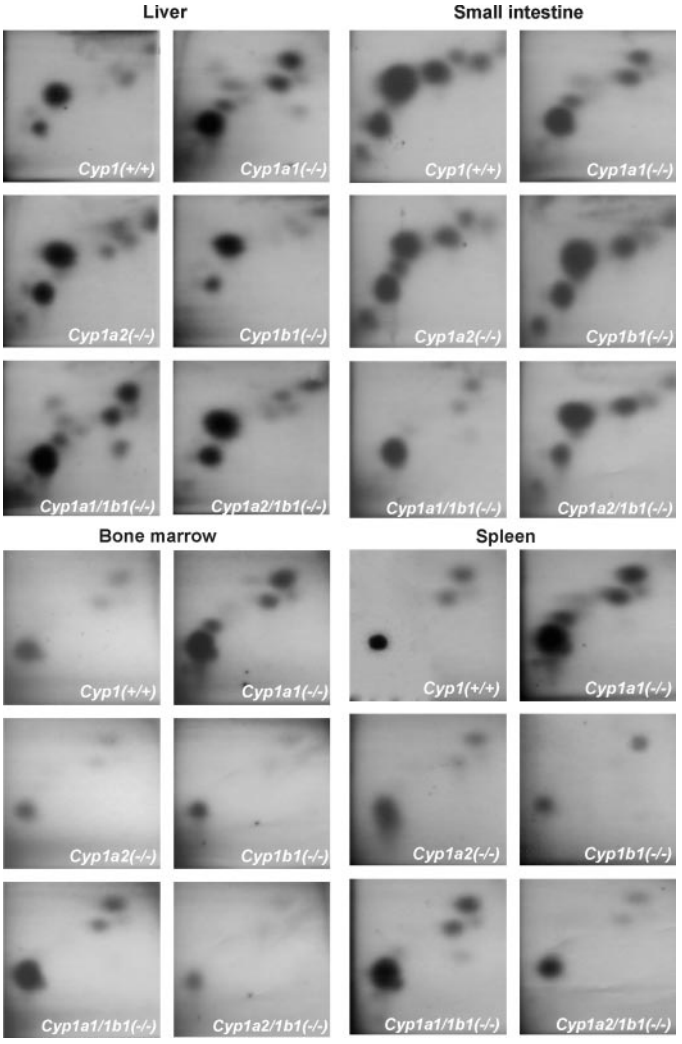

Fig. 9. Autoradiograms after two-dimensional chromatography of the benzo[*a*]pyrene-DNA adducts in liver, small intestine, spleen, and bone marrow from the six genotypes under study, after oral benzo[*a*]pyrene (125 mg/kg/day) for 18 days. Each is a representative blot of an adduct extract from a tissue that had been tested for benzo[*a*]pyrene-DNA adducts, combined from individual mice (n = 4 per group).

Because of the low amounts of adducts in ^{32}P-postlabeling experiments, mass spectroscopic analysis of "the most prominent spot", and perhaps some of the unique benzo[*a*]pyrene-DNA adducts (i.e., found in one genotype but not the other), is not technically feasible. However, the possibility exists that DNA repair products that correspond to these unique adducts could be detected in urine. This awaits future investigation.

Discussion

In the present study we have demonstrated that, compared with *Cyp1*$^{+/+}$ wild-type animals, mice having the genetic ablation of the *Cyp1a1* gene and receiving oral benzo[*a*]pyrene daily for 18 days exhibit striking weight loss, atrophy of the spleen and thymus, and increased liver weight (Fig. 4); elevated plasma AST and ALT levels, lowered hematocrit, and higher methemoglobin levels (Fig. 5); elevated peripheral blood neutrophils combined with leukocytopenia (Fig. 6); hypocellularity of the bone marrow (Figs. 6 and 7); higher benzo[*a*]pyrene-DNA adduct levels in liver, but strikingly higher adduct levels in the small intestine, spleen, and bone marrow (Fig. 8); and differences in benzo[*a*]pyrene-DNA adduct patterns and intensities (Fig. 9). In *Cyp1a1/1b1*$^{-/-}$ mice—which lack total-body CYP1B1, in combination with the absence of total-body CYP1A1—these parameters of tissue damage and benzo[*a*]pyrene-DNA adducts are in some cases markedly diminished. In stark contrast, all of these parameters are very similar in wild-type, *Cyp1a2*$^{-/-}$ and *Cyp1b1*$^{-/-}$ single-knockout, and *Cyp1a2/1b1*$^{-/-}$ double-knockout mice. We conclude that oral benzo[*a*]pyrene-inducible CYP1A1, presumably in small intestine and perhaps liver, is far more important in benzo[*a*]pyrene detoxication than in metabolic activation. In addition, these convergent data (with the three single- and two double-knockout lines) strongly suggest that basal and inducible CYP1B1, in immune tissues such as spleen and bone marrow, is more responsible for the metabolic activation of benzo[*a*]pyrene than for detoxication. This is in agreement with earlier studies showing that PAH-inducible CYP1B1 is detectable in bone marrow (Heidel et al., 1998; Galvan et al., 2003, 2005). Finally, we conclude that the role of basal or inducible CYP1A2, in liver or intestine, seems to play a negligible role during all of the observed oral benzo[*a*]pyrene-induced tissue damage.

CYP1B1 is known to predominate in formation of the diol-epoxide for 7,12-dimethylbenzo[*a*]anthracene (DMBA), whereas CYP1B1 function is similar to CYP1A1 function in activation of the benzo[*a*]pyrene diol-epoxide (Galvan et al., 2005). The fact that *Cyp1b1*-null mice are more resistant to DMBA-induced hematotoxicity than *Cyp1*$^{+/+}$ wild-type mice (Heidel et al., 1998) is consistent with the differential effects on bone marrow seen by DMBA versus benzo[*a*]pyrene (Galvan et al., 2006). However, the fact that *Cyp1b1*$^{-/-}$ mice are more resistant than wild-type mice to PAH-induced marrow damage whereas *Cyp1a1*$^{-/-}$ mice are far more sensitive than wild-type mice to PAH-induced marrow damage reflects two factors detailed in the experimental results of this study. First, in the absence of CYP1A1, benzo[*a*]pyrene accumulates in the blood, and the internal organs undergo long-term exposure to higher levels of benzo[*a*]pyrene. In the role of benzo[*a*]pyrene clearance, CYP1A1 is much more important than the other CYP1 family members. Second, CYP1B1 present in immune cells potentiates benzo[*a*]pyrene toxicity. Is metabolic potentiation an inherent function of CYP1B1? Data from this study do not answer this question, because body burden is always relatively low in the presence of inducible CYP1A1. To answer this question, we have generated conditional *Cyp1a1*$^{-/-}$ mice in which CYP1A1 can be preferentially eliminated only in the major organs involved in benzo[*a*]pyrene clearance (i.e., intestine and liver). With these mice, we should be able to assess the role of CYP1A1 and CYP1B1 at similar benzo[*a*]pyrene body burdens; such experiments will be the subject of future reports.

Lack of Correlation between Benzo[*a*]pyrene-Induced Tissue Damage and Benzo[*a*]pyrene Total-Body Burden. Clearance of benzo[*a*]pyrene (or any drug) is the volume of plasma from which the compound is eliminated from the body per unit of time per unit of body weight (Table 1). Clearance can also be calculated from a particular organ, instead of the whole body; e.g., "renal clearance" denotes the volume of plasma from which the chemical or drug is eliminated (or extracted) per unit of time as it passes through the kidneys. Likewise, "hepatic clearance" refers to the volume of plasma that is cleared per unit of time as it passes through the liver. If one sums all organ clearances, the result will be total-body clearance. In most cases, total-body clearance is calculated directly from a concentration-versus-time curve, after intravenous administration, by calculating the constant of elimination (k) for a first-order kinetics process and by calculating the apparent volume of distribution of the chemical. Total-body clearance is the best parameter by which to estimate true elimination of a given compound, because it takes into account not only the pure "elimination" processes of metabolism and excretion but also accounts for the volume of distribution of the compound throughout the entire body.

We had concluded previously that the effects of wasting, immunotoxicity, and DNA adduct formation are closely associated in *Cyp1a1*$^{-/-}$ mice with large increases in the AUC, decreases in the benzo[*a*]pyrene clearance rate, and a longer half-life of benzo[*a*]pyrene excretion (Uno et al., 2004a). However, in *Cyp1a1/1b1*$^{-/-}$ mice in which oral benzo[*a*]pyrene-induced damage has been reversed—because of the total-body absence of CYP1B1 as well as CYP1A1—the increases in the AUC, decreases in the benzo[*a*]pyrene clearance rate, and increases in half-life are even larger compared with that in *Cyp1a1*$^{-/-}$ mice (Table 1; Fig. 3). The magnitude of benzo[*a*]pyrene-induced tissue damage is therefore not correlated with benzo[*a*]pyrene total-body burden. This is an excellent example in the fields of pharmacokinetics and toxicokinetics: plasma clearance (or presumed total-body clearance) is not necessarily a good predictor of what might be occurring in any specific target tissue—with regard to the presence or absence of toxicity caused by any particular drug or other environmental toxicant.

Effects of Benzo[*a*]pyrene on Thymic Atrophy and Liver Hypertrophy Differ from Those on All Other Parameters. Compared with the effects seen in oral benzo[*a*]pyrene-treated *Cyp1a1*$^{-/-}$ knockout versus *Cyp1*$^{+/+}$ wild-type mice, the wild-type phenotype was more or less restored in benzo[*a*]pyrene-treated *Cyp1a1/1b1*$^{-/-}$ mice—except for thymus weight and liver weight (Fig. 4). Thymic atrophy (Staples et al., 1998; Hundeiker et al., 1999; Laiosa et al., 2003; Tomita et al., 2003; Nohara et al., 2005) and hepatocyte hypertrophy often measured by liver weight/total body

weight (Uno et al., 2004b) are two cardinal signs of long-term AHR activation. Thymic atrophy and hepatocyte hypertrophy are just as severe in *Cyp1a1/1b1*$^{-/-}$ as in *Cyp1a1*$^{-/-}$ mice (Fig. 4). Oral benzo[*a*]pyrene for 18 days obviously is able to continuously activate AHR, similar to what a single dose of TCDD is able to do: even though benzo[*a*]pyrene is metabolized many times more rapidly than TCDD, daily benzo[*a*]pyrene in the diet would serve to persistently activate the AHR. Thus, the presence or absence of CYP1B1 metabolism in the thymus or liver is independent of oral benzo[*a*]pyrene-induced thymic atrophy or hepatocyte hypertrophy, whereas it seems likely that long-term activation of the AHR is responsible for these tissue-specific effects.

Altered *Cyp1* Gene Expression in Oral Benzo[*a*]-pyrene-Treated Single- and Double-Knockout Mouse Lines. Strikingly altered CYP1 mRNA levels (Fig. 1), coupled with CYP1 protein levels (Fig. 2), were sometimes observed in oral benzo[*a*]pyrene-treated mice lacking one or more of the *Cyp1* genes. Two possible explanations must be considered. First, pharmacokinetics: changes in the tissue distribution and concentration of benzo[*a*]pyrene might lead to altered levels of CYP1 induction. Second, there might be as-yet-unappreciated compensatory changes at the *Cyp1* transcriptional, post-transcriptional, or post-translational level.

Compared with wild-type mice having inducible CYP1A1, a lack of the *Cyp1a1* gene resulted in large increases in CYP1B1 levels in all four tissues examined, and increased CYP1A2 in liver only. The oral benzo[*a*]pyrene-treated *Cyp1a1*$^{-/-}$ mouse clears benzo[*a*]pyrene from the body much more slowly than *Cyp1*$^{+/+}$ mice (Uno et al., 2004a). The observations in Figs. 1 and 2 thus are consistent with more benzo[*a*]pyrene, in these cell types, available for causing CYP1 induction via the AHR.

Compared with benzo[*a*]pyrene-treated wild-type mice, genetic ablation of the *Cyp1a2* gene led to significant decreases in CYP1A1 in liver and marrow only and decreased CYP1B1 in marrow only. Moreover, absence of the *Cyp1b1* gene resulted in large decreases in CYP1A1 and lowered CYP1A2 in all four tissues examined. There seems to be no satisfactory explanation for these observations in terms of pharmacokinetics; these effects might reflect compensatory changes in the genome or that benzo[*a*]pyrene metabolism by CYP1A2, or especially tissue-specific CYP1B1, produce the inducing chemical.

In *Cyp1a2*$^{-/-}$ mice, no effects on hepatic CYP1A1 mRNA levels, constitutive or inducible by intraperitoneal TCDD, were found (Liang et al., 1997). This observation, at variance with the present findings, might reflect differences in chemical stability or metabolism between benzo[*a*]pyrene and TCDD and/or differences in route-of-administration between oral and intraperitoneal treatment of the inducer.

Absence of both the *Cyp1a1* and *Cyp1b1* genes (Figs. 1 and 2) caused increased CYP1A2 in liver and small intestine, and especially spleen. These findings might be consistent with higher levels of the benzo[*a*]pyrene inducer, combined with available *Cyp1a2* gene inducibility, in these cell types.

Finally, absence of both the *Cyp1a2* and *Cyp1b1* genes caused increased CYP1A1 in spleen but no significant changes in liver, small intestine or bone marrow (Figs. 1 and 2). These data might be explained by higher benzo[*a*]pyrene levels in spleen, because of the absence of CYP1B1, again combined with available *Cyp1a1* gene inducibility, in the spleen cell types; in contrast, absence of CYP1B1 in marrow did not result in significant CYP1A1 increases in *Cyp1a2/1b1*$^{-/-}$ mice; these data thus suggest a compensatory genomic change in spleen CYP1A1

Benzo[*a*]pyrene-DNA Adduct Formation in Absence of Both CYP1A1 and CYP1B1. Among the best enzymes for the oxidation of benzo[*a*]pyrene to reactive metabolites are CYP1A1 and CYP1B1 (Shimada et al., 2001; Nebert et al., 2004). However, at the 12.5 mg/kg/day benzo[*a*]pyrene dose, *Cyp1a1/1b1*$^{-/-}$ mice exhibit substantial increases in benzo-[*a*]pyrene-DNA adduct formation over wild-type mice in liver and spleen, and a slight but significant increase in bone marrow (Fig. 8). What other enzymes in these tissues might be involved in such metabolism? PAHs such as benzo-[*a*]pyrene are known to induce one or more forms of CYP2A (Kimura et al., 1989), CYP2C (Fisslthaler et al., 1999), and CYP2S1 (Rivera et al., 2002) enzymes; whether these enzymes, in turn, are able to metabolize benzo[*a*]pyrene to oxygenated reactive intermediates is not yet known. PAHs such as benzo[*a*]pyrene are known to be potent substrates for cyclooxygenase-2 (Marnett et al., 1982) and CYP2C (Meehan et al., 1988), and CYP1A2 is able to oxygenate benzo-[*a*]pyrene to some limited degree (Shimada et al., 1996).

Conclusions

We have shown in the present study several lines of convergent data with oral benzo[*a*]pyrene, suggesting that inducible CYP1A1—probably in both intestine and liver—is most important in benzo[*a*]pyrene detoxication. Moreover, CYP1B1 in spleen and in bone marrow seems to be responsible for metabolic activation of benzo[*a*]pyrene, which results in immune damage in the genetic absence of CYP1A1. We also show evidence that both thymus atrophy and hepatocyte hypertrophy are independent of CYP1B1 metabolism; rather, these tissue-specific damages reflect long-term activation of the aryl hydrocarbon receptor. Finally, we have demonstrated that the magnitude of immune damage in *Cyp1b1*$^{-/-}$ and *Cyp1a1/1b1*$^{-/-}$ mice can be independent of plasma benzo[*a*]pyrene total-body burden and clearance.

It has been proposed (Nebert et al., 2004; Uno et al., 2004a) that phase I (CYP1) metabolism and phase II (conjugation) metabolism might be either tightly or loosely "coupled", depending on the tissue and cell type. Studies herein support further our hypothesis that oral benzo[*a*]pyrene-induced CYP1A1 in intestine and probably liver is beneficial to the mouse, whereas benzo[*a*]pyrene metabolism by CYP1B1 in the spleen and bone marrow is detrimental to the mouse. Using tissue- and cell-type-specific conditional knockouts of these various *Cyp1* genes, we hope in the near future to understand more fully this delicate balance between CYP1A1 and CYP1B1 during their functions of activation and detoxication.

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References

Alexandrov K, Cascorbi I, Rojas M, Bouvier G, Kriek E, and Bartsch H (2002) *CYP1A1* and *GSTM1* genotypes affect benzo[*a*]pyrene DNA adducts in smokers' lung: comparison with aromatic/hydrophobic adduct formation. *Carcinogenesis* **23:**1969–1977.

Buesen R, Mock M, Seidel A, Jacob J, and Lampen A (2002) Interaction between metabolism and transport of benzo[*a*]pyrene and its metabolites in enterocytes. *Toxicol Appl Pharmacol* **183:**168–178.

Buters JT, Sakai S, Richter T, Pineau T, Alexander DL, Savas U, Doehmer J, Ward JM, Jefcoate CR, and Gonzalez FJ (1999) Cytochrome P450 CYP1B1 determines susceptibility to 7, 12-dimethylbenz[*a*]anthracene-induced lymphomas. *Proc Natl Acad Sci USA* **96:**1977–1982.

Conney AH, Chang RL, Jerina DM, and Wei SJ (1994) Studies on the metabolism of benzo[*a*]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite. *Drug Metab Rev* **26:**125–163.

Dalton TP, Dieter MZ, Matlib RS, Childs N, Shertzer HG, Genter MB, and Nebert DW (2000) Targeted knockout in the *Cyp1a1* gene does not alter hepatic constitutive expression of other genes in the mouse [*Ah*] battery. *Biochem Biophys Res Commun* **267:**184–189.

Dey A, Jones JE, and Nebert DW (1999) Tissue- and cell type-specific expression of cytochrome P450 1A1 and cytochrome P450 1A2 mRNA in the mouse localized by in situ hybridization. *Biochem Pharmacol* **58:**525–537.

Ellard S, Mohammed Y, Dogra S, Wolfel C, Doehmer J, and Parry JM (1991) Use of genetically engineered V79 Chinese hamster cultures expressing rat liver CYP1A1, 1A2 and 2B1 cDNAs in micronucleus assays. *Mutagenesis* **6:**461–470.

Farin FM and Omiecinski CJ (1993) Regiospecific expression of cytochromes P-450 and microsomal epoxide hydrolase in human brain tissue. *J Toxicol Environ Health* **40:**317–335.

Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, and Busse R (1999) Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature (Lond)* **401:**493–497.

Foster JR, Idle JR, Hardwick JP, Bars R, Scott P, and Braganza JM (1993) Induction of drug-metabolizing enzymes in human pancreatic cancer and chronic pancreatitis. *J Pathol* **169:**457–463.

Galvan N, Jaskula-Sztul R, MacWilliams PS, Czuprynski CJ, and Jefcoate CR (2003) Bone marrow cytotoxicity of benzo[*a*]pyrene is dependent on CYP1B1 but is diminished by Ah receptor-mediated induction of CYP1A1 in liver. *Toxicol Appl Pharmacol* **193:**84–96.

Galvan N, Page TJ, Czuprynski CJ, and Jefcoate CR (2006) Benzo[*a*]pyrene and 7,12-dimethylbenzo[*a*]anthracene differentially affect bone marrow cells of the lymphoid and myeloid lineages. *Toxicol Appl Pharmacol*, in press.

Galvan N, Teske DE, Zhou G, Moorthy B, MacWilliams PS, Czuprynski CJ, and Jefcoate CR (2005) Induction of CYP1A1 and CYP1B1 in liver and lung by benzo-[*a*]pyrene and 7,12-dimethylbenzo[*a*]anthracene do not affect distribution of polycyclic hydrocarbons to target tissue: role of AHR and CYP1B1 in bone marrow cytotoxicity. *Toxicol Appl Pharmacol* **202:**244–257.

Garcia-Falcon MS, Gonzalez-Amigo S, Lage-Yusty MA, Lopez de Alda Villaizan MJ, and Simal-Lozano J (1996) Determination of benzo[*a*]pyrene in lipid-soluble liquid smoke (LSLS) by HPLC-FL. *Food Addit Contam* **13:**863–870.

Guengerich FP, Chun YJ, Kim D, Gillam EM, and Shimada T (2003) Cytochrome P450 1B1: a target for inhibition in anti-carcinogenesis strategies. *Mutat Res* **523–524:**173–182.

Heidel SM, Czuprynski CJ, and Jefcoate CR (1998) Bone marrow stromal cells constitutively express high levels of cytochrome P450 1B1 that metabolize 7,12-dimethylbenzo[*a*]anthracene. *Mol Pharmacol* **54:**1000–1006.

Hem A, Smith AJ, and Solberg P (1998) Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret and mink. *Lab Anim* **32:**364–368.

Hundeiker C, Pineau T, Cassar G, Betensky RA, Gleichmann E, and Esser C (1999) Thymocyte development in Ah-receptor-deficient mice is refractory to TCDD-inducible changes. *Int J Immunopharmacol* **21:**841–859.

Kim HS, Kwack SJ, and Lee BM (2000) Lipid peroxidation, antioxidant enzymes and benzo[*a*]pyrene-quinones in the blood of rats treated with benzo[*a*]pyrene. *Chem Biol Interact* **127:**139–150.

Kimura S, Kozak CA, and Gonzalez FJ (1989) Identification of a novel P450 expressed in rat lung: cDNA cloning and sequence, chromosome mapping and induction by 3-methylcholanthrene. *Biochemistry* **28:**3798–3803.

Knize MG, Salmon CP, Pais P, and Felton JS (1999) Food heating and the formation of heterocyclic aromatic amine and polycyclic aromatic hydrocarbon mutagens/carcinogens. *Advanc Exp Med Biol* **459:**179–193.

Laiosa MD, Wyman A, Murante FG, Fiore NC, Staples JE, Gasiewicz TA, and Silverstone AE (2003) Cell proliferation arrest within intrathymic lymphocyte progenitor cells causes thymic atrophy mediated by the aryl hydrocarbon receptor. *J Immunol* **171:**4582–4591.

Liang HC, Li H, McKinnon RA, Duffy JJ, Potter SS, Puga A, and Nebert DW (1996) *Cyp1a2*$^{-/-}$ null mutant mice develop normally, but show deficient drug metabolism. *Proc Natl Acad Sci USA* **93:**1671–1676.

Liang HC, McKinnon RA, and Nebert DW (1997) Sensitivity of CYP1A1 mRNA inducibility is the same in *Cyp1a2*$^{+/+}$ wild-type and *Cyp1a2*$^{-/-}$ null mutant mice. *Biochem Pharmacol* **54:**1127–1131.

Marnett LJ, Panthananickal A, and Reed GA (1982) Metabolic activation of 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene during prostaglandin biosynthesis. *Drug Metab Rev* **13:**235–247.

Meehan RR, Speed RM, Gosden JR, Rout D, Hutton JJ, Taylor BA, Hilkens J, Kroezen V, Hilgers J, Adesnik M, et al. (1998) Chromosomal organization of the cytochrome P450-2C gene family in the mouse: a locus associated with constitutive aryl hydrocarbon hydroxylase. *Proc Natl Acad Sci USA* **84:**2662–2666.

Miller KP and Ramos KS (2001) Impact of cellular metabolism on the biological effects of benzo[*a*]pyrene and related hydrocarbons. *Drug Metab Rev* **33:**1–35.

Nebert DW (1989) The *Ah* locus: genetic differences in toxicity, cancer, mutation and birth defects. *Crit Rev Toxicol* **20:**153–174.

Nebert DW, Dalton TP, Okey AB, and Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* **279:**23847–23850.

Nebert DW, Dalton TP, Stuart GW, and Carvan MJ III (2000a) "Gene-swap knock-in" cassette in mice to study allelic differences in human genes. *Ann NY Acad Sci* **919:**148–170.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, and Dalton TP (2000b) Role of the aromatic hydrocarbon receptor and [*Ah*] gene battery in the oxidative stress response, cell cycle control and apoptosis. *Biochem Pharmacol* **59:**65–85.

Nohara K, Pan X, Tsukumo S, Hida A, Ito T, Nagai H, Inouye K, Motohashi H, Yamamoto M, Fujii-Kuriyama Y, et al. (2005) Constitutively active aryl hydrocarbon receptor expressed specifically in T-lineage cells causes thymus involution and suppresses the immunization-induced increase in splenocytes. *J Immunol* **174:**2770–2777.

Pelkonen O and Nebert DW (1982) Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol Rev* **34:**189–222.

Pfeifer GP, Denissenko MF, Olivier M, Tretyakova N, Hecht SS, and Hainaut P (2002) Tobacco smoke carcinogens, DNA damage and p53 mutations in smoking-associated cancers. *Oncogene* **21:**7435–7451.

Puga A, Maier A, and Medvedovic M (2000) The transcriptional signature of dioxin in human hepatoma HepG2 cells. *Biochem Pharmacol* **60:**1129–1142.

Rivera SP, Saarikoski ST, and Hankinson O (2002) Identification of a novel dioxin-inducible cytochrome P450. *Mol Pharmacol* **61:**255–259.

Robinson JR, Felton JS, Levitt RC, Thorgeirsson SS, and Nebert DW (1975) Relationship between "aromatic hydrocarbon responsiveness" and the survival times in mice treated with various drugs and environmental compounds. *Mol Pharmacol* **11:**850–865.

Ryu DY, Levi PE, Fernandez-Salguero P, Gonzalez FJ, and Hodgson E (1996) Piperonyl butoxide and acenaphthylene induce cytochrome P450 1A2 and 1B1 mRNA in aromatic hydrocarbon-responsive receptor knock-out mouse liver. *Mol Pharmacol* **50:**443–446.

Sesardic D, Pasanen M, Pelkonen O, and Boobis AR (1990) Differential expression and regulation of members of the cytochrome P450 IA gene subfamily in human tissues. *Carcinogenesis* **11:**1183–1188.

Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP, and Sutter TR (1996) Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res* **56:**2979–2984.

Shimada T, Oda Y, Gillam EM, Guengerich FP, and Inoue K (2001) Metabolic activation of polycyclic aromatic hydrocarbons and other procarcinogens by cytochromes P450 1A1 and P450 1B1 allelic variants and other human cytochromes P450 in *Salmonella typhimurium* NM2009. *Drug Metab Dispos* **29:**1176–1182.

Staples JE, Murante FG, Fiore NC, Gasiewicz TA, and Silverstone AE (1998) Thymic alterations induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are strictly dependent on aryl hydrocarbon receptor activation in hemopoietic cells. *J Immunol* **160:**3844–3854.

Tatemichi M, Nomura S, Ogura T, Sone H, Nagata H, and Esumi H (1999) Mutagenic activation of environmental carcinogens by microsomes of gastric mucosa with intestinal metaplasia. *Cancer Res* **59:**3893–3898.

Tomita S, Jiang HB, Ueno T, Takagi S, Tohi K, Maekawa S, Miyatake A, Furukawa A, Gonzalez FJ, Takeda J, et al. (2003) T cell-specific disruption of aryl hydrocarbon receptor nuclear translocator (*Arnt*) gene causes resistance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced thymic involution. *J Immunol* **171:**4113–4120.

Uno S, Dalton TP, Derkenne S, Curran CP, Miller ML, Shertzer HG, and Nebert DW (2004a) Oral exposure to benzo[*a*]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol Pharmacol* **65:**1225–1237.

Uno S, Dalton TP, Shertzer HG, Genter MB, Warshawsky D, Talaska G, and Nebert DW (2001) Benzo[*a*]pyrene-induced toxicity: paradoxical protection in *Cyp1a1*$^{-/-}$ knockout mice having increased hepatic benzo[*a*]pyrene-DNA adduct levels. *Biochem Biophys Res Commun* **289:**1049–1056.

Uno S, Dalton TP, Sinclair PR, Gorman N, Wang B, Smith AG, Miller ML, Shertzer HG, and Nebert DW (2004b) *Cyp1a1*$^{-/-}$ male mice: protection against high-dose TCDD-induced lethality and wasting syndrome and resistance to intra-hepatocyte lipid accumulation and uroporphyria. *Toxicol Appl Pharmacol* **196:**410–421.

Walker NJ, Gastel JA, Costa LT, Clark GC, Lucier GW, and Sutter TR (1995) Rat CYP1B1: an adrenal cytochrome P450 that exhibits sex-dependent expression in livers and kidneys of TCDD-treated animals. *Carcinogenesis* **16:**1319–1327.

Wei C, Caccavale RJ, Kehoe JJ, Thomas PE, and Iba MM (2001) CYP1A2 is expressed along with CYP1A1 in the human lung. *Cancer Lett* **171:**113–120.

Winer J, Jung CK, Shackel I, and Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem* **270:**41–49.

Zhang QY, Dunbar D, and Kaminsky LS (2003) Characterization of mouse small intestinal cytochrome P450 expression. *Drug Metab Dispos* **31:**1346–1351.

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