Biochemical Pharmacology, Vol. 54, pp. 927–936, 1997.

ELSEVIER

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00268-2

Role of Nitroreductases but Not Cytochromes P450 in the Metabolic Activation of 1-Nitropyrene in the HepG2 Human Hepatoblastoma Cell Line

Kimberly J. Silvers, Letha H. Couch,† Ellen A. Rorke* and Paul C. Howard*†‡*

*Department of Environmental Health Sciences, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4940; and †National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079-9502, U.S.A.

ABSTRACT. 1-Nitropyrene is an environmental contaminant that is mutagenic in many prokaryotic and eukaryotic systems, including the hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) locus in the human hepatoma cell line HepG2. Metabolism and DNA adduct formation of [^{3}H]1-nitropyrene in the HepG2 were quantified to understand the role of nitroreduction and/or cytochrome P450-mediated C-oxidation of 1-nitropyrene in DNA adduct formation and mutagenicity. In uninduced HepG2 cells, 10 μM [^{3}H]1-nitropyrene was metabolized principally by nitroreduction to 1-aminopyrene (516 pmol/24 hr/10^6 cells), and by cytochrome P450-mediated C-oxidation to K-region *trans*-dihydrodiols (37 pmol/24 hr/10^6 cells), 1-nitropyren-3-ol (51 pmol/24 hr/10^6 cells), and 1-nitropyren-6-ol and 1-nitropyren-8-ol (77 pmol/24 hr/10^6 cells). Pretreatment of the HepG2 cells for 24 hr with 5 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in a complete change in the metabolism of [^{3}H]1-nitropyrene, with 1-nitropyren-6-ol and 1-nitropyren-8-ol formation (449 pmol/24 hr/10^6 cells) being 80-fold greater than 1-aminopyrene formation (6 pmol/24 hr/10^6 cells). This increase in C-oxidation of 1-nitropyrene was consistent with increased levels of cytochrome P450 1A. The only DNA adduct detected using the ^{32}P-postlabeling assay in the HepG2 cells administered 1-nitropyrene was *N*-(2′-deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP). Induction of C-oxidative metabolism through TCDD treatment resulted in a concomitant decrease in dG-C8-AP formation. DNA adducts for oxidized 1-nitropyrene metabolites were not detected in the TCDD-treated HepG2 cells administered 1-nitropyrene, which indicates that cytochrome P450-mediated C-oxidative pathways are detoxification pathways in HepG2 cells. BIOCHEM PHARMACOL **54**;8:927–936, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 1-nitropyrene; HepG2; nitroreductase; DNA adducts; metabolism; cytochromes P450; metabolic activation

Nitro-PAHs§ are important environmental pollutants that have been detected from a wide variety of sources. Nitro-PAHs are generated as a result of fuel combustion [1, 2], and their emission from factories and automobiles results in the contamination of urban atmospheres and sediments [3–5]. In addition, these compounds are generated from sources in the home environment, such as kerosene heaters, wood smoke, and grilled foods [2, 6]. The *in situ* formation of nitro-PAH in rodent lungs following administration of pyrene and exposure to nitrogen dioxide gas has also been reported [7, 8]. Therefore, the risk of long-term exposure to nitro-PAHs needs to be addressed in humans.

1-Nitropyrene, a prototypical nitro-PAH, is mutagenic in several prokaryotic and eukaryotic systems [5, 9, 10]. One pathway for the bioactivation of 1-nitropyrene is through nitroreduction to the nitroso and hydroxylamino species, with the subsequent formation of a C8 deoxyguanosine adduct, dG-C8-AP [11]. This adduct is responsible for 1-nitropyrene mutagenicity in *Salmonella typhimurium* [11, 12], Chinese hamster ovary cells [13], and cultured human foreskin fibroblasts [14].

A second route for the metabolism of 1-nitropyrene is through cytochrome P450-mediated C-oxidation to non-K-region phenols (1-nitropyren-3-ol, 1-nitropyren-6-ol, and 1-nitropyren-8-ol), and K-region oxides (1-nitropyrene-4,5-oxide and 1-nitropyrene-9,10-oxide) [15–21]. The K-region oxides are further metabolized to *trans*-dihydrodiols (1-nitropyrene-4,5-diol and 1-nitropyrene-9,10-diol) via epoxide hydrolase, or rearrange to phenols (1-nitropy-

‡ Corresponding author: Paul C. Howard, Ph.D., HFT-110, Division of Biochemical Toxicology, National Center for Toxicological Research, Food and Drug Administration, Jefferson, AR 72079-9502. Tel. (870) 543-7672; FAX (870) 543-7136; E-mail: PHOWARD@nctr.fda.gov.

§ *Abbreviations:* nitro-PAH, nitrated polycyclic aromatic hydrocarbon; HGPRT, hypoxanthine-guanosine phosphoribosyl transferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; dG-C8-AP, *N*-(2′-deoxyguanosin-8-yl)-1-aminopyrene; MEM, minimal essential medium; HI-FBS, heat-inactivated fetal bovine serum; HBSS, Hanks' Balanced Salts Solution; SET, 0.25 M sucrose, 0.05 M Tris–HCl, pH 7.4; 3MC, 3-methylcholanthrene; C-OXID/N-REDUC, ratio of 1-nitropyrene C-oxidation to nitroreduction; dG-C8-ANP, *N*-(2′-deoxyguanosin-8-yl)-1-amino-(6 or 8)-nitropyrene; EROD, ethoxyresorufin O-deethylase; AHH, benzo[*a*]pyrene hydroxylase activity; and CAT, chloramphenicol acetyltransferase.

Received 15 August 1996; accepted 20 May 1997.

ren-4-ol, 1-nitropyren-5-ol, 1-nitropyren-9-ol, or 1-nitropyren-10-ol). Many of these metabolites have been detected *in vitro* and *in vivo*, and several are mutagenic in *S. typhimurium* [19, 22]. A difference exists in the 1-nitropyrene metabolites formed in rodents versus man. All species that have been tested, with the exception of human, preferentially metabolize 1-nitropyrene to 1-nitropyren-6-ol and 1-nitropyren-8-ol [15–21]; however, human liver microsomes preferentially metabolize 1-nitropyrene to 1-nitropyren-3-ol [23, 24]. Therefore, humans may be at a greater mutagenic risk from 1-nitropyrene, since 1-nitropyren-3-ol is considerably more mutagenic than 1-nitropyren-6-ol or 1-nitropyren-8-ol [22].

One limitation of *in vitro* metabolism and mutagenicity experiments is that few cultured cells express the complete complement of enzymes necessary for the activation and/or detoxification of procarcinogens or promutagens. The use of exogenous activation systems often results in the production of reactive intermediates outside the cell, followed by translocation of the reactive intermediate into the cell, and formation of DNA adducts within the cells. Similarly, highly purified reactive intermediates have been added to cells to determine "direct-acting mutagenicity" of chemicals. Both of these approaches are constrained by the generation of the reactive intermediate outside the cell membrane. We have sought to validate the HepG2 human hepatoblastoma cell line as a model for human hepatocellular metabolism of 1-nitropyrene. HepG2 cells retain many characteristics of a well-differentiated hepatocyte, including cholesterol transport [25], albumin and glycogen synthesis [26], low-density lipoprotein synthesis [26, 27], bile canaliculus formation [28], and bile acid synthesis [28]. HepG2 cells have also been shown to bioactivate several types of procarcinogens [29–33], and possess the ability to metabolize 1-nitropyrene through both cytochrome P450-mediated C-oxidation and nitroreduction [34]. In the following studies, we have used the HepG2 cell to examine the relative role of nitroreduction and cytochrome P450-mediated C-oxidation in the metabolic activation of 1-nitropyrene to DNA-reactive species.

MATERIALS AND METHODS

Materials

[^{3}H]1-Nitropyrene (1 Ci/mmol; >98% radiochemical purity) and 1-nitropyrene (99.5%; 0.5% dinitropyrenes) were obtained from Chemsyn, Inc. (Lenexa, KS). The 1-nitropyrene C-oxidized metabolites, 1-nitropyren-3-ol, 1-nitropyren-6-ol, and 1-nitropyren-8-ol, were isolated as previously described [35, 36]. 3MC was purchased from the Aldrich Chemical Co. (Milwaukee, WI), and TCDD from Alltech Associates Inc. (Deerfield, IL). Dexamethasone and triacetyloleandomycin were from the Sigma Chemical Co. (St. Louis, MO). Unless otherwise indicated, all other reagents were the best available grade.

Cell Culture

HepG2 cells (American Type Culture Collection, Rockville, MD) were grown in continuous culture in minimal essential medium containing 10 U/mL penicillin, 10 μg/mL streptomycin, and 2 mM L-glutamine (MEM), and 10% (v/v) heat-inactivated (57°, 30 min) fetal bovine serum (MEM + 10% HI-FBS) [37]. The cells were maintained at 37° under 5% CO_2 and high humidity, and were subcultured at 1:2 splits every 3–4 days. All cell culture reagents were from Gibco (Grand Island, NY).

Metabolic Studies in Cell Culture

Inducers of metabolic enzymes, 3MC or TCDD, were added at the indicated concentrations (see text below) to HepG2 cells seeded at a density of 1×10^6 cells/100 mm^2 plate. The media containing the inducers was removed at the times indicated in the text, and changed to MEM + 2% HI-FBS. [^{3}H]1-Nitropyrene was added to a final concentration of 10 μM, and the cells were incubated with [^{3}H]1-nitropyrene for up to 24 hr. The medium was collected, the cells were washed with HBSS, and the monolayer was removed with 0.25% trypsin-EDTA. The medium and cells were combined and stored at −20°. A 1-mL aliquot of the cells and medium was incubated overnight at 37° following the addition of sodium acetate to 0.1 M, pH 5.1, 15 U/mL aryl sulfatase (Type V; Sigma), and 1500 U/mL β-glucuronidase (Type IXA; Sigma). Proteinase K (Sigma) was added to a final concentration of 1 mg/mL and incubated for another hour at 37°. Then samples were extracted twice with 1 mL chloroform, and the chloroform extracts were combined and dried under nitrogen. The [^{3}H]1-nitropyrene and its metabolites were dissolved in methanol and subjected to HPLC analysis as described below.

Preparation of HepG2 Cell Post-Mitochondrial Supernatant Fractions (S12)

HepG2 cells were removed from 100 mm^2 plates with a rubber policeman, combined, centrifuged at 275 *g* for 5 min, washed once with HBSS, and then resuspended in SET. The suspension was sonicated (Bransonic Cell Disrupter) and centrifuged at 12,000 *g* for 20 min. Aliquots of the HepG2 S12 were stored at −80°. Protein concentrations were determined by the method of Lowry *et al.* [38].

Metabolic Studies Using HepG2 S12

Samples were incubated under nitrogen at 37° in capped 13 × 100 mm test tubes in a final volume of 1 mL containing 8 U/mL glucose oxidase, 10 mM glucose, 40 U/mL catalase, 10 μM [^{3}H]1-nitropyrene, 0.05 M potassium phosphate, pH 7.4, 3 mM $MgCl_2$, 4 mM glucose-6-phosphate, and 0.1 U/mL glucose-6-phosphate dehydrogenase. Following addition of HepG2 S12 at 1 mg/mL, the tubes were purged with nitrogen, capped, and preincubated

at 37° for 5 min. Then NADP was added to 1 mM to initiate NADPH-dependent nitroreduction. Reactions were terminated after 60 min by the addition of an equal volume of chloroform:methanol (2:1). Following thorough mixing, the tubes were centrifuged at 180 *g* for 10 min, and the chloroform was removed. The aqueous component was reextracted with 1 mL chloroform, and the two chloroform fractions were combined and dried under nitrogen. Samples were then subjected to HPLC analysis as described below.

Quantitation of DNA Adducts

HepG2 cells were incubated with or without inducers (3MC, 2.5 μM; TCDD, 0.05 pM to 5 nM) for 24 hr. The media were changed to MEM + 2% HI-FBS, and 10 μM [^{3}H]1-nitropyrene was added for up to 24 hr. The medium was removed, the cells were washed with HBSS, and the monolayer was removed using 0.25% trypsin-EDTA. The cells were washed once with PBS, and resuspended in PBS. The DNA was purified from the cells using an anion-exchange column (Qiagen Corp., LaJolla, CA) following an overnight incubation of the DNA at 50° with 0.5 mg/mL proteinase K in 0.05 M Tris–HCl, pH 7, 1% sodium dodecyl sulfate. The DNA was precipitated with 0.5 M NaCl and 50% ethanol (−20°), washed with 70% ethanol, and resuspended in 5 mM bis-Tris–HCl, pH 7.1, 1 mM EDTA. The purity and concentration of the DNA were determined spectrophotometrically.

DNA adducts were quantified using the ^{32}P-postlabeling technique [39–41]. Typically, 1 μg of DNA from each sample was used for ^{32}P-postlabeling. DNA samples and standards were added to 10 mM sodium succinate buffer (pH 6.0), with 5 mM $CaCl_2$, and hydrolyzed with 10 μg spleen phosphodiesterase (Type II, Sigma) and 10 μg micrococcal nuclease (*Staphylococcus aureus*, Sigma) in a total volume of 10 μL at 37° for 3 hr. Following the addition of 40 μL containing 3.3 mM tetrabutylammonium chloride and 33 mM ammonium formate (pH 3.5), the samples were extracted twice with 50 μL *n*-butanol (water-saturated). The *n*-butanol extracts were combined, and back-extracted twice with 100 μL of water (*n*-butanol-saturated). To the *n*-butanol extracts were added 3 μL of 25 mM bicine, pH 9.5, 50 mM dithiothreitol, and the samples were dried *in vacuo* (Savant SpeedVac Concentrator, Farmingdale, NY). The nucleotides were dissolved in 25 μL of distilled water and stored at −70° overnight. The following day, 5 μL of a solution containing 180 mM bicine, pH 9.5, 60 mM $MgCl_2$, 60 mM dithiothreitol, 6 mM spermidine, 0.01 U T4 polynucleotide kinase (US Biochemicals, Cleveland, OH), and 200 μCi [γ-^{32}P]ATP (>2000 Ci/mmol, ICN Biochemicals, Costa Mesa, CA) was added to each sample, and incubated at 37° for 40 min. The kinase reaction was terminated by the addition of 0.03 U of potato apyrase (20 U/μL; Grade I, Sigma) to each tube, and the samples were incubated for another 30 min at 37°.

Each sample was applied to PEI-cellulose thin-layer chromatography sheets (10 × 10 cm; Scientific Absorbants, Atlanta, GA) and developed in the first direction in 0.9 M sodium phosphate, pH 6.8 (D1 buffer) onto a Whatman No. 1 paper wick (10 × 20 cm). The wick was removed, and the plates were washed with water, dried, and developed in 3.6 M lithium formate, 8.5 M urea, pH 3.5 (D2 buffer). After washing in water, the plates were rotated 90°, developed with 1.2 M lithium chloride, 0.5 M Tris–HCl, 8.0 M urea, pH 8.0 (D3 buffer), washed and dried, and then developed with D1 buffer onto a Whatman No. 1 paper wick (3 × 10 cm). For some experiments, plates were developed in the first direction in D1 buffer onto an 11 × 35 cm Whatman No. 1 paper wick, rotated 180°, and developed with D2 buffer. These directions are noted in the text. 5′-[^{32}P]Phospho-3′-phosphodeoxyribonucleotides were visualized by autoradiography, and the R_f values were compared with those of authentic dG-C8-AP. The ^{32}P-containing spots were removed and liquid scintillation counting was used to quantify the ^{32}P, comparing the samples to the dG-C8-AP standard.

The standard was made using [^{3}H]1-nitropyrene and *S. typhimurium* TA98. *S. typhimurium* TA98 cultures from overnight plates were harvested by centrifugation, washed with 0.067 M potassium phosphate, pH 7.4, resuspended in the same buffer at 100 mg/mL, and kept on ice. The bacteria were diluted to 10 mg/mL in 0.05 M potassium phosphate, pH 7.4, 0.08 M glucose, and a 9 μM concentration of the test compound ([^{3}H]1-nitropyrene, 1-nitropyren-3-ol, 1-nitropyren-6-ol, or 1-nitropyren-8-ol) was added. After shaking for 3 hr at 37°, the bacteria were harvested by centrifugation and washed, and the pellets were frozen at −75°. The DNA was isolated by the standard phenol/chloroform technique. ^{32}P-Postlabeling was performed as described above. The levels of dG-C8-AP were determined using the specific activity of the [^{3}H]1-nitropyrene. Calf-thymus DNA reacted with 1-nitropyrene-4,5-epoxide was provided by L. C. King (U.S. Environmental Protection Agency, Research Triangle Park, NC).

HPLC Analysis of [^{3}H]1-Nitropyrene Metabolism

Analysis of the metabolism of [^{3}H]1-nitropyrene was accomplished using previously described HPLC techniques [15–21, 23, 24]. Approximately 0.05 μCi of [^{3}H]1-nitropyrene and metabolites was injected onto a 10 μm μBondapak C_{18} reversed-phase column (0.34 × 25 cm; Waters Associates, Milford, MA) using a Varian 5500 HPLC (Varian Associates, Walnut Creek, CA) equipped with a Rheodyne 7125 injector. The metabolites were separated using a mobile phase at 1 mL/min of water:ethanol (54:46), increasing to water:ethanol (30:70) in 36 min (linear), followed by a linear increase to ethanol in 5 min. The ^{3}H was quantified using a flow-through scintillation counter (Flo-One β; Packard Instruments, Meridian, MA) with Ecolite "+" (ICN) as the scintillant at 2 mL/min. The counting efficiency for ^{3}H was approximately 34%. Non-radiolabeled 1-nitropyrene and metabolites were routinely co-injected, and retention was determined using UV-VIS absorbance [15–21, 23, 24].

TABLE 1. Comparison of [^{3}H]1-nitropyrene metabolism in untreated and TCDD-treated HepG2 cells

HepG2 pretreatment	1-Nitropyrene-4,5-diol and -9,10-diol	1-Nitropyren-6-ol and -8-ol	1-Nitropyren-3-ol	1-Aminopyrene	C-OXID/N-REDUC
	(pmol/24 hr/10^6 cells)				
None	37.47 ± 0.91	76.8 ± 2.9	50.5 ± 6.5	516.0 ± 32.5	0.32
TCDD, 5 nM	13.64 ± 2.09	449.1 ± 62.6	40.6 ± 4.6	6.3 ± 2.3	79.90

Cells were treated for 24 hr with TCDD or control media, the media was changed to MEM + 2% HI-FBS, and 10 μM [^{3}H]1-nitropyrene was added for 24 hr. The medium and cells were collected, and [^{3}H]1-nitropyrene metabolites were determined as described in Materials and Methods, using HPLC techniques. Results are expressed as the means ± SD for three separate experiments.

RESULTS

Effect of Cytochrome P450 Induction on [^{3}H]1-Nitropyrene Metabolism in HepG2 Cells

The incubation of HepG2 cells with [^{3}H]1-nitropyrene resulted in the production of 1-nitropyrene-4,5-diol, 1-nitropyrene-9,10-diol, 1-nitropyren-6-ol, 1-nitropyren-8-ol, 1-nitropyren-3-ol, and 1-aminopyrene (Table 1). This metabolism was linear for 24 hr (data not shown). In nonpretreated HepG2 cells, nitroreduction was responsible for the largest portion of [^{3}H]1-nitropyrene metabolism, with 1-aminopyrene being formed at three times the level of the C-oxidation metabolites [ratio of oxidative metabolites to 1-aminopyrene (C-OXID/N-REDUC) was 0.32]. In nonpretreated HepG2 cells, C-oxidation of 1-nitropyrene to 1-nitropyren-6-ol and 1-nitropyren-8-ol was preferred over the formation of 1-nitropyren-3-ol, 1-nitropyrene-4,5-diol, and 1-nitropyrene-9,10-diol. Pretreatment of the HepG2 cells with 5 nM TCDD 24 hr prior to the addition of [^{3}H]1-nitropyrene resulted in an approximately 6-fold increase in 1-nitropyren-6-ol and 1-nitropyren-8-ol formation, and an 82-fold decrease in 1-aminopyrene formation. The C-OXID/N-REDUC ratio increased from 0.32 in the untreated cells to 79.9 in TCDD-induced cells. The pretreatment of HepG2 cells with 2.5 μM 3MC, another cytochrome P450 inducer, resulted in a lower magnitude of induction of C-oxidation metabolism (3-fold) and a 6.6-fold decrease in aminopyrene formation (data not shown). With 3MC, the C-OXID/N-REDUC ratio increased from 0.6 in untreated HepG2 cells to 9 in 3MC-induced HepG2 cells (data not shown).

The induction of [^{3}H]1-nitropyrene C-oxidative metabolism in HepG2 cells was dependent on the TCDD concentration, with a maximum increase in 1-nitropyren-6-ol and 1-nitropyren-8-ol formation at 0.5 nM TCDD (Fig. 1). The increase in metabolism occurred solely in the formation of 1-nitropyren-6-ol and 1-nitropyren-8-ol, while the formation of 1-nitropyren-3-ol and 1-nitropyrene-4,5-diol and 1-nitropyrene-9,10-diol was unaffected. Specificity for 1-nitropyrene C-oxidation at the C6 and C8 positions is characteristic of cytochromes P450 1A, which are inducible by TCDD and 3MC [15, 23, 42].

The involvement of cytochrome P450 1A isozymes in the oxidative metabolism of 1-nitropyrene in HepG2 cells is further supported by α-napthoflavone inhibition of 1-nitropyrene metabolism. α-Naphthoflavone, a specific inhibitor of cytochromes P450 1A [43, 44], inhibited oxidative metabolism of 1-nitropyrene by 70% at 1 μM, 73% at 10 μM, and 93% at 100 μM (Fig. 2). The formation of 1-nitropyren-3-ol was affected only by the inclusion of 100 μM α-naphthoflavone, while the formation of 1-nitropy-

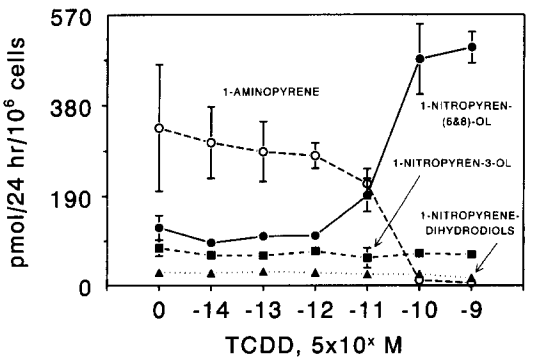

FIG. 1. Effect of TCDD pretreatment on the metabolism of [^{3}H]1-nitropyrene in HepG2 cells. HepG2 cells were incubated with the indicated concentrations of TCDD for 24 hr, washed, and then 10 μM [^{3}H]1-nitropyrene was added for another 24 hr. The cells and medium were collected, extracted as described in the text, and analyzed by HPLC techniques. The data are presented as the mean ± SD of three experiments. Where error bars are not present, the error is smaller than the symbol.

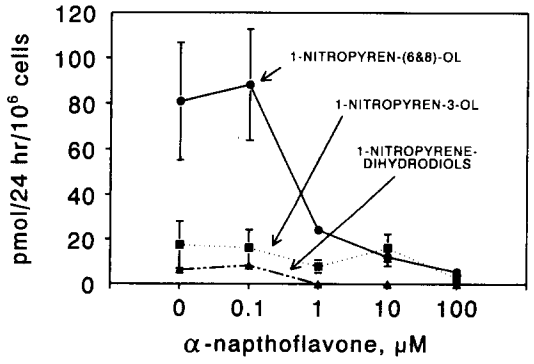

FIG. 2. Effect of α-naphthoflavone on the HepG2 metabolism of [^{3}H]1-nitropyrene. HepG2 cells were incubated with the indicated concentrations of α-naphthoflavone for 24 hr, washed, and then 10 μM [^{3}H]1-nitropyrene was added for another 24 hr. The cells and medium were collected, extracted as described in the text, and analyzed by HPLC techniques. The data are presented as the mean ± SD of three experiments. Where error bars are not present, the error is smaller than the symbol.

ren-6-ol, 1-nitropyren-8-ol, and the *trans*-dihydrodiols was inhibited by 1 μM α-naphthoflavone.

Previous studies have shown that cytochrome P450 3A isozymes are the predominant cytochrome P450s involved in human microsomal metabolism of 1-nitropyrene [23, 24]. To determine whether cytochrome P450 3A was involved in HepG2 cell metabolism of 1-nitropyrene, inducers and inhibitors of cytochrome P450 3A were incubated with HepG2 cells, and the effect on 1-nitropyrene metabolism was quantified. Dexamethasone is an inducer of several enzymes including cytochromes P450 3A *in vivo* and *in vitro* [45, 46]. Dexamethasone at concentrations from 1 to 10 μM did not induce C-oxidative metabolism of 1-nitropyrene, with or without charcoal pretreatment of the serum (data not shown). Triacetyloleandomycin is a mechanism-based inhibitor of cytochromes P450 3A [47], and has been shown to inhibit human liver microsomal 1-nitropyrene metabolism [24]. The incubation of HepG2 cells with 50–100 μM triacetyloleandomycin did not affect 1-nitropyrene metabolism (data not shown). Together, these results suggest that the cytochrome P450 3A subfamily does not participate in HepG2 cell metabolism of 1-nitropyrene.

The increase in 1-nitropyrene C-oxidative metabolism with both 3MC and TCDD treatment of HepG2 cells was accompanied by a concomitant decrease in 1-nitropyrene nitroreduction to 1-aminopyrene (Fig. 1). To determine whether this effect was due to the decreased expression of an enzyme involved in 1-nitropyrene nitroreductive metabolism, the subcellular location of the HepG2 1-nitropyrene nitroreductase was determined and the activity of the fraction in uninduced and TCDD-induced cells was measured. The 1-nitropyrene nitroreductase activity was associated with the cytosolic fraction (90% of total activity), while only 10% of the total 1-nitropyrene nitroreductase activity was present in the microsomal fraction (data not shown). HepG2 cells were then incubated, the cells were isolated and sonicated, and the nitroreductase activity of the subcellular fractions was measured under anaerobic conditions using 10 μM [^{3}H]1-nitropyrene as the substrate. Under these conditions, there was no difference in the ability of uninduced or TCDD-induced HepG2 S12 to nitroreduce [^{3}H]1-nitropyrene to 1-aminopyrene (2.7 ± 1.0 and 3.4 ± 2.3 pmol/min/mg protein, respectively).

These results suggest that the decrease in the ability of HepG2 cells to nitroreduce 1-nitropyrene under TCDD induction was not due to a change in the levels of nitroreductase enzyme(s), but was perhaps due to a competition of enzymes for limiting amounts of 1-nitropyrene when the oxidative pathway is induced. The decrease in nitroreduction of 10 μM 1-nitropyrene following TCDD treatment of the cells can be minimized by increasing the concentration of 1-nitropyrene (Fig. 3). HepG2 cells were preincubated for 24 hr with 5 nM TCDD, and incubated for another 24 hr with 10, 30, or 55 μM [^{3}H]1-nitropyrene. Increasing the [^{3}H]1-nitropyrene concentration increased the formation of [^{3}H]1-aminopyrene, indicating that the ability of the TCDD-induced HepG2 cells to nitroreduce 1-nitropyrene is due to limited levels of 1-nitropyrene in the cells.

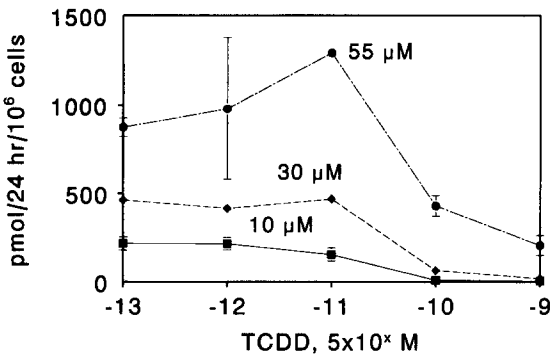

FIG. 3. Effect of [^{3}H]1-nitropyrene concentration on nitroreductive metabolism. Increasing concentrations of [^{3}H]1-nitropyrene were added to HepG2 cells to determine if the rate of nitroreduction was dependent on substrate concentration. The data are presented as the mean ± SD of three experiments. Where error bars are not present, the error is smaller than the symbol.

Effect of Cytochrome P450 Induction on 1-Nitropyrene DNA Adduct Formation in HepG2 Cells

We sought to determine the identity of the DNA adduct(s) formed by 1-nitropyrene in either uninduced or TCDD-pretreated HepG2 cells. Treatment of HepG2 cells with 1-nitropyrene resulted in the formation of one major DNA adduct, as shown in Fig. 4. The 1-nitropyrene DNA adduct from HepG2 cells co-migrated with the DNA adduct produced following incubation of 1-nitropyrene with *S. typhimurium*, which has been identified as dG-C8-AP [11]. The 1-nitropyrene used to generate the dG-C8-AP adduct in *S. typhimurium* was contaminated with 0.5% 1,6- or 1,8-dinitropyrene. This contamination resulted in the formation of a dinitropyrene DNA adduct(s) in the *S. typhimurium* DNA, which is indicated as dG-C8-ANP in Fig. 4; however, with the same 1-nitropyrene, no dG-C8-ANP adducts were detected in the DNA isolated from HepG2 cells exposed to 1-nitropyrene.

To generate DNA adduct standards for the phenolic metabolites of 1-nitropyrene, *S. typhimurium* TA98 cultures were treated with a 9 μM concentration of either 1-nitropyren-3-ol, 1-nitropyren-6-ol, or 1-nitropyren-8-ol, and the DNA was isolated. The migration of the corresponding DNA adducts of 1-nitropyren-3-ol, 1-nitropyren-6-ol, 1-nitropyren-8-ol, and 1-nitropyrene-4,5-oxide under our ^{32}P-postlabeling conditions was determined, and the results are indicated in Fig. 4. None of the phenolic DNA adducts co-migrated with dG-C8-AP. Identical DNA adducts were detected for each phenol when HepG2 cells were incubated separately with purified 1-nitropyren-3-ol, 1-nitropyren-6-ol, and 1-nitropyren-8-ol (data not shown). In neither the uninduced HepG2 cells nor the TCDD-treated HepG2 cells were DNA adducts of the phenols or epoxide detected following incubation with 1-nitropyrene.

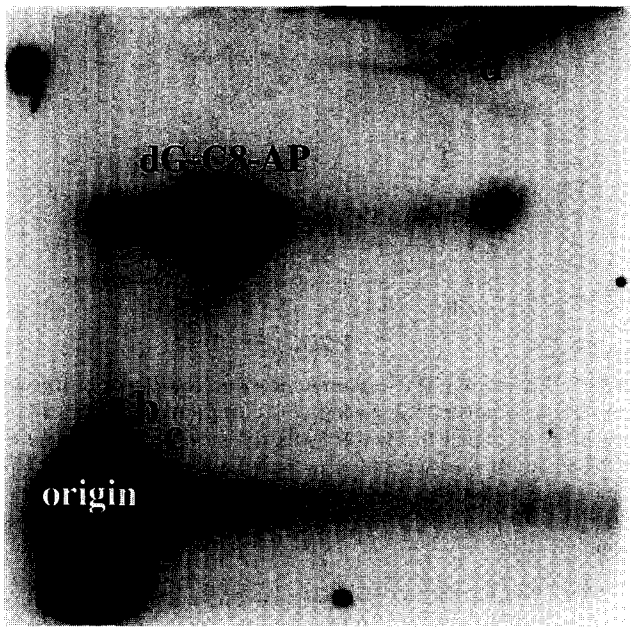

FIG. 4. DNA adduct formation following metabolism of 1-nitropyrene in HepG2 cells. DNA adduct formation was quantified by the ^{32}P-postlabeling technique as described in Materials and Methods. Shown is the chromatogram for HepG2 exposed to 1-nitropyrene, and the origin and location of dG-C8-AP are shown. The migration of the DNA adducts from 1,6-dinitropyrene (a), 1-nitropyren-3-ol (b), 1-nitropyren-6-ol (c), 1-nitropyren-8-ol (c), and 1-nitropyrene-4,5-oxide (d) is indicated in the figure.

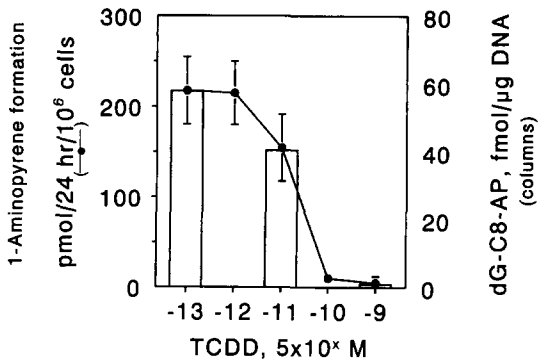

FIG. 5. Comparison of [^{3}H]1-nitropyrene nitroreduction and dG-C8-AP formation in HepG2 cells following TCDD pretreatment. Data (mean ± SD) are from three representative experiments measuring [^{3}H]1-nitropyrene nitroreduction and [^{3}H]1-nitropyrene DNA adduct formation following 24-hr TCDD induction and 24-hr exposure to 10 μM [^{3}H]1-nitropyrene. Where error bars are not present, the error is smaller than the symbol.

Pretreatment of HepG2 cells with TCDD resulted in an increase in the formation of 1-nitropyren-6-ol and 1-nitropyren-8-ol, and a decrease in the formation of 1-aminopyrene (Fig. 1). The DNA was isolated from TCDD-pretreated HepG2 cells incubated with [^{3}H]1-nitropyrene in order to determine the effect of this change in 1-nitropyrene metabolism on DNA adduct formation. The only DNA adduct detected by the ^{32}P-postlabeling method under these conditions was dG-C8-AP. TCDD treatment alone did not produce ^{32}P-postlabeling detectable DNA adducts (data not shown). TCDD pretreatment of the HepG2 cells resulted in a 99% reduction of the dG-C8-AP adduct in HepG2 cells (Fig. 5), which correlated with the loss of nitroreduction of [^{3}H]1-nitropyrene to 1-aminopyrene.

DISCUSSION

These experiments were initiated to determine the metabolism and DNA adduct formation of 1-nitropyrene in HepG2 cells, and to determine if the HepG2 cells might be a useful *in vitro* model for studying *in vivo* human metabolism and tumorigenicity of 1-nitropyrene.

We previously reported that HepG2 cells metabolize 1-nitropyrene to C-oxidized and nitroreduced metabolites, and that 1-nitropyrene is mutagenic in HepG2 cells [37]. We were unable at that time to determine the DNA adduct responsible for the mutagenicity in HepG2 cells at the HGPRT locus. In the present study, we determined that the only DNA adduct formed in HepG2 cells following treatment with 1-nitropyrene is dG-C8-AP. This DNA adduct is responsible for 1-nitropyrene mutagenicity in several other prokaryotic and eukaryotic cells [11–14], and evidently is responsible for the 1-nitropyrene mutagenicity reported in HepG2 cells [37]. Therefore, since dG-C8-AP formation is the result of 1-nitropyrene nitroreduction, the presence of a low C-OXID/N-REDUC ratio in cells or tissues would be consistent with an increased likelihood of DNA adduct formation and subsequent mutations.

The metabolism of 1-nitropyrene has been studied extensively using rodent and human microsomes, and in rodents *in vivo*. Most rodent species preferentially metabolize 1-nitropyrene to 1-nitropyren-6-ol and 1-nitropyren-8-ol, positions distal to the nitro group on pyrene. The ratio of 1-nitropyren-6-ol and 1-nitropyren-8-ol to 1-nitropyren-3-ol (proximal to nitro group) formation by rodent liver microsomes has been reported at 1.1 to 5.4 for Sprague–Dawley rats, CF1 mice, B6C3F1 mice, Hartley guinea pigs, Syrian golden hamsters, and New Zealand white rabbits [15–18]. Cytochromes P450 2B and 2C11 are predominantly responsible for untreated rat liver microsomal metabolism of 1-nitropyrene (Howard PC, unpublished observations); however, pretreatment of rats with CYP1A inducers such as 3MC, TCDD, or polychlorinated biphenyl mixtures (e.g. Aroclor-1254) results in increased metabolism of 1-nitropyrene by liver microsomes, and increased generation of 1-nitropyren-6-ol and 1-nitropyren-8-ol [15–18, 35, 42]. The metabolism of 1-nitropyrene by reconstituted rat CYP1A1 or 1A2 has not been reported; however, these data together indicate that although the cytochromes P450 1A are not involved in the C-oxidative metabolism in untreated rat livers, exposure of the rodents to CYP1A inducers results in CYP1A-dependent metabolism of 1-nitropyrene at the C6 and C8 positions forming 1-nitropyren-6-ol and 1-nitropyren-8-ol. These conclusions are consistent with our observations and those of others concerning the

role of CYP1A in 1-nitropyrene metabolism following treatment with CYP1A inducers [15–21, 35, 42, 48–50].

The metabolism of 1-nitropyrene by human cytochromes P450 has been reported with expressed proteins and with liver microsomal samples. Individual human cytochromes P450 were expressed in HepG2 cells using a *Vaccinia* virus expression system [23]. Of the twelve cytochromes P450 tested, cytochromes P450 3A3 and 3A4, and to a lesser extent 2B7, significantly increased the metabolism of 1-nitropyrene with the HepG2 cell lysates, with 1-nitropyren-3-ol being the major metabolite [23]. Cytochrome P450 1A2 did not display any catalytic activity towards 1-nitropyrene with the *Vaccinia* system. In additional studies using sixteen human liver microsomal samples, approximately 85% of the metabolism of 1-nitropyrene occurred through cytochrome P450 3A4, with 1-nitropyren-3-ol being the major metabolite [24]. Therefore, these studies demonstrate that human microsomal metabolism of 1-nitropyrene differs from that of rodents in that the cytochromes P450 3A are involved in the human samples and 1-nitropyren-3-ol is the predominant metabolite.

The levels of cytochrome P450 in untreated HepG2 cells are too low for detection by western or northern blot analysis [51, 52]; however, using reverse-transcription polymerase chain reaction, mRNAs for cytochrome P450 1A1, 1A2, and 3A3 have been detected in HepG2 cells [53]. Specific cytochrome P450 isozymes have also been detected in some studies by using inducers and/or growing the cells on specialized substrates using hormonally supplemented medium [54–56]. Using monoclonal human 3A antibodies and cDNA probes for CYP3A RNA, Schuetz *et al.* [57] showed that the only CYP3A isozyme present in HepG2 cells grown on Matrigel is 3A7, a fetal liver form. This enzyme was measured at levels of 17 pmol/mg microsomal protein, which was 10-fold lower than that detected in human liver. Cytochrome P450 3A7 is glucocorticoid-inducible and shares some substrate specificity with the adult forms of cytochromes P450 3A [58–62]; however, in HepG2 cells grown under our conditions, inducers and inhibitors of cytochrome P450 3A isozymes had little effect on 1-nitropyrene metabolism. This would indicate either that the 3A7 isozyme does not participate in 1-nitropyrene metabolism, as shown for *Vaccinia*-expressed human cytochrome P450 3A5 [23], or that the level of 3A7 expression in our system is too low to contribute significantly to 1-nitropyrene metabolism.

The cytochromes P450 in HepG2 cells are inducible by 3MC and TCDD, and are much less responsive to induction with phenobarbital [62–64]. The prototypical response used to measure CYP1A induction is an increase in either EROD activity or AHH activity. An increase in AHH activity from TCDD in HepG2 cells was initially shown by Cresteil *et al.* [65] who reported that the EC_{50} for TCDD induction was 1 nM. These results were confirmed by Labruzzo *et al.* [66] using similar concentrations of TCDD. Merchant *et al.* [67] demonstrated that a 100-fold increase in HepG2 cell EROD activity with 1 nM TCDD was accompanied by an increased CYP1A1 mRNA in HepG2 cells. While this study and another by McManus *et al.* [68] suggest that cytochrome P450 1A1 is the only 1A isozyme induced in HepG2 cells, a study by Fukuda *et al.* [53] has shown that CYP1A2 mRNA is detectable upon induction with 3MC. Doodstar *et al.* [62] have also suggested that both isozymes are induced by PAHs in HepG2 cells, since anti-cytochrome P450 1A1 antibodies inhibited 75% of activity in both untreated and benzanthracene-induced cells, while anti-cytochrome P450 1A2 antibodies inhibited EROD activity by 50% in HepG2 cell homogenates in untreated groups, and by 25% in benzanthracene-induced cells. Furthermore, transfection of the CYP1A2 promotor and CAT gene into HepG2 cells yielded a 2- to 3-fold induction of CAT activity using 2 nM 3MC [69]. These studies suggest that both isozymes are inducible in HepG2 cells, and the role of either in the 3MC- and TCDD-induced HepG2 cell metabolism of 1-nitropyrene cannot be established at this time.

The results from several studies, however, support the conclusion that CYP1A1 and not CYP1A2 is responsible for 1-nitropyrene C-oxidation in HepG2 and human cells. We determined that the rates of rabbit CYP1A1 and CYP1A2 catalyzed C-oxidation of 1-nitropyrene were 2.2 and 0.09 nmol/min/nmol P450, respectively [15]. A similar result was reported with a different nitro-PAH, 3-nitrofluoranthene, where the CYP1A1 and CYP1A2 catalyzed C-oxidation rates were 2.1 and 0.4 nmol/min/nmol P450, respectively [42]. Additionally, HepG2 cell lysates containing *Vaccinia*-expressed CYP1A2 did not catalyze the C-oxidation of 1-nitropyrene [23]. The role of CYP1A2 in human liver microsome C-oxidative metabolism must be minor since approximately 85% of human liver microsome metabolism of 1-nitropyrene is catalyzed by CYP3A4 [24]. Together these results suggest that the liver-specific CYP1A2 does not contribute to the C-oxidative metabolism of 1-nitropyrene; however, the CYP1A1 that is expressed in several extrahepatic tissues [70] could contribute to extrahepatic 1-nitropyrene C-oxidation.

1-Nitropyrene is an environmental contaminant that has shown marginal carcinogenic activity in rodents. Ohgaki *et al.* [71] originally reported 1-nitropyrene induced injection site sarcomas in adult male F344 rats; however, a later report indicated that the carcinogenicity was due to 0.8% contamination of the 1-nitropyrene with dinitropyrene isomers, and that administration of 40 mg of highly purified 1-nitropyrene was not tumorigenic [72]. 1-Nitropyrene was not tumorigenic when painted on 4- to 5-week-old Crl/CD-1(ICR)BR mice [73], and was not tumorigenic when peritoneally injected or skin-painted onto 7-week-old SENCAR mice [74]. 1-Nitropyrene did not produce tumors following subcutaneous injection in BALB/c mice [75], nor did it produce tumors when injected into the lungs of F344/DuCrj rats [76]. However, suprascapular injection of 1-nitropyrene in newborn S/D rats produced injection site histiocytomas in 28–32% of the rats, and mammary adenocarcinomas in the females [77, 78]. Both intraperitoneal

and subcutaneous injection of 1-nitropyrene into weanling female CD rats resulted in the development of mammary fibroadenomas [79], while liver carcinomas were detected in newborn B6C3F1 mice treated with 1-nitropyrene [80]. These studies suggest that 1-nitropyrene is tumorigenic when administered to newborn rodents; however, it is not tumorigenic when administered to adult rodents.

The liver cytochrome P450 content of rabbits does not increase until 4 weeks of age, and increases in the rat at day 15 [81–83]. This correlates with an increase in rat liver microsomal 1-nitropyrene C-oxidation at day 12 (Howard PC, unpublished observation). At this time, the ontogenic expression of the rodent nitroreductases has not been reported. Therefore, the reduced cytochrome P450 expression in the newborn (low C-OXID/N-REDUC) and the considerably higher cytochrome P450 expression in adult liver (high C-OXID/N-REDUC) offer one possible explanation for the difference in 1-nitropyrene tumorigenicity. In newborn rodents, low cytochrome P450 expression would allow more 1-nitropyrene to be reduced to the proximate carcinogenic form (hydroxylamine) than in the adult rodent where abundant cytochromes P450 are present. Therefore, as with the HepG2 cells, dG-C8-AP formation would be favored in the newborn and not in the adult rodent. While these studies suggest one explanation for the 1-nitropyrene tumorigenicity differences in newborn and adult rodents, further studies involving cytochrome P450 expression in newborn rats are required to substantiate this observation.

One of the goals of this study was to develop an *in vitro* model for the *in vivo* metabolism and mutagenicity of 1-nitropyrene. The TCDD-pretreated HepG2 may serve as a model for rodent metabolism of 1-nitropyrene, since cytochrome P450-mediated C-oxidation results predominantly in the formation of 1-nitropyren-6-ol and 1-nitropyren-8-ol [15–18]. The TCDD-pretreated HepG2 model may not be a good surrogate for human metabolism of 1-nitropyrene since human liver microsomal metabolism of 1-nitropyrene is through cytochrome P450 3A4, resulting in the formation of predominantly 1-nitropyren-3-ol [24]. In the present studies, 1-nitropyren-6-ol and 1-nitropyren-8-ol are predominantly formed in TCDD-pretreated HepG2. Our results have demonstrated that the formation of phenolic metabolites of 1-nitropyrene in the HepG2 does not result in DNA adduct formation, and only nitroreduction results in DNA adduct formation (Fig. 4). As a result, the TCDD-pretreated HepG2 may serve as a model for *in vivo* metabolism and mutagenicity, since the critical determinant in DNA adduct formation is the ratio of C-oxidation (detoxification) to nitroreduction (activation).

The authors would like to thank L. C. King, U.S. Environmental Protection Agency, for the 1-nitropyrene-4,5-oxide modified DNA. These studies were supported, in part, by PHS Grant ES-03648.

References

1. International Agency for Research on Cancer, Diesel and gasoline engine exhausts and some nitroarenes. *IARC Monogr Eval Carcinog Risks Hum* **46:** 1–418, 1989.
2. Lewtas J and Nishioka M, Nitroarenes: Their detection, mutagenicity and occurrence in the environment. In: *Nitroarenes: Occurrence, Metabolism and Biological Impact* (Eds. Howard PC, Hecht SS and Beland FA), pp. 73–84. Plenum Press, New York, 1990.
3. Beland FA, Heflich RH, Howard PC and Fu PP, The *in vitro* metabolic activation of nitro polycyclic aromatic hydrocarbons. In: *Polycyclic Hydrocarbons and Carcinogenesis* (Ed. Harvey RG), pp. 371–396. American Chemical Society, Washington, DC, 1985.
4. Zielinska B, Arey J and Atkinson R, The atmospheric formation of nitroarenes and their occurrence in ambient air. In: *Nitroarenes: Occurrence, Metabolism and Biological Impact* (Eds. PC Howard, SS Hecht and FA Beland), pp. 85–93. Plenum Press, New York, 1990.
5. Tokiwa H and Ohnishi Y, Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. *Crit Rev Toxicol* **17:** 23–60, 1986.
6. White CM, *Nitrated Polycyclic Aromatic Hydrocarbons*. Hurthig, Heidelberg, Germany, 1985.
7. Kanoh T, Fukuda M, Hayami E, Kinouchi T, Nishifuji K and Ohnishi Y, Nitro reaction in mice injected with pyrene during exposure to nitrogen dioxide. *Mutat Res* **245:** 1–4, 1990.
8. Kinouchi T, Kataoka K, Miyanishi K, Howard PC, Kanoh T and Ohnishi Y, Effect of nitrogen-dioxide exposure on formation of nitrated metabolites in animals treated with polycyclic aromatic hydrocarbons. *Environ Occupat Chem Hazards* **2:** 285–290, 1994.
9. Beland FA and Kadlubar FF, Metabolic activation and DNA adducts of aromatic amines and nitroaromatic hydrocarbons. In: *Chemical Carcinogenesis and Mutagenesis I* (Eds. Cooper CS and Grover PL), pp. 267–325. Springer, Berlin, 1990.
10. Rosenkranz HS and Mermelstein R, Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. *Mutat Res* **114:** 217–267, 1983.
11. Howard PC, Heflich RH, Evans FE and Beland FA, Formation of DNA adducts *in vitro* and in *Salmonella typhimurium* upon metabolic reduction of the environmental mutagen 1-nitropyrene. *Cancer Res* **43:** 2052–2056, 1983.
12. Heflich RH, Howard PC and Beland FA, 1-Nitrosopyrene: An intermediate in the metabolic activation of 1-nitropyrene to a mutagen in *Salmonella typhimurium* TA1538. *Mutat Res* **149:** 25–32, 1985.
13. Heflich RH, Fullerton NF and Beland FA, An examination of the weak mutagenic response of 1-nitropyrene in Chinese hamster ovary cells. *Mutat Res* **161:** 99–108, 1986.
14. Howard PC, Gerrard JA, Milo GE, Fu PP, Beland FA and Kadlubar FF, Transformation of normal human skin fibroblasts by 1-nitropyrene and 6-nitrobenzo[a]pyrene. *Carcinogenesis* **4:** 353–355, 1983.
15. Howard PC, Reed KA and Koop DR, Oxidative metabolism of 1-nitropyrene by rabbit liver microsomes and purified microsomal cytochrome P450 isozymes. *Cancer Res* **48:** 4261–4269, 1988.
16. Djurić Z, Fifer EK, Howard PC and Beland FA, Oxidative microsomal metabolism of 1-nitropyrene and DNA-binding of oxidized metabolites following nitroreduction. *Carcinogenesis* **7:** 1073–1079, 1986.
17. Rosenkranz HS and Howard PC, Structural basis of the activity of nitrated polycyclic aromatic hydrocarbons. In: *Carcinogenic and Mutagenic Effects of Diesel Engine Exhaust*

(Eds. Ishinishi I, Koizumi A, McClellan RO and Stöber W), pp. 141–168. Elsevier Science, Amsterdam, 1986.
18. Howard PC, DeMarco GJ, Consolo MC and McCoy GD, Differing effects of chronic ethanol consumption by mice on liver microsomal metabolism of xenobiotics: 1-nitropyrene, nicotine, aniline, and *N*-nitrosopyrrolidine. *Mol Toxicol* **1:** 177–189, 1987.
19. El-Bayoumy K and Hecht SS, Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. *Cancer Res* **43:** 3132–3137, 1983.
20. El-Bayoumy K and Hecht SS, Metabolism of 1-nitro[*U*-4,5,9,10-^{14}C]pyrene in the F344 rat. *Cancer Res* **44:** 4317–4322, 1984.
21. El-Bayoumy K, Reddy B and Hecht SS, Identification of ring oxidized metabolites of 1-nitropyrene in the feces and urine of germfree F344 rats. *Carcinogenesis* **5:** 1371–1373, 1984.
22. Consolo MC, Anders M and Howard PC, Mutagenicity of the phenolic microsomal metabolites of 3-nitrofluoranthene and 1-nitropyrene in strains of *Salmonella typhimurium*. *Mutat Res* **210:** 263–269, 1989.
23. Howard PC, Aoyama T, Bauer SL, Gelboin HV and Gonzalez FJ, The metabolism of 1-nitropyrene by human cytochromes P450. *Carcinogenesis* **11:** 1539–1542, 1990.
24. Silvers KJ, Chazinski T, McManus ME, Bauer SL, Gonzalez FJ, Gelboin HV, Maurel P and Howard PC, Cytochrome P-450 3A4 (nifedipine oxidase) is responsible for the C-oxidative metabolism of 1-nitropyrene in human liver microsomal samples. *Cancer Res* **52:** 6237–6243, 1992.
25. Faust RA, Cheung MC and Albers JJ, Secretion of cholesteryl ester transfer protein–lipoprotein complexes by human HepG2 hepatocytes. *Atherosclerosis* **77:** 77–82, 1989.
26. Wong SH, Fisher EA and Marsh JB, Effects of eicosapentaenoic and docosahexaenoic acids on apoprotein B mRNA and secretion of very low density lipoprotein in HepG2 cells. *Arteriosclerosis* **9:** 836–841, 1989.
27. Pinkerton FD, Spilman CH, Via DP and Schroepfer GJ Jr, Differing effects of three oxysterols on low density lipoprotein metabolism in HepG2 cells. *Biochem Biophys Res Commun* **193:** 1091–1097, 1993.
28. Everson GT and Polokoff MA, HepG2: A human hepatoblastoma cell line exhibiting defects in bile acid synthesis and conjugation. *J Biol Chem* **261:** 2197–2201, 1986.
29. Diamond L, Cherian K, Harvey RG and DiGiovanni J, Mutagenic activity of methyl- and fluoro-substituted derivatives of polycyclic aromatic hydrocarbons in a human hepatoma (HepG2) cell-mediated assay. *Mutat Res* **136:** 65–72, 1984.
30. Bhatt TS, Coombs M, DiGiovanni J and Diamond L, Mutagenesis in Chinese hamster cells by cyclopenta(*a*)phenanthrenes activated by a human hepatoma cell line. *Cancer Res* **43:** 984–986, 1983.
31. DiGiovanni J, Singer JM and Diamond L, Comparison of the metabolic activation of 7,12-dimethylbenz(*a*)anthracene by a human hepatoma cell line (HepG2) and low passage hamster embryo cells. *Cancer Res* **44:** 2878–2884, 1984.
32. Chang G, Jacobson-Kram D and Williams JR, Use of an established human hepatoma cell line with endogenous bioactivation for gene mutation studies. *Cell Biol Toxicol* **4:** 267–279, 1988.
33. Dearfield KL, Jacobson-Kram D, Brown NA and Williams JR, Evaluation of a human hepatoma cell line as a target cell in genetic toxicology. *Mutat Res* **108:** 437–449, 1983.
34. Silvers KJ, Eddy EP, McCoy EC, Rosenkranz HS and Howard PC, Pathways for the mutagenesis of 1-nitropyrene and dinitropyrenes in the human hepatoma cell line HepG2. *Environ Health Perspect* **102** (Suppl 6)**:** 95–200, 1994.
35. Howard PC, Flammang TJ and Beland FA, Comparison of the *in vitro* and *in vivo* hepatic metabolites of the carcinogen 1-nitropyrene. *Carcinogenesis* **6:** 243–249, 1985.
36. Howard PC, Beland FA and Cerniglia CE, Reduction of the carcinogen 1-nitropyrene to 1-aminopyrene by rat intestinal bacteria. *Carcinogenesis* **4:** 985–990, 1983.
37. Eddy EP, Howard PC, McCoy GD and Rosenkranz HS, Mutagenicity, unscheduled DNA synthesis and metabolism of 1-nitropyrene in the human hepatoma cell line HepG2. *Cancer Res* **47:** 3163–3168, 1987.
38. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
39. Gupta RC, Reddy MV and Randerath K, ^{32}P-Postlabeling analysis of non-radioactive aromatic carcinogen–DNA adducts. *Carcinogenesis* **3:** 1081–1092, 1982.
40. Lu L-JW, Disher RM, Reddy MV and Randerath K, ^{32}P-Postlabeling assay in mice of transplacental DNA damage induced by the environmental carcinogens safrole, 4-aminobiphenyl, and benzo(*a*)pyrene. *Cancer Res* **46:** 3046–3054, 1986.
41. Smith BA, Korfmacher WA and Beland FA, DNA adduct formation in target tissues of Sprague–Dawley rats, CD-1 mice and A/J mice following tumorigenic doses of 1-nitropyrene. *Carcinogenesis* **11:** 1705–1710, 1990.
42. Howard PC, McManus ME and Koop DR, The role of cytochrome P450 form 3b (P450 2C3) in untreated rabbit liver microsomal metabolism of 1-nitropyrene and 3-nitrofluoranthene. *Biochem Toxicol* **9:** 71–78, 1994.
43. Johnson EF, Schwab GE and Muller-Eberhard U, Multiple forms of cytochrome P-450: Catalytic differences exhibited by two homogeneous forms of rabbit cytochrome P-450. *Mol Pharmacol* **15:** 708–718, 1979.
44. Murray M and Reidy GF, Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. *Pharmacol Rev* **42:** 85–101, 1990.
45. Prichard L, Fabre I, Fabre G, Domergue J, Saint Aubert B, Mourad G and Maurel P, Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metab Dispos* **18:** 595–606, 1990.
46. Daujat M, Pichard L, Dalet C, Larroque C, Bonfils C, Pompon D, Li D, Guzelian PS and Maurel P, Expression of five forms of microsomal cytochrome P-450 in primary cultures of rabbit hepatocytes treated with various classes of inducers. *Biochem Pharmacol* **36:** 3597–3606, 1987.
47. Delaforge M, Jaouen M and Mansuy D, The cytochrome P-450 metabolite complex derived from troleandomycin: Properties *in vitro* and stability *in vivo*. *Chem Biol Interact* **51:** 371–376, 1984.
48. Saito K, Kamataki T and Kato R, Participation of cytochrome P450 in reductive metabolism of 1-nitropyrenes by rat liver microsomes. *Cancer Res* **44:** 3169–3173, 1984.
49. Mitchell CE, Effect of aryl hydrocarbon hydroxylase induction on the *in vivo* covalent binding of 1-nitropyrene, benzo[*a*]pyrene, 2-aminoanthracene, and phenanthridone to mouse lung deoxyribonucleic acid. *Biochem Pharmacol* **34:** 545–551, 1985.
50. Beliasario MA, Carrano L, de Giulio A and Buonocore V, Role of rat liver inducible enzymes in *in vitro* metabolic transformation of 1-nitropyrene. *Toxicol Lett* **32:** 89–96, 1986.
51. Sassa S, Sugita O, Galbraith RA and Kapp A, Drug metabolism by the human hepatoma cell line, HepG2. *Biochem Biophys Res Commun* **142:** 52–57, 1987.
52. Fukuda Y and Sassa S, Suppression of cytochrome P450IA1 by interleukin-6 in human HepG2 hepatoma cells. *Biochem Pharmacol* **47:** 1187–1195, 1994.
53. Fukuda Y, Ishida N, Noguchi T, Kappas A and Sassa S,

Interleukin-6 down regulates the expression of transcripts encoding cytochrome P450 IA1, IA2, and IIIA3 in human hepatoma cells. *Biochem Biophys Res Commun* **184:** 960–965, 1992.
54. Sirica AE and Pitot HC, Drug metabolism and effects of carcinogens in cultured hepatic cells. *Pharmacol Rev* **31:** 205–228, 1979.
55. Doodstar H, Duthie SJ, Burke MD, Melvin WT and Grant MH, The influence of culture medium composition on drug metabolizing enzyme activities of the human liver derived HepG2 cell line. *FEBS Lett* **241:** 15–18, 1988.
56. Guillouzo A, Morel F, Fardel O and Meunier B, Use of human hepatocyte cultures for drug metabolism studies. *Toxicology* **82:** 209–219, 1993.
57. Schuetz EG, Schuetz JD, Strom SC, Thompson MT, Fisher RA, Molowa DT, Li D and Guzelian PS, Regulation of human liver cytochromes P-450 in family 3A in primary and continuous culture of human hepatocytes. *Hepatology* **18:** 1254–1262, 1993.
58. Molowa DT, Schuetz EG, Wrighton SA, Watkins PB, Kremers P, Mendez-Picon G and Parker A, Complete cDNA sequence of a cytochrome P-450 inducible by glucocorticoids in human liver. *Proc Natl Acad Sci USA* **83:** 5311–5315, 1986.
59. Kitada M, Kamataki T, Itahashi K, Rikihisa T and Kanakubo Y, P-450 HFLa, a form of cytochrome P-450 purified from human fetal livers, is the 16-α-hydroxylase of dehydroepiandrosterone 3-sulfate. *J Biol Chem* **262:** 13534–13537, 1987.
60. Wrighton SA, Molowa DT and Guzelian PS, Identification of a cytochrome P-450 in human fetal liver related to glucocorticoid-inducible cytochrome P-450HLp in the adult. *Biochem Pharmacol* **37:** 3053–3055, 1988.
61. Wrighton SA and Vandenbranden M, Isolation and characterization of human fetal liver cytochrome P-450HLp2: A third member of the P450III gene family. *Arch Biochem Biophys* **268:** 144–151, 1989.
62. Doodstar H, Grant MH, Melvin WT, Wolf CR and Burke MD, The effects of inducing agents on cytochrome P450 and UDP-glucuronyltransferase activities in human HepG2 hepatoma cells. *Biochem Pharmacol* **46:** 629–635, 1993.
63. Dawson JR, Adams DJ and Wolf CR, Induction of drug metabolizing enzymes in human liver cell line HepG2. *FEBS Lett* **183:** 219–222, 1985.
64. Belisario MA, Arena AR, Pecce R, Borgia R, Staiano N and DeLorenzo F, Effect of enzyme inducers on metabolism of 1-nitropyrene in human hepatoma cell line HepG2. *Chem Biol Interact* **78:** 253–268, 1991.
65. Cresteil T, Jaiswal AK and Eisen HJ, Transcriptional control of human cytochrome P_1-450 gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in human tissue culture cell lines. *Arch Biochem Biophys* **253:** 233–240, 1987.
66. Labruzzo P, Yu XF and Dufresne MJ, Induction of aryl hydrocarbon hydroxylase and demonstration of a specific nuclear receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in two human hepatoma cell lines. *Biochem Pharmacol* **38:** 2339–2348, 1989.
67. Merchant M, Morrison V, Santostefano M and Safe S, Mechanism of action of aryl hydrocarbon receptor antagonists: Inhibition of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced *CYP1A1* gene expression. *Arch Biochem Biophys* **298:** 389–394, 1992.
68. McManus ME, Burgess WM, Veronese ME, Huggett A, Quattrochi LC and Tukey RH, Metabolism of 2-acetylaminofluorene and benzo[*a*]pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. *Cancer Res* **50:** 3367–3376, 1990.
69. Quattrochi LC and Tukey RH, The human cytochrome CYP1A2 gene contains regulatory elements responsive to 3-methylcholanthrene. *Mol Pharmacol* **36:** 66–71, 1989.
70. Shimada T, Hayes CL, Yamazaki H, Amin S, Hecht SS, Guengerich FP and Sutter TR, Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res* **56:** 2979–2984, 1996.
71. Ohgaki H, Matsudura N, Morino K, Kawachi T, Sugimura T, Morita K, Tokiwa H and Hirota T, Carcinogenicity in rats of the mutagenic compounds 1-nitropyrene and 3-nitrofluoranthene. *Cancer Lett* **15:** 1–17, 1982.
72. Ohgaki H, Hasejawa H, Kato T, Negishi C, Sato S and Sugimura T, Absence of carcinogenicity of 1-nitropyrene, correction of previous results, and new demonstration of carcinogenicity of 1,6-dinitropyrene in rats. *Cancer Lett* **25:** 239–245, 1985.
73. El-Bayoumy K, Hecht SS and Hoffman D, Comparative tumor initiating activity on mouse skin of 6-nitrobenzo[*a*]pyrene, 6-nitrochrysene, 3-nitroperylene, 1-nitropyrene, and their parent hydrocarbons. *Cancer Lett* **16:** 333–337, 1982.
74. Nesnow S, Triplett JL and Slaga TJ, Tumor initiating activity of 1-nitropyrene and its nitrated products in SENCAR mice. *Cancer Lett* **23:** 1–8, 1984.
75. Tokiwa H, Otofuji T, Horikawa K, Kitamori S, Otsuka H, Manabe Y, Kinouchi T and Ohnishi Y, 1,6-Dinitropyrene: Mutagenicity in *Salmonella* and carcinogenicity in BALB/c mice. *JNCI* **73:** 1359–1363, 1984.
76. Maeda T, Izumi K, Otsuka H, Manabe Y, Kinouchi T and Ohnishi Y, Induction of squamous cell carcinoma in the rat lung by 1,6-dinitropyrene. *JNCI* **76:** 693–697, 1986.
77. Hirose M, Lee M-S, Wang CY and King CM, Induction of rat mammary gland tumors by 1-nitropyrene, a recently recognized environmental mutagen. *Cancer Res* **44:** 1158–1162, 1984.
78. El-Bayoumy K, Rivenson A, Johnson B, DiBello J, Little P and Hecht SS, Comparative tumorigenicity of 1-nitropyrene, 1-nitrosopyrene, and 1-aminopyrene administered by gavage to Sprague–Dawley rats. *Cancer Res* **48:** 4256–4260, 1988.
79. Imaida K, Hirose M, Tay L, Lee MS, Wang CY and King CM, Comparative carcinogenicities of 1-, 2- and 4-nitropyrene and structurally related compounds in the female CD rat. *Cancer Res* **51:** 2902–2907, 1991.
80. Wislocki PG, Bagan ES, Lu AYH, Dooley KL, Fu PP, Han-Hsu H, Beland FA and Kadlubar FF, Tumorigenicity of nitrated derivatives of pyrene, benzo[*a*]anthracene, chrysene, and benzo[*a*]pyrene in the newborn mouse assay. *Carcinogenesis* **7:** 1317–1322, 1986.
81. Maeda K, Tetsuya K, Nagai T and Kato R, Postnatal development of constitutive forms of cytochrome P-450 in liver microsomes of male and female rats. *Biochem Pharmacol* **33:** 509–512, 1984.
82. Cresteil T, Beaune P, Celier C, Leroux JP and Guengerich FP, Cytochrome P-450 isoenzyme content and monooxygenase activities in rat liver: Effect of ontogenesis and pretreatment by phenobarbital and 3-methylcholanthrene. *J Pharmacol Exp Ther* **236:** 269–276, 1986.
83. Bonfils C, Combalbert J, Pineau T, Angevin J, Larroque C, Derancourt J, Capony JP and Maurel P, Ontogenesis of rabbit liver cytochrome P450: Evidence for a cytochrome P450-IIe (3a)-related form prevailing during the post-natal period. *Eur J Biochem* **188:** 187–194, 1990.