Biotransformation in Carcinogen-induced Diploid and Polyploid Hepatocytes Separated by Centrifugal Elutriation

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ABSTRACT

Biotransformation in carcinogen-induced diploid and polyploid hepatocytes was studied using isozone-selective substrates for several enzyme pathways. Diploid hepatocytes were induced by partial hepatectomy, a single injection of diethylnitrosamine, and 4 weeks of 2-acetylaminofluorene (2-AAF) feeding. Then, after an additional 3-5 weeks on the control diet, diploid and polyploid hepatocytes were separated from freshly isolated hepatocytes by centrifugal elutriation. Benzo(a)pyrene hydroxylase, ethoxyresorufin O-deethylase, and methoxycoumarin O-demethylation activities were approximately 15-40% lower in the diploid hepatocyte fraction than in the polyploid cell fraction. Activities of 1-chloro-2,4-dinitrobenzene, glutathione S-transferase, 3-hydroxy-benzo(a)pyrene or 4-hydroxybiphenyl UDP-glucuronosyltransferase, and DT-diaphorase were not different in the two cell fractions. Determination of activity during the 2-AAF treatment indicated that 2-AAF increased 7-ethoxyresorufin O-deethylase and 3-hydroxy-benzo(a)pyrene glucuronosyltransferase activities by 300 and 200%, respectively, in both the diploid and polyploid hepatocyte fractions. Administration of phenobarbital for 4 days at the end of the control diet period increased ethoxyresorufin and methoxycoumarin dealkylations by 2- and 4-fold, and 3-hydroxybenzo(a)pyrene glucuronidation and 1-chloro-2,4-dinitrobenzene conjugation with glutathione by 1.5- to 2-fold in both hepatocyte fractions. Slight increases in benzo(a)pyrene hydroxylation and 4-hydroxybiphenyl glucuronidation were also evident in diploid cells. Although there is a slight decrease in cytochrome P-450-dependent monooxygenase activities, these data indicate that carcinogen-induced diploid hepatocytes do not show the typical toxicant-resistant phenotype observed in preneoplastic hepatocytes of altered liver foci, which are characterized by large decreases in monooxygenase biotransformations as well as increased activities of several phase II enzymes. This finding is compatible with the hypothesis that 2-AAF-induced nonploidizing growth of diploid hepatocytes is caused by nontoxic mechanisms in the present experimental paradigm. In addition, carcinogen-induced diploid cells respond to phenobarbital in a manner similar to that of polyploid hepatocytes.

INTRODUCTION

Chemical carcinogenesis is a multihit, multistage process characterized by transformation of normal cells into initiated cells, and their progeny into tumor cells (1-3). The early intermediate cell stages in this sequence are termed preneoplastic; chemicals with tumor-promoting activity cause the clonal expansion of preneoplastic cells and raise the probability of further carcinogenic events leading to malignant tumor cells (1, 4, 5). In the liver, putatively preneoplastic hepatocytes can be identified as focal groups of phenotypically altered hepatocytes (1, 3, 6). These cells, as well as neoplastic liver nodules, frequently exhibit great changes in the activity of drug-metabolizing enzymes, including decreased activities of cytochrome P-450-dependent monooxygenases and increased activities of UDP-glucuronosyltransferases, epoxide hydrolase, and DT-

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3 The abbreviations used are: DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's minimal essential medium; PB, sodium phenobarbital; PBS, phosphate-buffered saline.
mation in diploid and polyploid hepatocytes were also determined.

MATERIALS AND METHODS

Chemicals. Bovine pancreatic RNase A (90 units/mg), dimcarol, glutathione, reduced nicotinamide adenosine dinucleotide phosphate, phenobarbital, polyvinylpyrrolidone (Mw, ~40,000) and trizma base were obtained from Sigma Chemie (Deisenhofen, Germany); EGTA and phenol reagent came from Merck (Darmstadt, Germany); CDNB, 4-hydroxybiphenyl, and 7-methoxyxoumarin were purchased from Aldrich Chemie (Steinheim, Germany); benzo(a)pyrene was obtained from Roth KG (Karlsruhe, Germany); Brij 58 came from Atlas Chemie (Essen, Germany); collagenase, bovine serum albumin, bovine pancreatic DNase I (grade 2), 7-ethoxysorufin, and UDP-glucuronic acid came from Boehringer Mannheim (Mannheim, Germany); penicillin/streptomycin (10,000 U/mL) and MEM amino acids and vitamins from Biochrom KG (Berlin, Germany). 3-Hydroxybenzo(a)pyrene was obtained from the Carcinogen Standard Reference Repository, National Cancer Institute (Bethesda, MD). DEN and DMEM were purchased from Serva Feinbiochemika (Heidelberg, Germany). All buffer materials and organic compounds were the highest quality commercially available. Double-distilled water was used for all aqueous solutions.

Animals. Male Wistar rats approximately 4 weeks old (70–80 g) were obtained from the GSF breeding colony and were given free access to food and water throughout the experimental period. In the standard protocol, i.e., the classic regimen for the production of carcinogen-induced diploid hepatocytes (11, 15), rats were anesthetized with diethyl ether, and a two-thirds partial hepatectomy was performed. Twenty hours later, DEN was administered by gavage at 50 mg/kg in water. One week after the partial hepatectomy, the rats were provided the standard pellet diet containing 0.02% 2-AAF (Altromin, Lage, Germany) for 2 weeks. Then the rats were switched to the standard diet without 2-AAF for 3–5 weeks before the hepatocytes were isolated, elutriated, and stored in liquid nitrogen until use. The second group of rats (during 2-AAF treatment), used to determine drug-metabolizing enzyme activities in early carcinogen-induced diploid hepatocytes and to study whether 2-AAF had an effect on xenobiotic biotransformation enzymes, underwent the partial hepatectomy, DEN injection, and 22–24 days of the 0.02% 2-AAF diet before hepatocytes were isolated. Finally, a third group of rats (additional PB treatment), which had been subjected to a partial hepatectomy and treated with 50 mg DEN/kg and then 4 weeks of the 2-AAF diet and 4 weeks of standard diet, was treated with PB (80 mg/kg i.p. in water on day 1 and 0.1% in the drinking water for 3 days) to determine whether phenobarbital induction was similar or different in diploid versus polyploid cells. Twenty-four hours after the last dose of PB, hepatocytes were prepared as described below.

Isolation of Hepatocytes. After anesthesia with sodium pentobarbital (100 mg/kg; 4 mL/kg in water), the abdominal cavity was opened by a midline incision, and the liver was perfused via the portal vein with approximately 200 mL of Ca²⁺-free modified Hanks' solution containing 100 μM EGTA at a flow rate of 40 mL/min followed by 200 mL of the same Ca²⁺-free medium without EGTA. Next, the liver was perfused for 10–15 min with 100 mL of DMEM containing 1.8 mM CaCl₂ and 100 units/ml of collagenase before Glisson's capsule was opened and the cells gently dispersed in DMEM. After filtration through 80-μm and subsequently 40-μm nylon mesh filters, the cells were washed three times in DMEM at 50 × g for 5 s. Viability, as determined by exclusion of 0.4% trypan blue, was greater than 85%.

Centrifugal Elutriation. Cells (125 × 10⁹) were loaded into the Beckman JE-6B rotor at 1700 rpm, 5°C, and a flow rate of 19 mL/min. The medium contained 137 mM NaCl, 5.6 mM KCl, 1 mM MgSO₄, 7H₂O, 1 mM CaCl₂, 2H₂O, 0.9 mM KH₂PO₄, 2.1 mM Na₂HPO₄, 2H₂O, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mM glucose, MEM amino acids and vitamins, 1% polyvinylpyrrolidone, and 50 μg DNase 1/mL, pH 7.4. The last two agents were included in the medium to protect the cells and improve flow conditions and to prevent cell aggregation, respectively. Four 200-ml fractions were obtained by stepwise increments in the flow rate at 1700 rpm. Cellular debris and dead cells eluted between 19 and 27 mL/min, predominately diploid hepatocytes between 27 and 33 mL/min, mixed fraction of diploid and polyploid cells at 33–50 mL/min, and finally the polyploid hepatocytes at 50–60 mL/min and decreasing to 1550 rpm. The diploid and polyploid fractions were saved for further experimentation; the latter fraction contains mononucleolated and binucleolated polyploid hepatocytes. The cells were concentrated by centrifugation in a Sorval RC-2B with a GSA rotor at 800 rpm for 45 s. The medium was aspirated, and the cells were washed in 2 mL of PBS and then resuspended in 2 mL PBS.

Aliquots were removed for determination of cell number and viability as well as flow cytometry, and the remaining cells were stored in liquid nitrogen until use. Viability of diploid and polyploid cell fractions was 88 ± 2% and 89 ± 2%, respectively, after elutriation.

Flow Cytometric Analysis. Cells were fixed by adding 2 mL of −20°C methanol for 10 min. After centrifugation at 50 × g for 1 min, the supernatant was removed, and the cells were washed with PBS, then resuspended in PBS, and gently mixed at 4°C overnight. Next, the cells were incubated in 2 mL PBS at 37°C for 45 min with 200 μg heat-inactivated RNase A. After addition of 60 μM propidium iodide, DNA content of hepatocytes in the parent cell suspension and elutriated fractions (diploid and polyploid) was determined by fluorescence-activated cell sorter analysis (Becton-Dickinson). Volume was determined by electrical resistance pulse sizing using a 75- or 100-μm orifice. DNA content of hepatocytes was then determined by flow cytometric analysis (Becton-Dickinson Consort 30 Program version E 12/86 and corrected for cell aggregates as described by Klose et al. (22).

Determination of Enzyme Activity. Cells removed from the liquid nitrogen were allowed to thaw on ice for approximately 30 min before dilution in ice-cold PBS and resuspension with five to seven strokes of a Potter-Elvehjem homogenizer. Protein concentrations were determined for all cell homogenate preparations by the method of Lowry et al. (23), with bovine serum albumin as the standard. Cytchrome P-450 monooxygenase activity was determined fluorometrically with the following substrates and methods. Demethylation of 50 μM 7-ethoxysorufin was determined with 100–500 μg protein in a 30-min incubation (24). Hydroxylation of 100 μM benzo(a)pyrene was measured with 100–300 μg protein after a 15-min incubation (25). The demethylation of 400 μM 7-methoxyxoumarin was quantitated after a 10-min incubation of 75–150 μg hepatocyte protein in 100 μl of cell suspension in 1 mL of 50 mM Tris-HCl, pH 7.6, containing 2.5 mM MgSO₄ and 0.5 mM NADPH at 37°C (26). The reaction was terminated by addition of 150 μL 15% trichloroacetic acid and extraction into 3 mL chloroform. The chloroform phase was then extracted with 3 mL of 1 M NaCl plus 0.01 M NaOH. After separation of the phases, the fluorescence of the aqueous phase was determined by 368 nm excitation and 456 nm emission. UDP-glucuronosyltransferase activities were assayed fluorometrically in 0.1 M Tris-HCl, pH 7.5, with 5 mM MgCl₂ at 37°C in the presence of 0.01% Brij 58 with 50 μM 3-hydroxybenzo(a)pyrene and 20–100 μg protein (27) and 500 μM 4-hydroxibiphenyl with 300–900 μg hepatocyte protein (28). Conjugation of 2 μM CDNB by glutathione S-transferase was measured at 25°C in 0.1 M potassium phosphate, pH 6.5, with 20–75 μg protein using the spectrophotometric method of Habig and Jakoby (29). DT-Diaphorase was assayed by incubating 50–100 μg protein in 0.05 M sodium phosphate buffer (pH 7.8) containing 0.07% bovine serum albumin and 0.2 mM NADPH (30). The reaction was started by addition of 40 μM 2,6-dichlorophenolindophenol, and the reduction of 2,6-dichlorophenolindophenol was followed spectrophotometrically at 600 nm for 2–3 min. To subtract other activities not related to DT-diaphorase, the reaction was run additionally in the presence of 1–3 μM dicumarol for about 10 min. The dicumarol-sensitive part of the activity is a measure of DT-diaphorase activity.

All enzyme reactions were performed in duplicate under conditions of initial velocity with appropriate blanks and were proportional to incubation time and protein concentration.

Statistics. Means and SE were generated for all data, which were analyzed by a one-way analysis of variance and Duncan's new multiple-range test to compare the means. All differences between diploid and polyploid cell fractions that are discussed in the text are significant at
RESULTS

Ploidy distributions of the parent hepatocyte suspensions are shown in Table 1 for untreated 15-week-old rats; for rats undergoing the standard Seglen protocol, consisting of a partial hepatectomy, DEN treatment, and 4 weeks of standard diet (standard protocol); for rats around the end of the 2-AAF feeding, i.e., after 22-24 days of 2-AAF (during 2-AAF treatment); and for rats undergoing the Seglen protocol followed by phenobarbital (additional PB). The data represent cellular ploidy distributions; i.e., “tetraploid hepatocytes” comprise 4n and 2 × 2n and “octaploid hepatocytes” comprise 8n and 2 × 4n cells, respectively. Normally, rats between 10 and 15 weeks of age have very small populations of diploid hepatocytes. However, the regimen of partial hepatectomy, DEN, and 2-AAF increases the percentage of diploid hepatocytes and reduces the percentage of tetraploid and octaploid cells. There was no difference in ploidy distribution between cells isolated from rats during 2-AAF treatment or from rats 4 weeks after termination of 2-AAF treatment. Additional treatment with phenobarbital increased slightly, but not significantly, the percentage of polyploid hepatocytes. There are no reports on phenobarbital increases polyploidization within 5 days in mice (31), but only a slight increase in polyploidization was observed after 15 days in rats (32). However, these animals were not subjected to our experimental paradigm of two-thirds partial hepatectomy plus DEN followed by 2-AAF in the diet.

Table 2 lists ploidy distributions of the diploid and polyploid cell fractions as well as the parent cell suspension. Centrifugal elutriation provided a diploid fraction that contained 90% diploid hepatocytes and a polyploid fraction with 77% tetraploid and 3% octaploid cells. Cell viability after elutriation was 88 ± 2% and 89 ± 2% in diploid and polyploid cell fractions, respectively, indicating that centrifugal elutriation did not reduce cell viability.

Fig. 1 shows cytochrome P-450-dependent monooxygenase activity toward benzo(a)pyrene (BP), ethoxyresorufin (ER), and 7-methoxycoumarin (MC) in carcinogen-induced diploid (DIP) and polyploid (POLY) hepatocytes obtained by centrifugal elutriation, and in the parent isolated hepatocyte suspension (SUS). Enzyme activities were determined in hepatocytes isolated from rats treated with the Seglen protocol (C), during the 2-AAF treatment of the standard protocol (after 22-24 days of 0.02% 2-AAF in the diet) (D), or after additional treatment with phenobarbital (80 mg/kg i.p. once with 0.1% phenobarbital in the drinking water for 3 subsequent days). Values are means ± SE for 4–6 rats. All differences between diploid and polyploid cell fractions that are discussed in the text are significant at P < 0.05. *, differences between standard protocol and 2-AAF treatment; +, differences between standard protocol and PB.
of rats which have undergone the standard Seglen protocol increased methoxyxoumarin O-demethylase and ethoxyresorufin O-deethylase by 4.2- and 1.8-fold, respectively, compared to the enzyme activities determined after the standard protocol without additional phenobarbital treatment. There was no difference in phenobarbital induction between diploid or polyploid hepatocytes, except for the 50% increase in benzo(a)pyrene hydroxylase activity in diploid hepatocytes. A nearly 2-fold increase in 3-hydroxybenzo(a)pyrene glucuronidation, a 1.5-fold increase in CDNB conjugation with glutathione, and a slight although not significant increase in DT-diaphorase are illustrated in Fig. 2 for the diploid and polyploid cell fractions and the parent cell suspension. There was a 70% increase in 4-hydroxybiphenyl glucuronidation in diploid hepatocytes after phenobarbital.

**DISCUSSION**

Sequential treatment of rats with two-thirds partial hepatectomy and DEN followed by 0.02% 2-AAF in the diet for 4 weeks and then 4 weeks of standard diet, otherwise called the Seglen protocol (standard protocol), induces the emergence of a population of small diploid hepatocytes (Table 1) and the subsequent appearance of neoplastic nodules and hepatomas (11, 14–16). Although their origin is still unknown, small diploid hepatocytes could be derived from either bile duct epithelial cells (oval cells) (33–35) or from a dividing diploid hepatocyte colony (11, 14, 16, 35). The oval cells represent a somewhat heterogeneous cell population capable of metamorphosing into differentiated hepatocytes (36, 37), which may be implicated in liver tumor formation (34, 38, 39). On the other hand, Schwarze et al. and Saeter et al. (11, 14, 16) favor the idea of a selective proliferation of diploid hepatocyte stem cells. Regardless of origin, diploid cells are clearly parenchymal liver cells, as indicated by their morphological appearance, their size (11, 15), and the responsiveness to phenobarbital (the present study). The cells in the elutriated diploid cell fraction have diameters of 17.3 µm, while the largest nonparenchymal liver cell, the oval cell, is significantly smaller, with its diameter of 13 µm (40). In terms of cell volume, which is proportional to the small diploid hepatocytes and oval cells differ by a factor of almost 2.5. Thus, contamination of the diploid hepatocyte fraction with nonparenchymal liver cells is highly unlikely.

It has been suggested that in the Seglen protocol the arylamine 2-AAF induces the growth of diploid hepatocytes and promotes tumor formation via nontoxic, probably hormonelike mechanisms (14, 19). This would imply that carcinogen-induced diploid liver cells need not necessarily exhibit the toxicant-resistant phenotype in order to proliferate during 2-AAF treatment. In contrast, this toxicant-resistant phenotype, which is characterized by great changes in the activity of many drug-metabolizing enzymes, is apparently a prerequisite in treatment protocols where 2-AAF exerts its tumor-promoting activity via toxic mechanisms (2, 3, 18). To determine whether carcinogen-induced diploid and polyploid hepatocytes differ in their capacity to metabolize xenobiotics, the present study quantitated phase I and phase II enzyme activities in these cell fractions. In this connection it is without bearing for the present study that the polyploid cell fraction contains both binucleated and mononucleated hepatocytes; both are thought to represent stages of hepatocellular differentiation. Cytochrome P-450-dependent monoxygenases were assayed using 7-ethoxyresorufin, which is selectively metabolized by poly cyclic aromatic hydrocarbon-inducible isoenzyme forms (24, 41); 7-methoxyxoumarin, a preferred substrate of constitutive and PB-inducible enzyme forms in the liver (26, 42); and benzo(a)pyrene, which measures constitutive, poly cyclic aromatic hydrocarbon-inducible and, to a lesser degree, PB-inducible isoenzyme forms (43, 44). With respect to UDP-glucuronosyltransferases, two aglycones were used; 3-hydroxybenzo(a)pyrene is preferentially metabolized by poly cyclic aromatic hydrocarbon-inducible and, to a lesser degree, PB-inducible isoenzyme forms (43, 44). In contrast to studying these multienzyme families with several substrates, glutathione S-transferases were assayed with only CDNB, which has broad substrate specificity, giving high activities toward many of the purified isoenzymes (47).

In rats which have undergone the standard protocol (Fig. 1), the activities of benzo(a)pyrene hydroxylase, ethoxyresorufin O-deethylase, and methoxyxoumarin O-demethylase were approximately 30–40% lower in carcinogen-induced diploid hepatocytes than in polyploid cells 3–5 weeks after 2-AAF exposure. These alterations in monoxygenase activity are significant but rather modest when compared to the up to 95% decreases of cytochrome P-450-dependent hydroxylations that have been observed in preneoplastic hepatocyte foci and neo-
plastic nodules (2, 10, 48). The phase II enzymes CDNB glutathione S-transferase, 3-hydroxybenzo(a)pyrene or 4-hydroxybiphenyl UDP-glucuronosyltransferases, and DT-diaphorase (Fig. 2) were not different between the two elutriated cell fractions, whereas preneoplastic nodules have been shown to express significantly higher activities of these enzymes (except for 4-hydroxybiphenyl UDP-glucuronosyltransferase) when compared to the surrounding liver (UDP-glucuronosyltransferase, 2–5-fold; CDNB glutathione S-transferase, 3-fold; DT-diaphorase, 6–13-fold) (3, 48–50). The present data indicate that carcinogen-induced diploid hepatocytes do not express all aspects of the toxicant-resistant phenotype, which is characterized by large decreases in cytochrome P-450-dependent monooxygenase activities and significant increases in the activities of several phase II enzymes. Nevertheless, the decrease in the biotransformation of ethoxyresorufin may be taken as an indication of somewhat less N-hydroxylation, which is catalyzed by the cytochrome A2 form and represents the first step in the activation of 2-AAF (51).

It is interesting to note that the enzyme profile of rat bile ductular epithelial cells indicates no detectable amidopyrine demethylation but demonstrable activities of p-nitrophenol UDP-glucuronosyltransferase and 1,2-dichloro-4-nitrobenzene glutathione S-transferase, which were similar to those of hepatocytes obtained from the bile duct-ligated animals (52). To our knowledge, no data have been published on the drug-metabolizing activities in oval cells per se. The fact that the enzyme activity profile of the diploid hepatocytes isolated in the present study resembles that of differentiated hepatocytes may be taken as a further indication that carcinogen-induced diploid cells isolated by centrifugal elutriation are in fact parenchymal hepatocytes. If they were derived from bile ductular epithelial cells/oval cells, then they rapidly lost their oval cell characteristics during the process of transformation to hepatocytes. This would be indicated by the fact that already during the feeding of 2-AAF, i.e., during the development of the diploid cells, activities of drug-metabolizing enzymes are expressed that are similar to those in parenchymal hepatocytes.

Drug metabolism by diploid and polyploid hepatocytes was also analyzed in cells isolated during 2-AAF treatment of the animals (Figs. 1 and 2). Increased ethoxyresorufin O-deethylase and 3-hydroxybenzo(a)pyrene UDP-glucuronosyltransferase during 2-AAF treatment support previously published data (48, 50). Although DT-diaphorase is reportedly induced by polycyclic aromatic hydrocarbons (53, 54), the slight increases after 2-AAF treatment (55) more closely resemble the lack of significant change in DT-diaphorase activity observed in the 2-AAF group (Fig. 2) in the present study. No other enzyme activities were different from those in hepatocyte fractions isolated 3–5 weeks after the 2-AAF treatment (standard protocol). This latter finding suggests that the drug-metabolizing enzymes studied do not show developmental changes during the first weeks of 2-AAF exposure in carcinogen-induced cells because the emerging young diploid hepatocytes (2-AAF group) exhibited enzyme activities similar to those of the diploid cells that were isolated 3–5 weeks after the treatment of the animals (standard protocol).

Administration of phenobarbital is also known to induce several biotransformation enzymes. Methoxyxocamarin O-deethylase activity is selectively increased by exposure of hepatocytes to phenobarbital (26, 42) and was increased to about 430% of control (Fig. 1) in the parent hepatocyte suspension. There was also a slight increase to 170% (Fig. 1) in ethoxyxocamarin O-deethylase activity, which was similar to the 200% observed by Thompson et al. (56). Also in agreement with Thompson et al. (56), benzo(a)pyrene hydroxylase activity was not increased in hepatocytes isolated from the phenobarbital-treated rats. The increased glucuronidation (Fig. 2) of 3-hydroxybiphenylpyrene by approximately 180% in all cell fractions was expected (27, 45). It is not clear why 4-hydroxybiphenyl glucuronidation was increased only in the diploid cell fraction but not in the parent cell suspension or the polyploid hepatocytes (Fig. 2), as has been shown by others (50). The conjugation of CDNB in the diploid and the polyploid cell fractions and the parent hepatocyte suspension after phenobarbital administration is similar to that reported by Thompson et al. (56). In summary, the data in Figs. 1 and 2 indicate that the diploid hepatocytes respond to phenobarbital in a manner similar to that of the polyploid cells. In addition, none of the studies presenting conflicting data on enzyme induction used our experimental paradigm of partial hepatectomy plus DEN followed by 2-AAF in the diet for 4 weeks.

Carcinogen-induced diploid and tetruploid hepatocytes that were isolated by centrifugal elutriation after treatment of the rats with the Seglen protocol do not show the typical toxicant-resistant phenotype. There is a slight decrease in monooxygenase activity but little change in the phase II biotransformation reactions that were examined. Our data are compatible with the theory that the proliferation and promotion of diploid hepatocytes by 2-AAF are caused by nontoxic mechanisms and that diploid hepatocytes can respond to 2-AAF and to phenobarbital in a manner that is similar to that observed with polyploid hepatocytes.

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