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Lack of association between CYP2A5 induction and apoptosis in mouse primary hepatocytes

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Abstract

Upregulation of mouse hepatic cytochrome P450 2A5 (CYP2A5) is a process closely associated with hepatocellular damage and formation of liver tumours. 2-Aminopurine, a protein kinase inhibitor modulating cell cycle control, was recently shown to strongly induce CYP2A5 in mouse hepatocytes. The objective of this study was to determine the association between CYP2A5 induction and apoptosis in mouse primary hepatocytes. Five well-characterised CYP2A5 inducers were tested for their ability to affect apoptosis rate, determined by immunohistochemical *in situ* 3′-end-labelling technique, in a primary mouse hepatocyte model. Transforming growth factor beta (TGFβ) was used as a positive (proapoptotic) control. Phenobarbital, pyrazole and the mitogen-activated protein kinase inhibitor PD98059 did not significantly affect apoptosis rate in hepatocytes. Norcocaine induced apoptosis at 6 hr (1.8-fold) and 2-aminopurine 12 hr (1.4-fold) after treatment, which is considerably earlier than peaks in the amount of CYP2A5 mRNA. TGFβ reduced CYP2A5 marker activity, coumarin 7-hydroxylase by 74%. These results indicate that in a primary hepatocyte model (a) there is no systematic correlation between apoptosis and CYP2A5 induction; (b) phenobarbital does not significantly affect the rate of apoptosis; and (c) the induction of apoptosis caused by the chemicals tested occurs considerable earlier than elevation of CYP2A5 expression. Thus, no causal link appears to exist between induction of CYP2A5 and apoptotic rate. © 2002 Published by Elsevier Science Inc.

Keywords: Apoptosis; Cytochrome P450; CYP; Coumarin; 2-Aminopurine

1. Introduction

The mouse cytochrome P450 2A5 (CYP2A5) enzyme and its human orthologue CYP2A6 metabolise many toxic substrates, such as nicotine, nitrosamines, and aflatoxins [1,2]. CYP2A5 and CYP2A6 are predominantly expressed in hepatocytes, but they are also present in some extrahepatic tissues, especially nasal mucosa [3].

The expression of CYP2A5 is induced by stimuli that usually repress CYP forms, including hepatocellular damage caused by hepatotoxic chemicals such as cocaine [4], pyrazole [5], porphyrinogenic agents [6], solvents [4], and heavy metals [7]. An induction of CYP2A5 can be elicited also by activators of cAMP [8,9]. An elevated CYP2A protein level in hepatocytes is associated with the development of liver tumours in mice [10] and humans [11]. In addition, biological insults upregulate mouse hepatic CYP2A5 *in vivo* [12,13]. In human liver, the amount of CYP2A6 is increased in conjunction with liver cirrhosis caused by tumours [11] or ethanol consumption [14].

The exact relationship between CYP2A5 upregulation and perturbation of hepatocyte cell cycle control is unknown presently. We showed recently that several chemicals modulating intracellular phosphorylation status are capable of inducing CYP2A5 in mouse primary hepatocytes [15]. A particularly interesting finding was that 2-aminopurine, a non-specific inhibitor of several protein kinases, is a potent inducer of CYP2A5 [15]. 2-Aminopurine is a compound known to elicit an override of mitotic checkpoints and exit from mitosis [16,17]. 2-Aminopurine inhibits several serine/threonine protein kinases, including

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Abbreviations: CYP, cytochrome P450; TGFβ, transforming growth factor β.

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the double-stranded RNA-dependent protein kinase [18] and MAP kinases [19].

Several chemical inducers of hepatic CYP2A5, such as carbon tetrachloride [20], cadmium [21], thioacetamide [22], and cocaine [23] have been shown to elicit cell death and apoptosis in hepatocytes *in vivo*. Thus, there appears to be a connection between induction of hepatocyte CYP2A5 and cell cycle regulation, including apoptosis. To date, no studies have been carried out addressing CYP2A5 induction and apoptosis simultaneously. Thus, the objective of this study was to determine the relationship between induction of CYP2A5 and apoptosis in a primary hepatocyte model. Several established chemical inducers of CYP2A5 were used was to find out if CYP2A5 upregulation is associated with increased or decreased apoptotic rate in primary hepatocytes.

2. Materials and methods

2.1. Reagents

Phenobarbital, TGFβ, dexamethasone, Williams medium E, and ITS (insulin–transferrin–selenate media supplement) were obtained from Sigma. 2-Aminopurine and PD98059 were purchased from Calbiochem.

2.2. Hepatocyte cultures and treatments

Male DBA/2 mice, aged between 7 and 10 weeks, were used in this study. To isolate hepatocytes, mouse livers were subjected to collagenase perfusion according to previously published protocols [8,15,24]. After filtration and centrifugation (50 *g*, 2 min), the isolated hepatocytes were dispersed in William's medium E containing dexamethasone (20 ng/mL), ITS (insulin 5 mg/L, transferrin 5 mg/L, sodium selenate 5 μg/L), gentamicin (10 μg/mL) and 10% FCS at a density of 5×10^6 cells/90 mm uncoated plastic dish (Falcon 3003). The cultures were maintained at 37° in humidified incubator. After a 2 hr incubation, non-attached cells were discarded by aspiration, followed by changing the medium to Williams E without FCS. The compounds to be studied were added to the serum-free medium. Control dishes received the respective vehicle only (in most cases DMSO). Test compounds in this study were phenobarbital (1.5 mM), 2-aminopurine (10 mM), pyrazole (10 mM), TGFβ (2 ng/mL), PD98059 (20 μM) and norcocaine (10 μM). After 1, 3, 6 and 12 hr in culture, cells were washed, scraped, slightly centrifuged and 500 μL 10% formalin were added. Samples were embedded in paraffin.

2.3. In situ 3′-end labelling

In situ 3′-end labelling of apoptotic DNA was performed by using the ApopTag Peroxidase Kit (Oncor) following the manufacturers instructions with a few modifications. Briefly, cell samples were embedded in paraffin. Five micrometer thick sections were cut from cell samples to the slides (Super Frost Plus, Menzel Gläser). After dewaxing and rehydration, the sections were stripped from proteins by incubating the slides in 20 μg/mL proteinase K (Boehringer Mannheim) at room temperature for 15 min. Endogenous peroxidase activity was consumed by incubating in 2% hydrogen peroxide in phosphate-buffered saline, pH 7.2 for 20 min. Terminal transferase enzyme was used to catalyse the addition of digoxigenin labelled nucleotides to the 3′-OH ends of the fragmented DNA. After that, antidigoxigenin-peroxidase solution was applied on the slides. Colour reaction was developed with diaminobenzidine with hydrogen peroxide. Finally, sections were counterstained with Methyl Green. Negative controls did not contain the terminal transferase enzyme.

2.4. Evaluation of apoptosis in hepatocytes

The extent of apoptosis in hepatocytes was evaluated by the labelling pattern of apoptotic cells and cells detected by *in situ* 3′-end labelling. The number of apoptotic cells and bodies per 500 cells were counted to indicate the extent of apoptosis. Only views with apoptotic activity were counted. Counting was done blindly. Three samples from three independent primary cultures were analysed. As a positive control we used TGFβ, which is an inducer of apoptosis in mouse primary hepatocytes [25]. The apoptotic index is given as the percentage of stained cells relative to the total number of cells.

2.5. Cytotoxicity

Because many compounds used in this study are toxic to liver *in vivo*, the direct cytotoxicity was tested with lactate dehydrogenase leakage analysis using an automatic analysator (Cobas Integra 700, Roche Diagnostics). Before scraping, 1 mL of the culture medium was stored at −20° for later analysis. The test was repeated four times for each chemical.

2.6. RNA blot analysis

Adherent cells were scraped, washed and homogenised in 400 μL of extraction buffer. mRNA was purified by Quick-Prep Micro mRNA Purification Kit (Pharmacia). Purified mRNA was separated on a 1% agarose gel and transferred to Qiabrane nylon membrane (Qiagen). RNA was fixed by UV-cross-linking in UV Stratalinker 1800 (Stratagene) and the membrane was hybridised to a [^{32}P]dCTP-labelled *Cla*I-cut fragment of CYP2A5 cDNA. Mouse CYP2B10 and CYP1A2 cDNAs were the same as used previously in our laboratory [24]. Tyrosine aminotransferase (TAT) cDNA was amplified using rat tyrosine aminotransferase (TAT) gene specific oligonucleotides

and cloned into pCRII vector (Invitrogen). Purified TAT inserts were used as probes in mRNA blot experiments. Radioactivity was measured with PhosphorImager SI equipment (Molecular Dynamics).

2.7. Statistical analysis

The values given in immunohistochemistry results represent the means of three separate experiments and four separate experiments in cytotoxicity. Student's *t*-test was used and *P*-values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Effect of 2-aminopurine on CYP and TAT mRNA

We have demonstrated earlier that 2-aminopurine, a nonspecific inhibitor of several protein kinases, increases CYP2A5 marker activity (coumarin 7-hydroxylase) in cultured murine hepatocytes up to 25-fold, which is the highest induction elicited by any single compound [15]. To demonstrate that this induction involves mRNA accumulation, primary hepatocytes were treated with 2-aminopurine for 48 hr and the amounts of mRNAs of CYP2A5, CYP2B10, and CYP1A1/2 were determined. As shown in Fig. 1, 2-aminopurine increased CYP2A5 mRNA levels 56-fold. The amount of tyrosine aminotransferase (TAT) mRNA, serving as an indicator of a gene responsive to changes in intracellular phosphorylation status, was elevated 74-fold by 5 mM 2-aminopurine. CYP2B10 mRNA increased up to 13-fold and a modest increase of CYP1A1/2 mRNA (2.4-fold) was also observed (Fig. 1).

3.2. Effect of selected CYP2A5 inducers on apoptosis

A series of compounds (phenobarbital, pyrazole, 2-aminopurine, PD98059, and norcocaine) that have previously been shown to induce CYP2A5 was tested for their ability to influence apoptosis in primary hepatocytes. These agents typically induce CYP2A5 so that peaks of CYP2A5 mRNA concentration and catalytic activity (coumarin 7-hydroxylation) are observed at 24 and 48 hr, respectively, after administration of the inducers [4,7,15,24,26]. The well characterised proapoptotic compound TGFβ was used as a positive control.

Fig. 2 shows the result of immunohistochemical *in situ* 3′-end labelling of hepatocytes 6 hr after treatment with vehicle, TGFβ, 2-aminopurine, and norcocaine. A clear increase in the number of stained hepatocytes was observed especially in response to TGFβ (panel b) and norcocaine treatment. The consistent elevation of the apoptotic index by TGFβ shows that the model used performs properly with attached hepatocytes being capable of responding to proapoptotic stimuli. An increase in apoptosis index at the 6 hr time-point occurred also in response to treatment with norcocaine (panel d), whereas 2-aminopurine (panel c) did not affect apoptosis at this time-point.

The changes in hepatocyte apoptosis index as a function of time is shown in Fig. 3. An apoptotic index of 12% was observed at time 0, i.e. 2 hr after the hepatocytes were plated on dishes. In dishes treated with the vehicle only, the proportion of apoptotic hepatocytes increased to 30% in 12 hr. TGFβ (2 ng/mL) elicited a significant increase in apoptosis rate at every time-point examined, the proportion of apoptotic cells rising to 46% by 6 hr (Fig. 3). After the 12 hr time-point, determination of apoptotic index was not feasible.

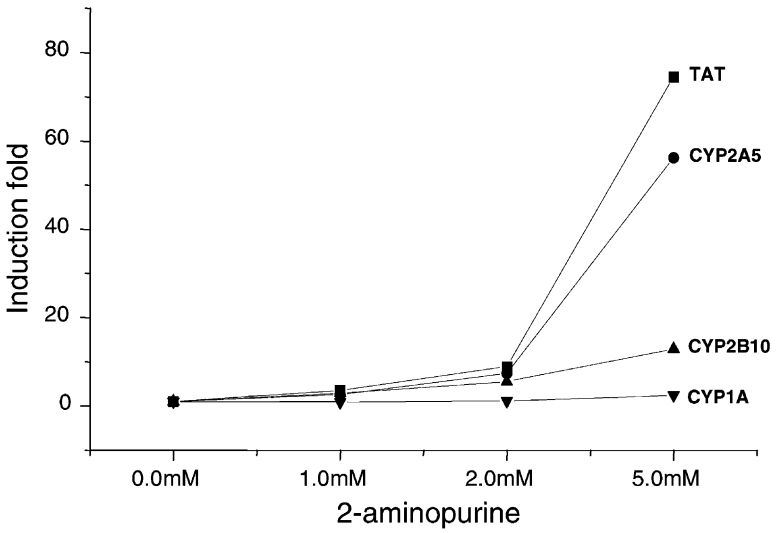

Fig. 1. 2-Aminopurine dose–response curves of CYP2A5, CYP2B10, CYP1A1/2 and TAT mRNA. The values represent the means of a minimum of three separate primary cultures from different animals and are given relative to the control values, which were normalised to 1.0. Coefficients of variation ranged from 0.08 to 0.69. All treatments were for 48 hr.

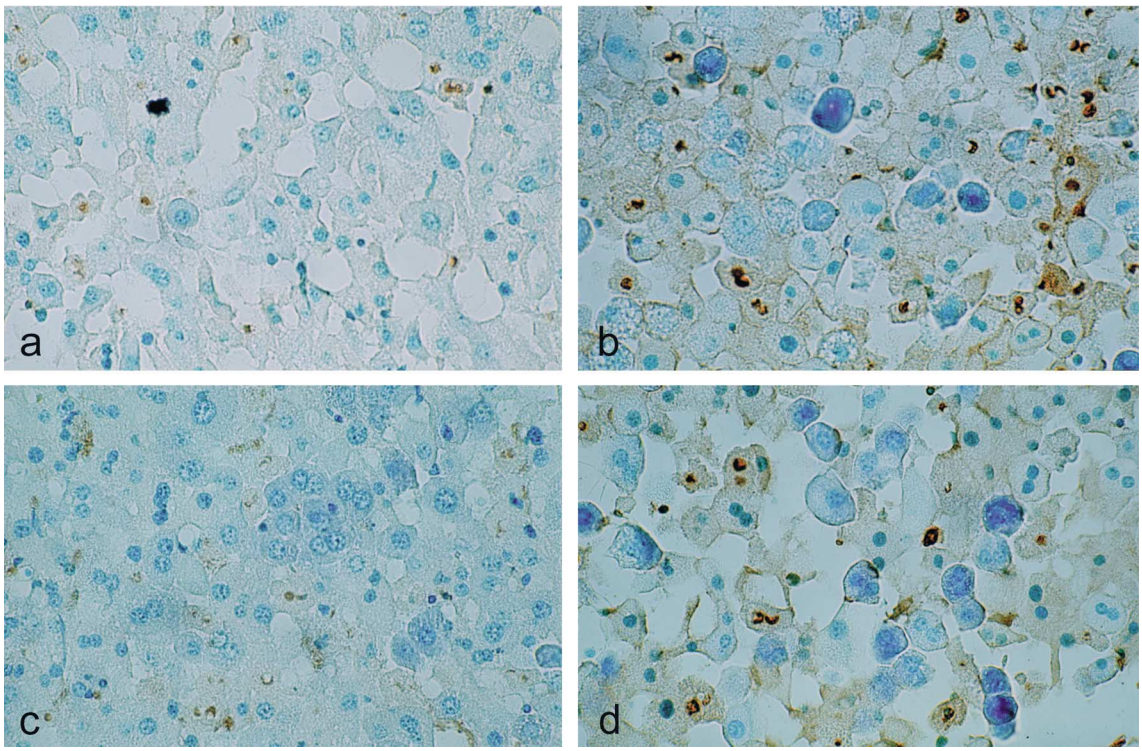

Fig. 2. Immunohistochemical *in situ* 3′-end labelling of hepatocytes 6 hr after treatment with vehicle only (a), TGFβ (b), 2-aminopurine (c), and norcocaine (d).

Figs. 4 and 5 illustrates the relative changes in apoptotic index elicited by the tested chemicals. Norcocaine produced a rapid rise in apoptosis rate peaking at a value of 55% (control value 31%) at 6 hr and then declining. 2-Aminopurine increased the apoptosis index to a value of 45% at 12 hr (control 32%). Phenobarbital, pyrazole and PD98059 did not show statistically significant changes in the proportion of apoptotic hepatocytes at any time point.

In independent experiments, inclusion of TGFβ (2 ng/mL) to cell culture medium reduced hepatocyte coumarin 7-hydroxylase activity to 26% of control value (data not shown).

3.3. Cytotoxicity

Leakage of lactate dehydrogenase from hepatocytes to the culture medium was used as an indicator of direct

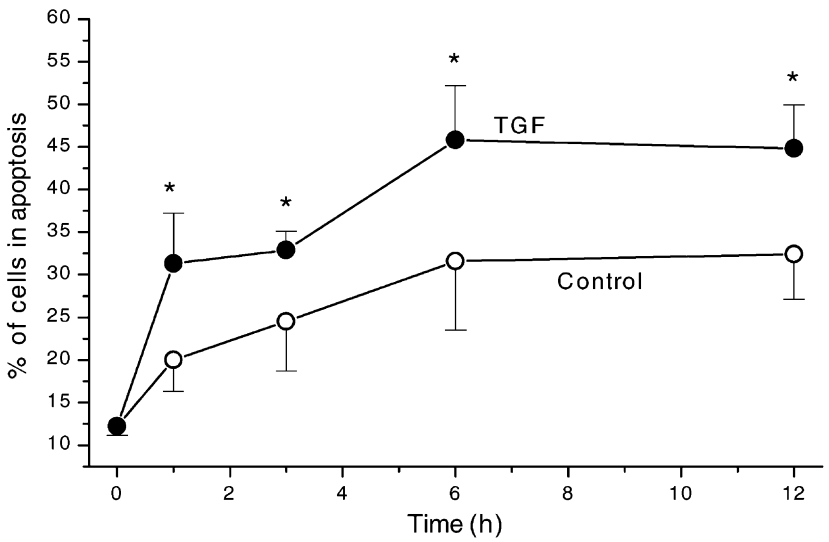

Fig. 3. Time course of apoptosis in control and TGFβ (2 ng/mL) treated hepatocytes. Apoptosis index is expressed as the percentage of apoptotic cells and bodies per 500 cells detected at 1, 3, 6 and 12 hr. The means were calculated from three independent primary cultures from different animals. The symbol (∗) indicates a statistically significant value ($P < 0.05$). Error bars represent standard deviations.

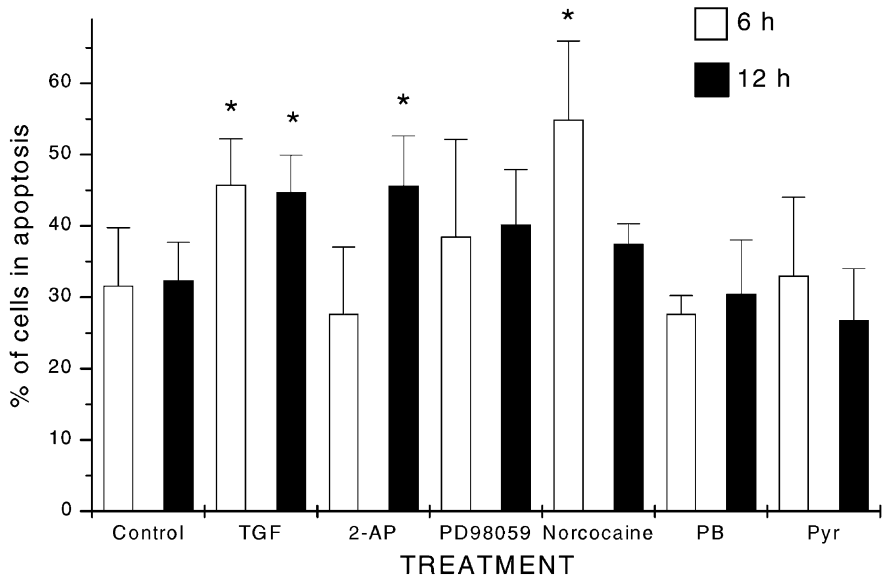

Fig. 4. Apoptosis in control hepatocytes or after treatment with TGFβ, 2-aminopurine (2-AP), PD98059, norcocaine, phenobarbital (PB) and pyrazole (Pyr) 6 and 12 hr after treatment. The symbol (∗) indicates a statistically significant value ($P < 0.05$). Error bars represent standard deviations.

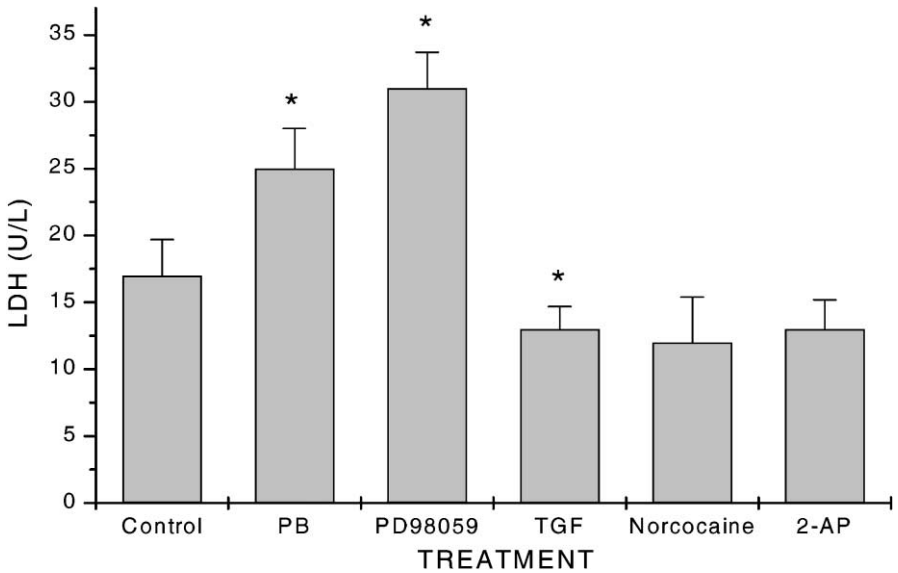

Fig. 5. Lactate dehydrogenase (LDH) leakage to the culture medium at 12 hr as a marker of direct cytotoxicity. The mean of control sample was 19 U/L. The symbol (∗) indicates a statistically significant value ($P < 0.05$). Error bars represent standard deviations.

cytotoxicity. The mean level of lactate dehydrogenase in the medium of untreated control sample at the 12 hr time-point was 19 U/L. Phenobarbital and PD98059 elicited statistically significant elevations in LDH levels. TGFβ caused a slight but significant reduction in lactate dehydrogenase levels. Pyrazole has previously been shown to be non-cytotoxic in these conditions [26].

4. Discussion

There appears to be an association between altered hepatocyte growth control and induction of CYP2A5. The most compelling example of this is the sequential increase in the amount of CYP2A5 protein in preneoplastic foci, adenomas, and carcinomas in murine liver [10]. The ability of several inducers of hepatic CYP2A5, such as carbon tetrachloride, cadmium, thioacetamide, and cocaine [4,20–23] to induce apoptosis in hepatocytes is a striking association. Cocaine and its metabolite norcocaine are known to cause liver damage in humans [27] and the mouse [28,29]. As shown in this study, however, apoptosis and CYP2A5 expression in mouse hepatocytes are not correlated to each other.

The primary hepatocyte model presented here for determination of altered apoptosis rate allows testing of agents that cannot be administered *in vivo*, due to their systemic toxicity or prohibitively high cost. In this system, hepatocytes

in culture responded readily to TGFβ, indicating that proapoptotic signalling pathways have remained operative in the cells. In addition, the relatively high basal rate on apoptosis (from 12 to 30% during the 12 hr observation period) makes it possible to study also anti-apoptotic effects. In this model apoptosis occurred quickly, peaking at 6–12 hr, while the induction of CYP2A5 takes substantially longer, usually from 24 to 96 hr [15]. If a low basal rate of apoptosis is desirable, culture plates can be coated with an extracellular matrix, e.g. collagen [25].

The present study was prompted by the finding that the cell cycle perturbing agent 2-aminopurine strongly upregulates CYP2A5 in hepatocytes. The results of this study show that out of the five well-defined CYP2A5 inducers tested, only 2-aminopurine consistently increased the rate of apoptosis, while the rest of the inducers did not significantly affect it. On the other hand, the most efficient inducer of apoptosis, TGFβ, strongly suppressed CYP2A5 catalytic activity. Thus, although many CYP2A5 inducers are proapoptotic *in vivo*, this study showed that there are also CYP2A5 inducers that have no significant effect on apoptosis in primary hepatocytes. This study extends the previous findings [15] by showing 2-aminopurine treatment dramatically elevates the amount of steady-state CYP2A5 mRNA, indicating either increased *Cyp2a-5* gene transcription or mRNA stabilisation [30].

Phenobarbital decreases the rate of cell death both in normal liver and hepatic tumours, thus, accelerating tumour progression [31]. The mechanism of apoptotic inhibition by phenobarbital is thought to be by increasing the levels of bcl-2 protein which is an inhibitor of apoptosis [25,32]. Interestingly, TGFβ-induced apoptosis can be inhibited by phenobarbital [25]. We have previously demonstrated that CYP2A5 is induced by phenobarbital in unique way compared to other CYP enzymes, like CYP2B10 and CYP1A1 [15].

These results indicate that in a primary hepatocyte model (a) there is no systematic correlation between apoptosis and CYP2A5 induction; (b) phenobarbital does not affect the rate of apoptosis; and (c) the induction of apoptosis caused by the chemicals tested occurs considerable earlier than elevation of CYP2A5 enzyme activity. Thus, no causal link appears to exist between induction of CYP2A5 and apoptotic rate. This does not rule out the possibility that these two phenomena are co-regulated at some common level. Our current studies are aimed at clarifying such mechanisms.

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