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ZONATION OF ACETAMINOPHEN METABOLISM AND CYTOCHROME P450 2E1-MEDIATED TOXICITY STUDIED IN ISOLATED PERIPORTAL AND PERIVENOUS HEPATOCYTES

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Abstract—To study the mechanism of centrilobular damage developing in the centrilobular region after high doses of acetaminophen (APAP), its metabolism and toxicity were compared in periportal and perivenous hepatocytes isolated by digitonin/collagenase perfusion. Contrary to earlier reports, based on perfusions, no evidence for a periportal dominance of APAP sulfation could be observed. Glucuronidation, the dominant pathway of conjugation at high (5 mM) APAP concentration, was faster in perivenous cells. During primary culture, prolonged exposure (≥ 24 hr) to 5 mM APAP damaged perivenous cells, with a higher P450 2E1 level than periportal cells. When cells were isolated from ethanol-pretreated rats, to induce P450 2E1 levels specifically in the perivenous region, perivenous hepatocytes exhibited enhanced APAP vulnerability and extensive glutathione depletion. In contrast, corresponding periportal cells retained good viability. Isoniazid, an inhibitor of cytochrome P450 2E1, protected cells against APAP toxicity and prevented glutathione depletion. Induction of P450 2E1 also caused a 3-fold increase in the covalent binding of reactive intermediates from [^{14}C]APAP, and this increase was mainly confined to perivenous cells. These results indicate that in rat liver there is only slight perivenous zonation of APAP conjugation and suggest that zone-specific APAP activation, mediated by the regional expression of ethanol-inducible cytochrome P450 2E1, is responsible for the characteristic centrilobular liver damage elicited by APAP.

Hepatic failure after an overdose of the analgesic acetaminophen (APAP,‡ paracetamol) continues to be a major problem, especially in the U.S. and the U.K., where this drug is widely used. Although the mechanisms of APAP-induced damage have been studied extensively [1–3], the enzyme systems responsible for the initiation processes are not well understood. APAP toxicity develops e.g. when detoxification mechanisms, mainly glutathione (GSH)-mediated, become insufficient to cope with the toxic insult. A regional imbalance between activation and detoxication capacities probably forms the basis for the centrilobular damage typically elicited by APAP. In order to study these relations, we have compared conjugation and activation mechanisms in hepatocytes isolated from the periportal (pp) or the perivenous (pv) (centrilobular) part of rat liver. About 80% of an ingested dose of APAP is conjugated with glucuronic acid and sulfate before being excreted [3]. Less than 5% may be activated via a cytochrome P450-catalysed reaction to the highly reactive toxic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [2, 4]. This electrophile binds covalently to macromolecules and causes GSH depletion, oxidative stress and changes in the calcium and/or thiol status [5–7]. The importance of the cellular GSH status in APAP toxicity is evident from studies demonstrating that interventions which deplete GSH or inhibit its redox cycling potentiate toxicity, whereas compounds that sustain cellular GSH metabolism, such as the clinically used *N*-acetylcysteine, protect against toxicity [8–10].

In humans primarily the 2E1 and 1A2 forms of cytochrome P450 appear to be responsible for APAP bioactivation [11]. The importance of the ethanol-inducible 2E1 form is reflected in the observation that chronic alcohol consumption leads to enhanced APAP toxicity [12, 13]. *In vitro* studies, in which normally resistant hepatocytes exhibited a toxic response after induction of P450 by phenobarbital [8] suggested a role for the 2B form, but according to other studies the capacity of the 2B1 form for APAP oxidation is very low [14]. Knowledge of the relative involvement of different P450 isozymes is still incomplete and is also complicated by differences between species.

In this study we have taken advantage of the recently acquired knowledge on the regional distribution of the expression of various cytochrome P450 isozymes [15–17]. We have focussed on the ethanol-inducible P450 2E1 form, since it appears to be of special toxicological importance [18, 19] and since it is almost exclusively expressed in the centrilobular region [15]. The influence of a possible heterogenous detoxification capacity in the regioselective toxicity of APAP was also evaluated by comparing rates of sulfation and glucuronidation

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‡ Abbreviations: APAP, acetaminophen; GSH, glutathione; NAPQI, *N*-acetyl-*p*-benzoquinone imine; LDH, lactate dehydrogenase; BSO, buthionine sulfoximine; pp, periportal; pv, perivenous.

in isolated pp and pv hepatocytes. Previous data on zonation of sulfation are conflicting [20–22], possibly depending on the experimental approach and on substrate specificity, whereas studies on glucuronidation suggest that this process may preponderate in pericentral areas [20, 21].

In the present study we have compared isolated pp or pv hepatocytes with respect to events of importance for regional toxicity expression. The results show that cytochrome P450 2E1 plays an important role in the initiation of APAP toxicity in the pv region, while differences in conjugation activities seem to be of minor importance.

MATERIALS AND METHODS

Male Wistar rats (150–220 g body weight, given food and water *ad lib.*) were used throughout. Some animals were provided with ethanol (3–12% v/v) in the drinking water for 2 weeks as described previously [23]. The mean daily intake of ethanol was 8.3 ± 1.2 g and weight gain was normal. A moderate ethanol treatment was chosen to avoid any cellular damage that could complicate the interpretation of the culture experiments. For short-term experiments, P450 2E1 induction was obtained by giving acetone (5 mL orally/kg) once daily for 2 days to rats starved during this period (48 hr).

Hepatocytes from the pp or pv region were isolated by the digitonin–collagenase perfusion technique described in detail elsewhere [24, 25]. Viability as determined by exclusion of 0.05% eosin was typically about 90% immediately after isolation. Cells were seeded at a density of 6×10^4 cells/cm^2 on 1.5 diameter culture dishes in Krebs–Ringer buffer supplemented with fetal calf serum (5%), newborn calf serum (5%), insulin (80 ng/mL), glucagon (1.8 ng/mL), dexamethasone (0.1 μM), gentamicin (50 μg/mL) and nystatin (1 μg/mL). After 4 hr of preincubation in the humidified atmosphere of air:CO_2 (95:5%), unattached cells were removed by exchanging the plating medium to serum-free buffer. Acetaminophen (Sigma Chemical Co., St Louis, MO, U.S.A.), isoniazid and ethanol were added at indicated concentrations. Cell viability was assessed from dye exclusion (0.05% eosin) and from lactate dehydrogenase (LDH) release into the medium. Activity of glutamate synthetase and alanine aminotransferase was determined as described previously [26].

Intracellular GSH content of cultured hepatocytes and of cell suspensions was quantitated fluorometrically [27]. Formation of APAP glucuronide and sulfate conjugates was assayed during incubation in Krebs–Ringer buffer of a suspension of freshly isolated pp or pv cells (20 mg/mL). Aliquots were periodically withdrawn, deproteinized with 3 N perchloric acid and metabolites analysed by HPLC [28]. The covalent binding of [^{14}C]APAP (9.7 mCi/mmol, Sigma) to total cell protein was measured during incubations of pp or pv cells with 5 mM APAP and 2 μCi/mL of [^{14}C]APAP. Aliquots precipitated with 3 N perchloric acid were washed with methanol, methanol–ether, ethylacetate and methanol again, and then dissolved in NaOH [29]. Protein was measured (see Ref. 30) and the amount of radioactivity counted by liquid scintillation.

All experiments were performed at least three times. Data presented are means ± SD and statistical significance was determined by Student's *t*-test (non-matched pairs).

RESULTS

The separate acinar origins of the two cell populations were ascertained by means of marker enzymes [15]. The activity of the pp marker alanine aminotransferase was 2.1-fold higher in pp cells than in pv cells. The pv marker glutamine synthetase had a 78-fold higher activity in pv cells as compared to pp cells. These results are in agreement with previous reports [15, 19], and demonstrate a good separation of pp and pv cell preparations.

Rates of sulfation and glucuronidation of APAP were compared in freshly isolated pp or pv cells. Generally, pv cells appeared initially to conjugate APAP faster (Fig. 1). At a low APAP concentration (0.05 mM), sulfate conjugation exceeded glucuronidation in both pp and pv cells. Conversely, at high APAP concentration (5 mM), glucuronidation was faster than sulfation in both pp and pv cells. Sulfate conjugation appeared to be already maximal at APAP doses of 0.05 mM.

During the first 15 min of incubation, APAP sulfation was significantly ($P \leq 0.01$) faster in pv cells both at 0.05 and 5 mM concentrations while glucuronidation was faster only with 5 mM APAP. Upon prolonged incubation both sulfation and glucuronidation appeared to proceed at approximately equal rates in pp and pv cells, and no significant differences could be observed.

Cultured pp and pv cells isolated from either control or ethanol-pretreated rats and exposed to 2 mM APAP showed no loss of viability for up to 24 hr of incubation (Fig. 2). Cytotoxic effects of APAP were observed when the dose was increased to 5 mM, but only in pv cells. Increasing the concentration of APAP to 10 mM resulted in the development of damage within 24 hr in both pv and pp cells.

Chronic treatment of rats with ethanol results in induction of cytochrome P450 2E1, seen both at the protein and enzyme level [15, 17]. Ethanol pretreatment by addition to the drinking water for 2 weeks, as used in the present study, results in an approximately 3-fold increase in the amount of 2E1 protein [9, 31]. In the present experiments induction was measured catalytically as demethylation of nitrosodimethylamine in isolated microsomes [32], and was found to be about 3-fold: 0.84 ± 0.40 (nmol/min/mg microsomal protein) in control livers and 2.28 ± 0.97 in liver from ethanol-pretreated rats. Analysis of western blots of lysates from pp and pv cell preparations confirmed our earlier observations [15, 19, 31] that the ethanol-induced induction was confined primarily to the pv region (data not shown).

Ethanol pretreatment seemed to selectively sensitize pv cells towards APAP cytotoxic effects. During culture, pv cells were severely damaged after 15 hr of exposure to 5 mM APAP (Fig. 2). After 24 hr of APAP exposure, about 70% of pv cells from

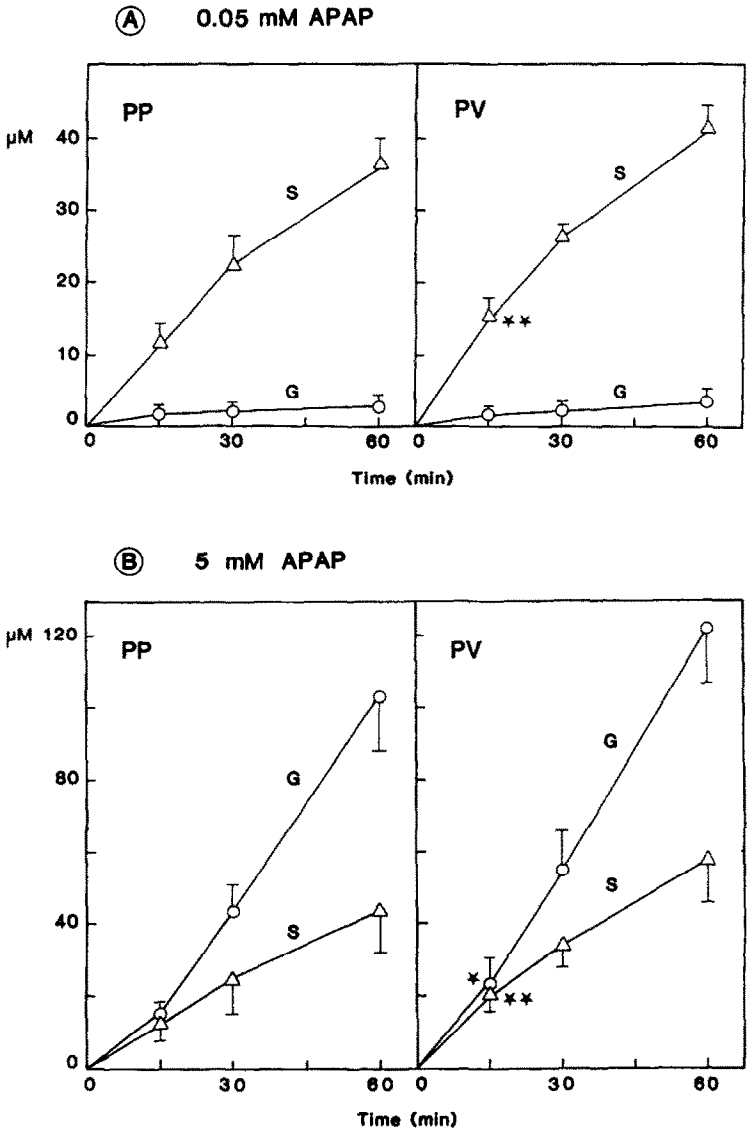

Fig. 1. Glucuronidation and sulfation of APAP in isolated pp and pv cells. Glucuronide (G) and sulfate (S) conjugates of 0.05 mM APAP (upper panels) and 5 mM APAP (lower panels) were assayed by HPLC. Means ± SD of incubations with 4–6 preparations of pp and pv cells are shown. *$P \leq 0.05$ and **$P \leq 0.01$ for pp/pv difference.

ethanol-pretreated rats were dead, while only about 25% of correspondingly treated control pv cells were killed. Under these conditions cells isolated from the pp region remained viable.

The differential toxicity of APAP in pp and pv cells was also reflected in the extent of intracellular GSH depletion. While 2 and 5 mM APAP had little effect on GSH levels in pp cells, an extensive depletion of GSH was observed in pv cells (Fig. 3). However, when cells were exposed to 10 mM APAP the GSH content was lowered to almost the same extent in both pp and pv cells. The effect of APAP exposure on GSH levels appeared to be similar in hepatocytes isolated from control and ethanol-pretreated rats, although GSH levels in pp cells from ethanol-treated animals appeared to be somewhat lowered also by exposure to 2 and 5 mM APAP.

Isoniazid (2 mM), an inhibitor of the cytochrome P450 2E1 form [33], completely prevented APAP toxicity, and counteracted the decrease in GSH levels observed primarily in pv cells from both control and ethanol-pretreated rats (Fig. 4). Ethanol (50 mM), which may act as a competitive inhibitor of P450 2E1 [34], delayed the onset of APAP toxicity but had no effect on the extent of GSH depletion (Fig. 4).

The relationship between GSH metabolism and APAP toxicity was investigated in further detail by treating cells with buthionine sulfoximine (BSO, an inhibitor of glutathione synthetase [35]), which

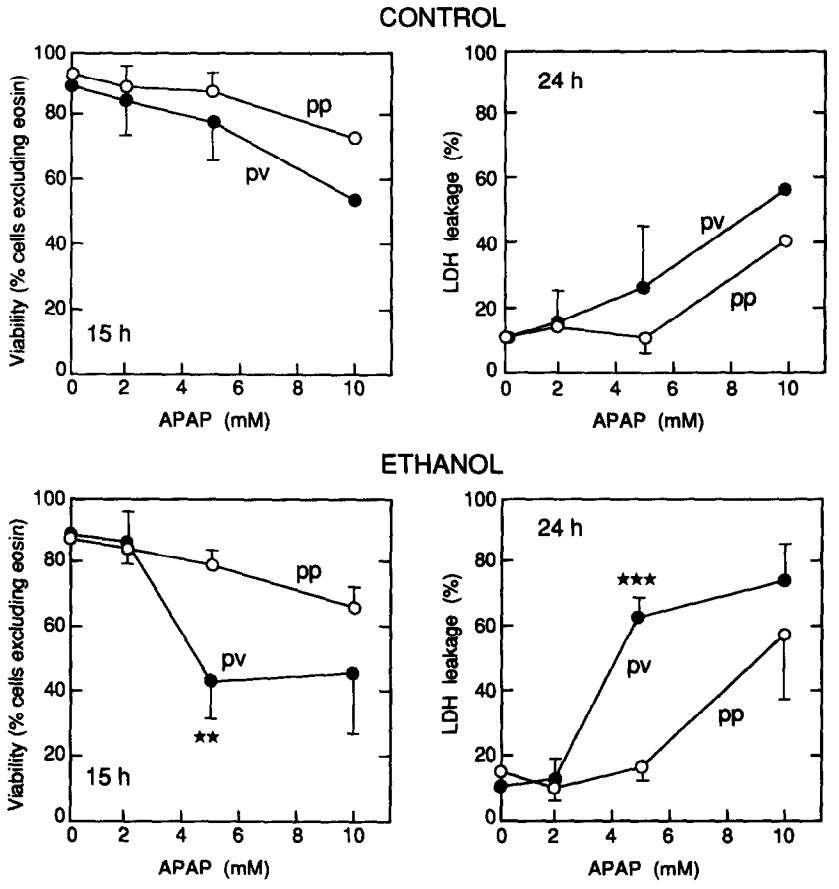

Fig. 2. Concentration-dependent cytotoxicity of APAP in cultured pp and pv cells. Isolated pp or pv cells were exposed to APAP for 15 (left panels) or 24 hr (right panels) during primary culture. Cell integrity was expressed as LDH leakage or as percentage of cells excluding eosin. These two parameters of viability correlated well. The data are means ± SD from 3 or 4 different cell preparations obtained from control (upper panels) or ethanol-treated animals (lower panels). **P ≤ 0.01 and ***P ≤ 0.001 for pp/pv difference.

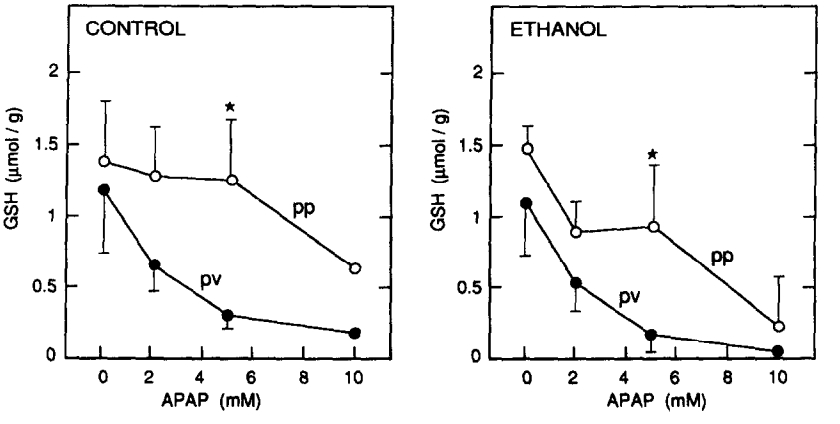

Fig. 3. APAP-induced depletion of GSH in cultured pp and pv cells. Cells isolated from control or ethanol-pretreated rats were exposed to APAP for 24 hr in primary culture. The data are means ± SD of 3–4 experiments. *P ≤ 0.05 for pp/pv difference.

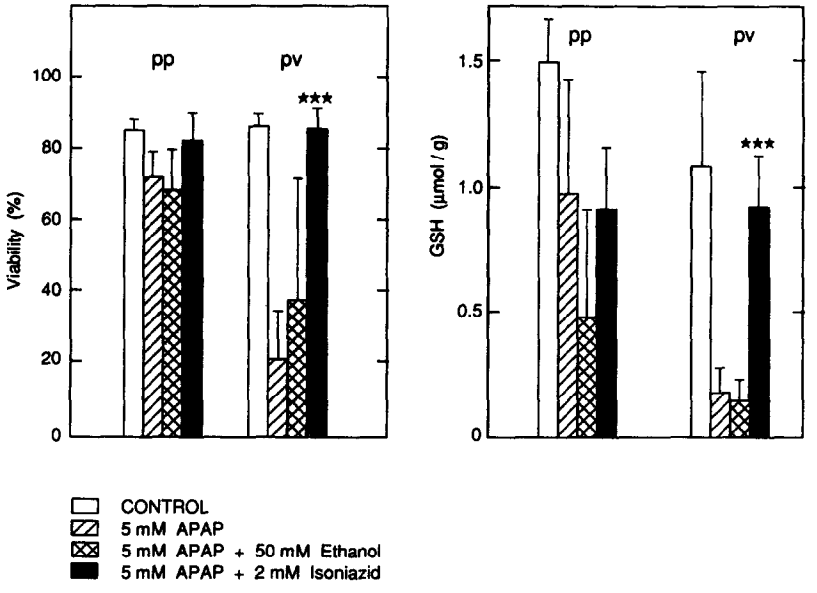

Fig. 4. Effect of isoniazid and ethanol on APAP-induced cell damage and GSH levels. Cells isolated from ethanol-pretreated animals were exposed to APAP and ethanol or isoniazid for 24 hr in primary culture. Means ± SD (bars) of 4 experiments are given. ***$P \leq 0.001$ for effect of isoniazid.

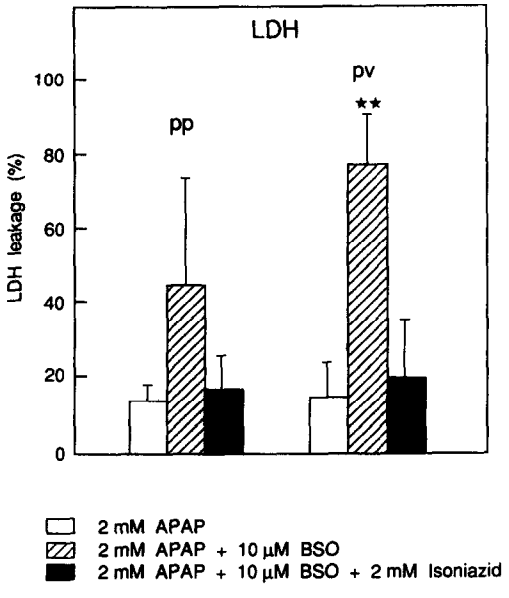

Fig. 5. Enhancement by BSO of APAP cytotoxicity in pp and pv cells. The effect of BSO or isoniazid on APAP toxicity during culture for 24 hr was investigated in cells from control rats. Means ± SD (bars) of 3 experiments. **$P \leq 0.01$ for the potentiating effect of BSO and for the counteraction by the additional presence of isoniazid.

rapidly depleted GSH levels in both pp and pv cells by about 90%. In the presence of BSO the toxicity of APAP was potentiated in pv as well as in pp cells, seen both as an extensive leakage of LDH (Fig. 5) and with phase contrast light microscopy (Fig. 6). In pv cells exposure to APAP and BSO caused heavy granulation of hepatocytes and many cells had lost or reduced attachment to the matrix (Fig. 6) while large areas of corresponding preparations of pp cells exhibited normal morphology. After 24 hr with 2 mM APAP about 40% of pp cells were dead compared to 80% of pv cells while in the absence of BSO cells remained viable (Fig. 5). Additional experiments demonstrated a similar effect of BSO in pp and pv cells isolated from ethanol-pretreated rats (data not shown). The sensitizing effect of BSO was completely prevented by the addition of isoniazid. No increase in LDH leakage was observed, and both pp and pv cells excluded eosin and looked morphologically similar to control cells (Fig. 6).

The relationship between induction of P450 levels and the production of reactive APAP intermediates was also investigated by measuring covalent binding to total protein in control and in induced pp and pv cells. For this purpose, cytochrome P450 2E1 levels were induced with a short treatment with acetone, which caused an about 5–6-fold increase in 2E1 protein levels. Covalent binding was significantly enhanced in both pp and pv cells as compared to corresponding cells from control animals (Fig. 7). The extent of this covalent binding was about 30% higher in pv than in pp cells from acetone-induced rats. Isoniazid (2 mM) inhibited the covalent binding of reactive APAP metabolites by about 30 and 50% in pp and pv cells, respectively.

DISCUSSION

In the present study we have used preparations of intact hepatocytes isolated from the pp or pv region

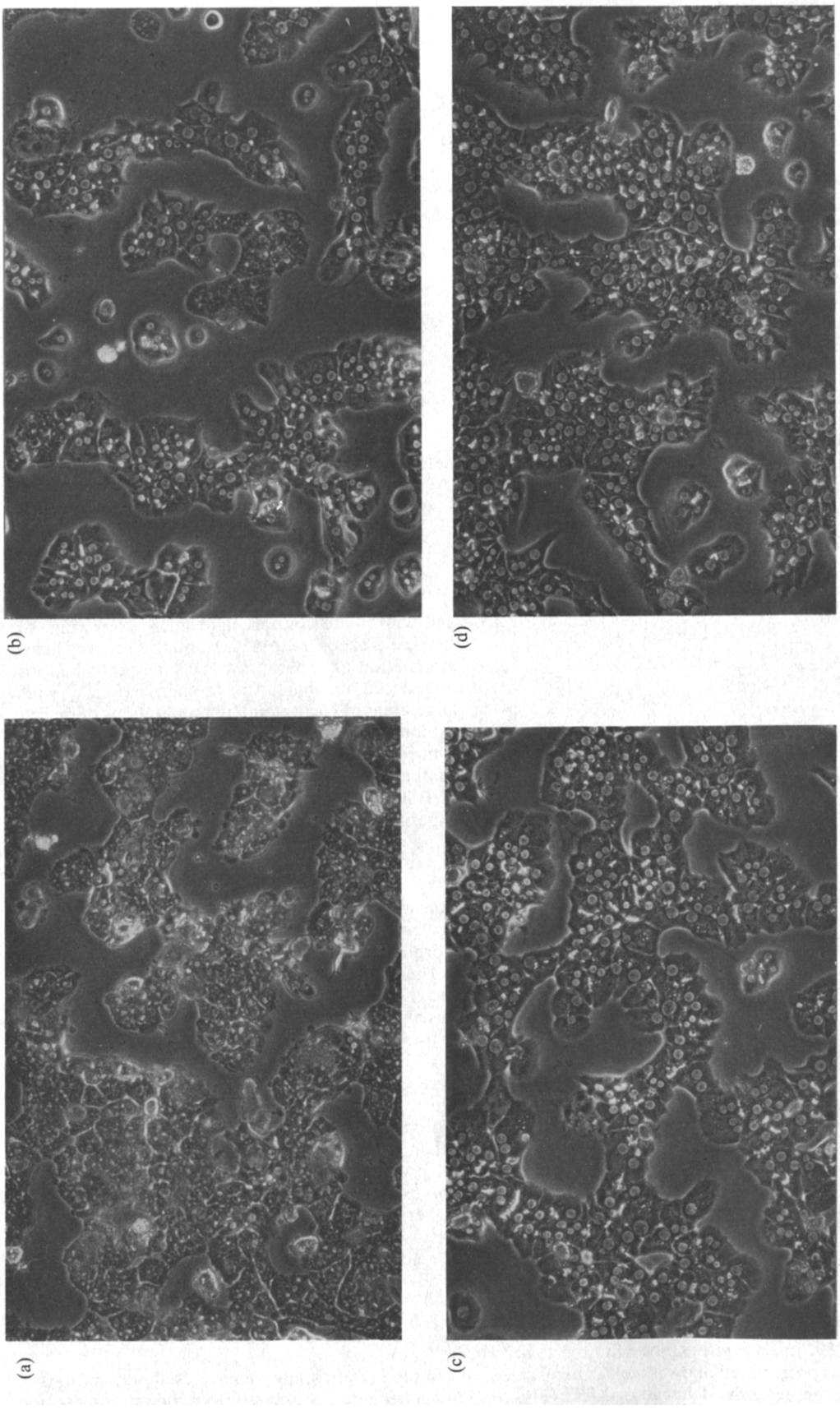

Fig. 6. Phase contrast light micrographs of pp and pv cells in culture. Protection against BSO-potentiated APAP toxicity by isoniazid. After exposure of cultures to 2 mM APAP and 10 μM BSO for 24 hr only about 20% of pv hepatocytes excluded eosin; most cells appeared granulated with diffuse boundaries and had started to disattach (A). In contrast, similarly treated pp cells were much less damaged and most cells retained distinct nuclei and cellborders (B). Addition of isoniazid prevented these changes both in pv (C) and pp (D) cells.

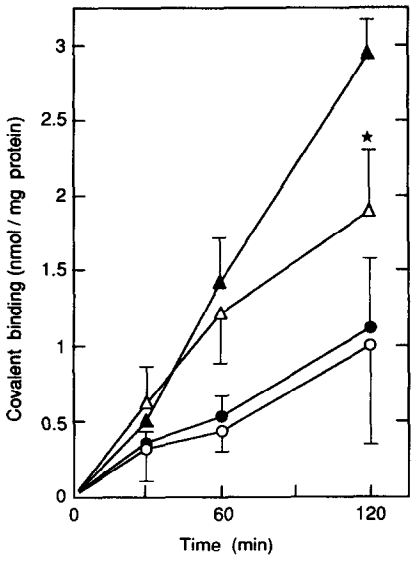

Fig. 7. Effect of induction of cytochrome P450 2E1 on covalent binding of APAP to protein in pp and pv cells. Cells from either the perivenous (filled symbols) or periportal (open symbols) region were isolated from control rats (circles) or from acetone-treated rats (triangles) to induce P450 2E1 and then incubated with 5 mM ^{14}C-labelled APAP. Means ± SD of 3–4 experiments. *$P \leq 0.05$ for pp/pv difference.

as a way to compare directly the capacity of activation and conjugation of APAP in the pp and pv liver region. While neither sulfation nor glucuronidation seemed to exhibit any major zonation, several pieces of evidence indicate a crucial role for cytochrome P450 2E1 in activation and initiation of damage in the pv region. Thus, hepatocytes isolated from the pv region, which have a higher expression of several P450 forms, including 2E1 [16–18], are more sensitive to damage from exposure to APAP than corresponding cells isolated from the pp region. The difference in vulnerability most probably reflects differences in the rate of activation of APAP to the highly reactive toxic intermediate NAPQI. That this activation of APAP is mediated largely via P450 2E1 is suggested from the fact that the difference in vulnerability between pv and pp cells is enhanced when cells are isolated from chronically ethanol-pretreated rats. Chronic administration of ethanol not only causes specific induction of the 2E1 form, but this induction occurs almost exclusively in the pv or centrilobular region. Furthermore, the damage by APAP could be prevented by the addition of isoniazid, which has been demonstrated to act as a rather specific inhibitor of the P450 2E1 form [33]. Addition of ethanol (50 mM) also protected against APAP toxicity, although less efficiently than isoniazid. Ethanol may protect not only by competitively inhibiting P450 2E1 [34], but also by modulating the redox state in the cell [36]. Interestingly, ethanol, which by itself did not affect GSH, did not, in contrast to isoniazid, prevent the depletion of GSH associated with APAP toxicity, suggesting that protection by ethanol indeed occurs partly by different mechanisms as compared to isoniazid.

Although the present data suggest a crucial role for the ethanol-inducible 2E1 form, the involvement of other P450 forms should not be ignored and large species differences in P450 isozyme patterns exist. In human liver microsomes, which resemble rat liver microsomes with respect to the P450 expression pattern, the 1A2 form, in addition to the 2E1 form, activated APAP [10]. However, the affinity of the 1A form for APAP may be lower than that of the 2E1 form, but in rabbit livers the 2A1 form has a higher affinity for APAP than the 2E1 form [34]. Although several, but not all [37] studies indicate that pretreatment of rats with phenobarbital potentiates APAP toxicity, the purified P450 2B1 form has a low capacity for oxidation of APAP [14], suggesting the involvement of confounding factors after phenobarbital treatment. It is obvious that isozyme specificity and affinity for APAP oxidation varies a lot between species and that the role of the 2E1 form may be particularly important in rats and humans.

Local differences in vulnerability to APAP toxicity should reflect a regional imbalance between toxification and detoxification reactions. Although removal of NAPQI may be the most critical detoxification event, quantitatively sulfation and glucuronidation of APAP represent the most important reactions.

In the present experiments, using both high and low concentrations of APAP, we saw no evidence for a periportal dominance of sulfation. This finding is at variance with previous studies based on the perfused liver model [20, 22], but confirms previous observations from our laboratory [21]. Because the quantitative contribution of nonparenchymal cells to APAP conjugation appears to be low [38], the present hepatocyte model should reflect the zonation in the intact liver and afford a more direct comparison as compared to indirect data on uptake and conjugation kinetics in the perfused organ. However, data on the zonal distribution of key sulfation enzymes would aid in estimating the zonation of sulfation processes. At therapeutic low doses of drugs, the high-affinity sulfation system will compete efficiently with glucuronidation, while the latter may dominate at high and toxic concentrations of substrate. Our present results indicate that glucuronidation of APAP may not be significantly zonated at low concentrations, while at high concentrations a low-affinity pv glucuronidation system may become the dominating pathway. Pv dominance of glucuronidation has been observed previously, using hydroxycoumarin [20], methylumbelliferone [21] or resorufin [39] as substrate.

In addition to glucuronidation and sulfation of APAP, the GSH-mediated conjugation and detoxification pathways are important [9]. Pv cells replenish GSH slower than pp cells [40]. This may reinforce the zonal APAP toxicity due to the pv expression of P450 2E1. The potentiation of APAP toxicity by BSO was more evident in pv cells, a finding that also demonstrates the interdependence between GSH status and toxicity. GSH is reduced

mainly by conjugating to NAPQI, and the rate of depletion of GSH therefore probably reflects the extent of APAP activation. The fact that the pv cells exhibited faster covalent binding of APAP as compared to pp cells only after 2E1 induction suggests involvement of alternative pathways. Glutathione *S*-transferase activity is higher in the pv region [41, 42], and GSH depletion by APAP is faster in pv cells, suggesting that faster NAPQI conjugation with GSH could counteract faster production in uninduced cells. The similar pattern of GSH depletion in cells from control and ethanol-pretreated animals suggests that the potentiation of pv cell death after ethanol pretreatment reflects the induction of P450 2E1 and is secondary to differences in GSH metabolism. We conclude that the regiospecific expression and induction of cytochrome P450 2E1 is of major importance in explaining the centrilobular damage typically elicited by a high dose of APAP.

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