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Cocaine hepatotoxicity: a study on the pathogenesis of periportal necrosis

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Summary. Cocaine is reported to produce either periportal or mid-zonal necrosis in mice pretreated with the enzyme inducer phenobarbitone (James *et al.* 1987; Powell *et al.* 1991; Charles & Powell 1992). Dose–response and time course experiments were performed in phenobarbitone treated male DBA/2Ha mice to study the pathogenesis of this unusual cocaine induced lesion. An increase in the dose of cocaine from 60 to 90 or 120 mg/kg produced more extensive and severe periportal and linking portal damage and elevated plasma aspartate (AST) and alanine (ALT) aminotransferases in a dose dependent manner.

Scattered hepatocyte degeneration began at the edge of the periportal region and was detectable by electron microscopy within 30 minutes of administration of 60 mg/kg of cocaine, with conspicuous disorganization of the endoplasmic reticulum being one of the earliest changes. Significant elevations of plasma AST and ALT were observed 3 hours after cocaine administration and were sustained for 12 hours, at which time progressive hepatocyte damage had developed into a network of confluent necrosis at the periphery of the periportal region.

The rapidity of organelle derangement and subsequent cell death, and absence of any effect on total cytochrome P-450 or FAD-mono-oxygenase levels, appear to distinguish this periportal lesion from previous reports of cocaine induced centrilobular necrosis in non-enzyme induced mice, suggesting that the two types of damage may develop by different mechanisms. The observation that periportal lesions commence at the periphery of the periportal area, progressing portalwards with increasing dose and time, offers an explanation for the previously conflicting reports of cocaine induced mid-zonal and/or periportal lesions in phenobarbitone treated mice.

Keywords: cocaine, hepatotoxicity, periportal necrosis, mid-zonal necrosis, liver damage

Cocaine abuse is escalating in the UK, across Europe and in the USA, where 30 million people are estimated to have been exposed to the drug (Ivor-Povenmire & House 1989). Liver damage in cocaine abusers has often been attributed to viral hepatitis or alcoholism, but clinical evidence has been presented to show that large doses of cocaine alone can be responsible (Perino *et al.* 1987;

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Wanless *et al.* 1990; Kanel *et al.* 1990). In contrast, the hepatotoxicity of cocaine in the mouse is well established (Shuster *et al.* 1977; Evans & Harbison 1978), with the DBA/2Ha strain being particularly susceptible (Kloss *et al.* 1982).

The histological pattern of liver damage induced experimentally by cocaine is unusually variable and has been termed 'shifting necrosis', a feature which has probably contributed to uncertainty in the clinical recognition of cocaine hepatotoxicity. A single dose of 60 mg/kg cocaine i.p. produces centrilobular necrosis (acinar zone 3) in male DBA/2Ha mice (Kloss *et al.* 1982), but following the administration of the same dose after pretreatment with the liver enzyme inducer sodium phenobarbitone, the lesions which develop are either mid-zonal (zone 2) (Powell *et al.* 1991; James *et al.* 1987) or periportal (zone 1) (Evans & Harbison 1978; James *et al.* 1987; Roth *et al.* 1992; Thompson *et al.* 1984) and exclude centrilobular hepatocytes. In DBA/2Ha mice pretreated with phenobarbitone we noted that 60 mg/kg cocaine produced necrosis in a narrow but confluent band of cells, usually twice as far from central veins as from portal tracts, that is, at the junction of acinar zones 1 and 2 (Powell *et al.* 1991). The aim of the present study was to reconcile reports of mid-zonal and/or periportal cocaine induced damage. Dose–response and time course studies were employed to examine the initial cellular target, and to determine whether the pathogenesis of the lesion might offer an explanation for earlier discrepant reports.

The time frame and sampling interval used in time course studies are crucial. Most previous studies of cocaine hepatotoxicity have only been made 24 hours after a single dose, a time at which liver damage would be expected to be well developed. Our initial objective was to determine the *first* cellular target of cocaine hepatotoxicity and to characterize the early biochemical and ultrastructural changes which accompany it. An advantage of early examinations is that they minimize the complication of secondary and inflammatory effects.

A preliminary report of some of these experiments was presented at the VIth International Congress of Toxicology (Charles & Powell 1992).

Materials and methods

Animals

Male DBA/2Ha mice (Charles River Ltd, Margate, Kent, UK) 20–24 g body weight, were allowed powdered food (SDS Ltd, Witham, Essex) and water *ad libitum*. Animals were randomly allocated to experimental groups and were allowed to acclimatize to the following environmental conditions prior to the commencement of any treatment: 20 ± 2°C, relative humidity 50 ± 10%, a 12-hour light/dark cycle and 10–12 air changes per hour.

Experimental design

In a preliminary experiment, 4 groups, each of 3 mice, received daily intraperitoneal doses of sodium phenobarbitone (80 mg/kg, BDH, Poole, Dorset, UK) dissolved in saline, for 3 days, while a further group of 3 received an equivalent volume of saline (0.9% w/v). Twenty-four hours later, 3 of the 4 sodium phenobarbitone treated groups received a single intraperitoneal dose of 60, 90 or 120 mg/kg (177, 265 or 353 mmoles/kg) cocaine hydrochloride (purity > 99%, Sigma Chemical Co., Poole, Dorset, UK). The two remaining groups received an equivalent volume of saline and served as phenobarbitone or saline treated controls. Twenty-four hours after cocaine or saline administration, animals were killed by CO_2 asphyxiation and terminal blood and liver samples were taken. Plasma was separated and AST and ALT levels were measured using a Cobas Bio Autoanalyser, with kits from Roche Diagnostics. Samples of each of the four major liver lobes were fixed in 10% neutral buffered formalin, processed into paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin. In a subsequent experiment, 9 groups, each of at least 6 mice, were similarly pretreated with sodium phenobarbitone i.p. once daily for 3 days, while a further group of 6 received an equivalent volume of saline (0.9% w/v). Twenty-four hours later, 8 of the 9 sodium phenobarbitone treated groups received a single i.p. dose of 60 mg/kg cocaine hydrochloride. The two remaining groups received an equivalent volume of saline, and served as phenobarbitone or saline treated controls. One group of cocaine treated animals was killed after 30 minutes by CO_2 asphyxiation, and the remaining groups were killed after 1, 2, 3, 4, 6, 9 or 12 hours, animals from both control groups being killed either 4 or 9 hours after the last administration of saline. Samples of blood and liver were taken for plasma enzyme analysis and histopathology respectively, as before. In addition, liver samples were embedded in resin, sectioned at 1 μm, and stained with toluidine blue, to be surveyed before selected areas of liver were processed for electron microscopy. Hepatic microsomes were prepared from the remaining liver of pairs of mice (Mann *et al.* 1985), and the protein content estimated (Lowry *et al.* 1951). Cytochrome P-450 content was measured by comparing the reduced, carbon monoxide complexed form giving a spectral maximum at 450 nm, with the reduced uncomplexed

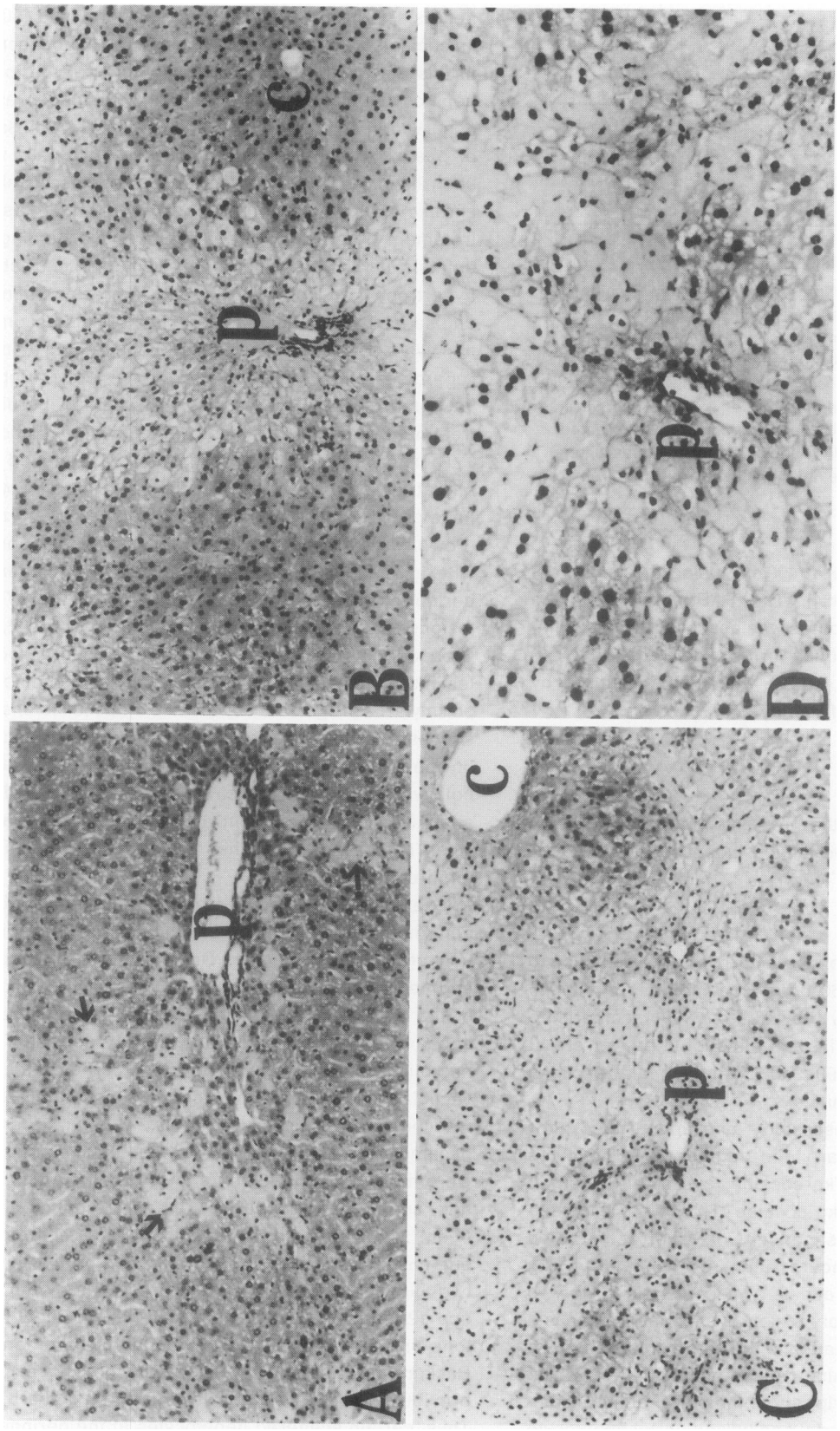

Figure 1. The dose-related progression from 'peripheral portal' to periportal hepatocyte necrosis 24 hours after i.p. administration of cocaine to phenobarbitone treated DBA/2Ha mice. A, 60 mg/kg cocaine, necrosis of scattered, pale hepatocytes (arrowed) was visible at the periphery of the periportal area, i.e. peripheral portal necrosis. B, 90 mg/kg cocaine, more extensive vacuolar degeneration and necrosis of periportal cells was seen, leaving centrilobular hepatocytes largely intact. C, 120 mg/kg cocaine, extensive periportal necrosis with pale, anucleate hepatocytes extending at least halfway across the lobule, with only slight vacuolation of centrilobular hepatocyte cytoplasm. D, Enlargement of a periportal area similar to that in (C) showing pale, swollen, necrotic hepatocytes lacking nuclei, but sinusoidal cells with nuclei present. p, Portal tract; c, central vein. Haematocylin and eosin stained sections. A, B and C × 32, D × 64.

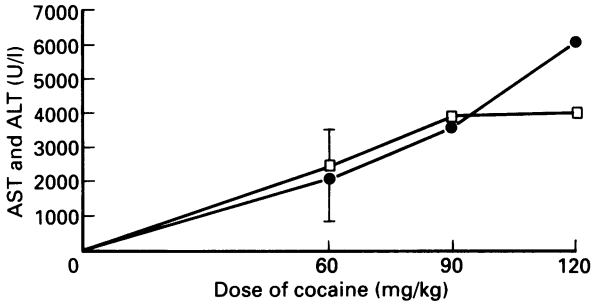

Figure 2. Response of plasma ●, aspartate and □, alanine aminotransferases (AST and ALT) following i.p. administration of 60, 90 or 120 mg/kg cocaine to phenobarbitone treated DBA/2Ha mice. Values are the means, ± s.e.m. where possible, for at least 2 mice per dose level. Mean control values ± s.e.m. for AST and ALT were 41.2 ± 9.1 and 20.8 ± 3.1 U/l respectively for saline treated mice, and 31.7 and 23.4 U/l respectively for phenobarbitone treated mice. Values were not significantly different from either control group.

form giving a baseline at 490 nm, using the method of Omura and Sato (1964). The FAD-mono-oxygenase activity of liver microsomes was assessed by determining their ability to produce an *N*-oxide from *N*-ethyl-*N*-methylaniline *in vitro*, which was then measured by HPLC by the method of Hadley *et al.* (1993).

Statistical analysis

The significance of the intergroup differences was evaluated using the two-tailed Student's *t*-test.

Results

The clinical reactions of phenobarbitone treated mice to the administration of 60 mg/kg cocaine were similar to those noted previously (Powell *et al.* 1991), comprising hyperactivity and ataxia, and although more severe at the higher doses of 90 and 120 mg/kg, all reactions subsided within 60 minutes.

In the dose–response study, one animal died after receiving only phenobarbitone, another died 10 minutes after receiving 90 mg/kg cocaine, and a third died 10 minutes after 120 mg/kg cocaine. They were excluded from subsequent investigation. One other animal died between 8 and 24 hours after receiving 120 mg/kg cocaine. The cardiac effects of cocaine were likely to have been a contributory factor, even in the latter case.

Microscopic examination of the liver confirmed that a 60 mg/kg dose of cocaine caused necrosis in scattered hepatocytes at the periphery of the portal area, accompanied by some periportal and linking portal hepatocyte necrosis (Figure 1A). The higher doses of 90 and 120 mg/kg cocaine produced more extensive confluent necrosis in this area with substantial involvement of periportal hepatocytes (Figure 1B–D). Hepatocyte damage was not observed in any livers from animals treated with saline or phenobarbitone alone. Glycogen replete cells were seen in mid-zonal to periportal areas and occasional chronic inflammatory foci were seen regardless of treatment. Neither of these were unusual findings in animals of this age and strain. In addition, centrilobular hypertrophy, an increase in mitotic figures and minimal diffuse microvacuolation, suggestive of lipid accumulation, were seen in most phenobarbitone treated animals, regardless of any subsequent treatment. These observations are common characteristics of phenobarbitone treatment, and are consistent with enzyme induction.

Thus, cocaine induced lesions which developed initially at the periphery of the periportal area, and which have previously been described by some as mid-zonal (Powell *et al.* 1991; James *et al.* 1987), extended portally with increasing dose to produce a typical pattern of periportal necrosis after 120 mg/kg cocaine. These lesions, at the junction of acinar zones 1 and 2, will subsequently be referred to as 'peripheral portal' lesions. In animals given lower doses of cocaine, the portalwards progression of cocaine induced lesions was suggested by the location of degenerating hepatocytes, on the portal side of the necrotic lesions at 24 hours. Following cocaine administration, plasma levels of AST and ALT were elevated in a dose dependent manner when compared with either group of control animals (Figure 2).

In a subsequent study, the first light microscopic abnormalities were observed 2 hours after administration of 60 mg/kg cocaine. Degeneration, eosinophilia and necrosis of scattered hepatocytes at the periphery of the periportal area could be seen, while adjacent hepatocytes remained morphologically normal. These necrotic hepatocytes were typically enlarged, with pale, vacuolated cytoplasm and frequently contained karyorrhectic or karyolytic nuclei (Figure 3A). Hepatic damage became more extensive with time, so that necrotic cells were more abundant 6 hours after cocaine administration, and had developed into ribbon-like bands of confluent 'peripheral portal' and linking portal necrosis, one-third of a lobule width from the portal tract and two-thirds of a lobule width from the central vein, by 9 and 12 hours (Figure 3B). Treatment with phenobarbitone or saline alone did not produce hepatic damage. Background pathology was similar to that described previously, as was that attributable to phenobarbitone. Thus, certain scattered hepatocytes at the periphery of

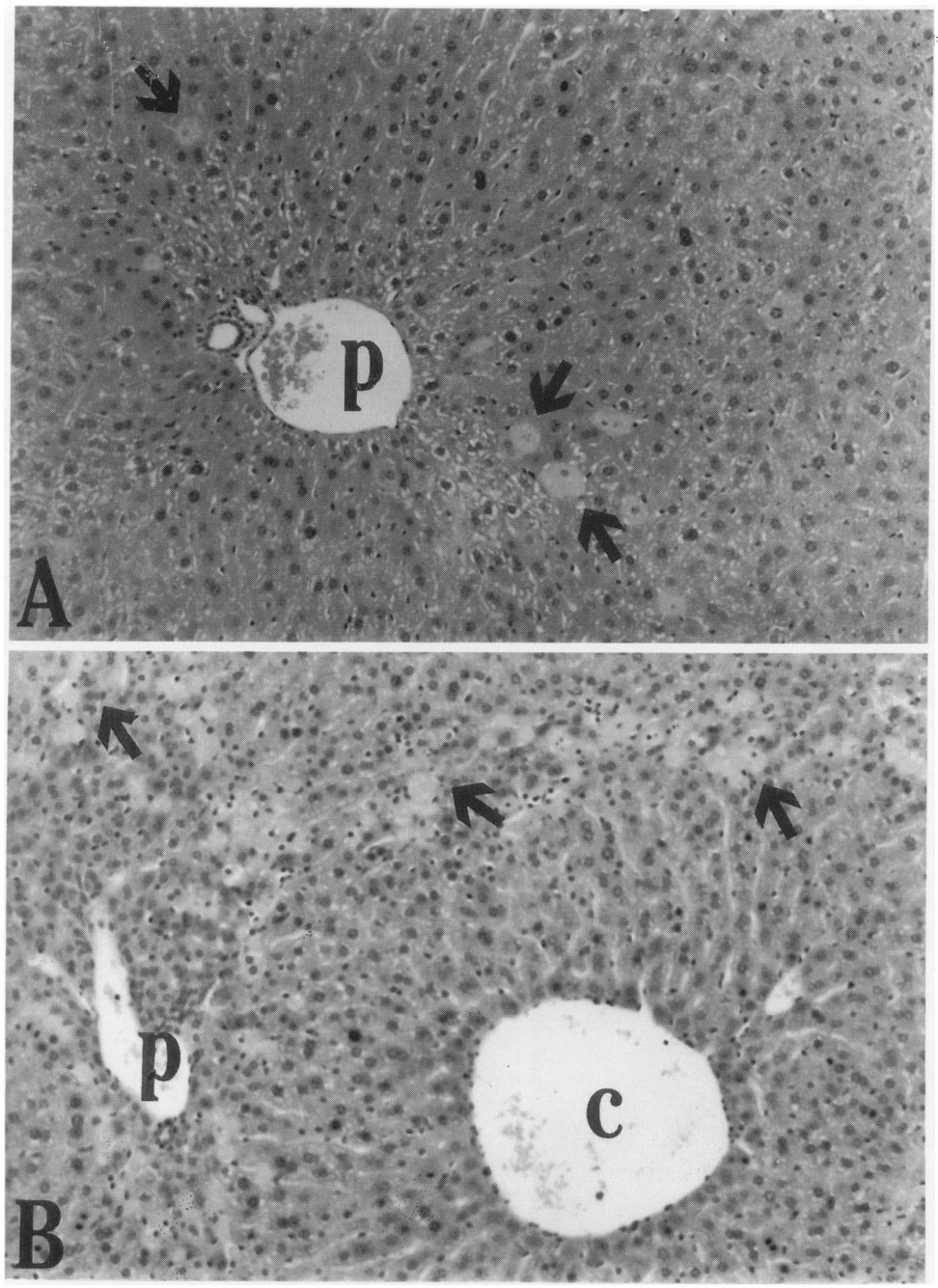

Figure 3. The initial development of 'peripheral portal' hepatocyte necrosis after i.p. administration of 60 mg/kg cocaine to phenobarbitone treated DBA/2Ha mice. A, After 2 hours, degeneration and necrosis of scattered 'peripheral portal' hepatocytes (arrowed) was visible. B, After 12 hours, ribbons of confluent 'peripheral portal' and linking portal hepatocyte necrosis were seen. Ribbons of necrotic cells (arrowed) connected areas of 'peripheral portal' necrosis while several rows of hepatocytes adjacent to portal tracts survived. p, Portal tract; c, central vein. Haematoxylin and eosin stained sections. A and B ×32.

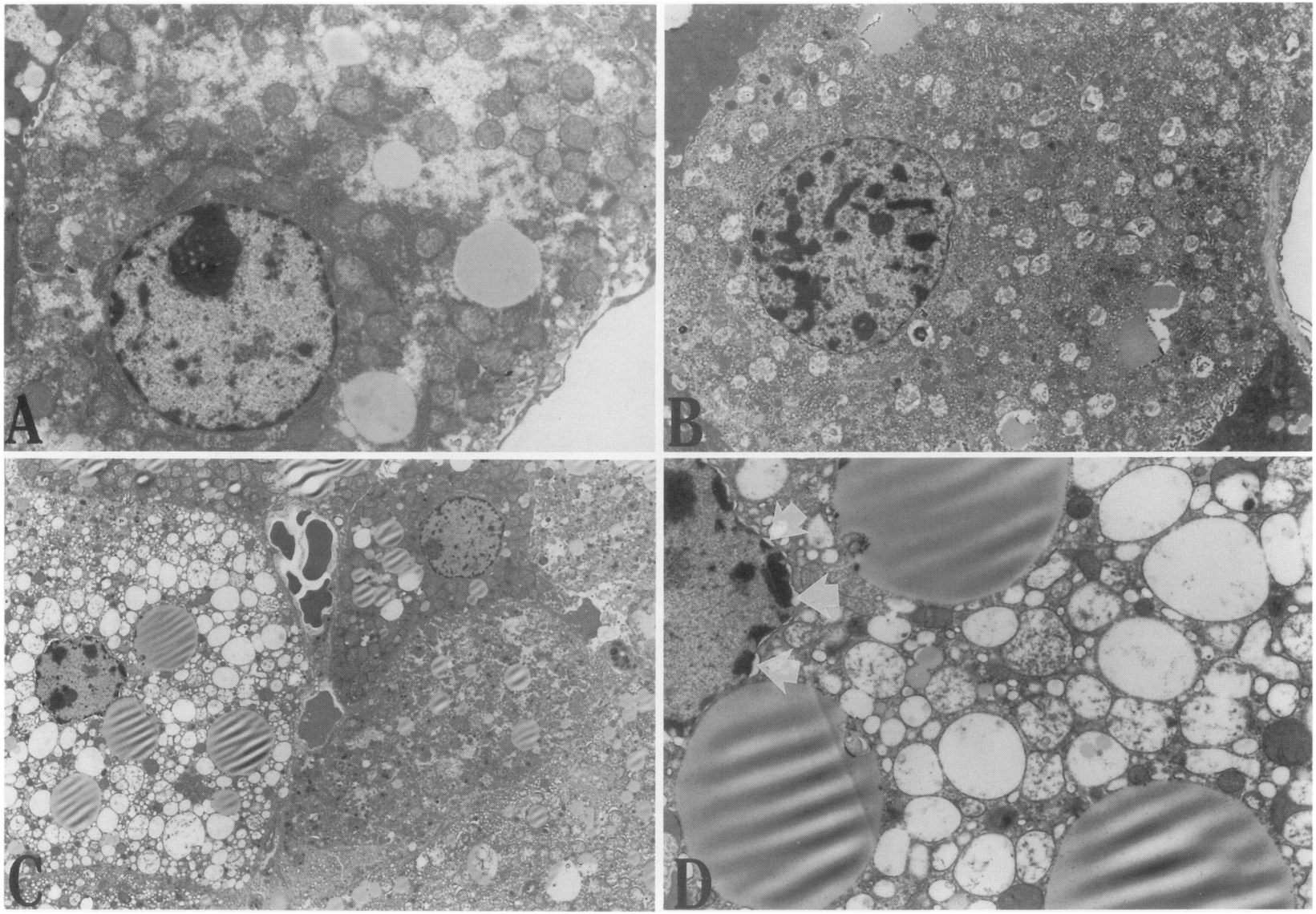

Figure 4. Ultrastructural detail of the development of damage from 60 mg/kg cocaine i.p. in 'peripheral portal' hepatocytes of phenobarbitone treated DBA/2Ha mice. A, Representative cell from phenobarbitone treated control liver, showing typical cytology. B, After 30 minutes, cytoplasmic and nuclear degenerative changes were conspicuous in scattered cells, with uniform microvacuolation and dilatation of endoplasmic reticulum, mitochondrial swelling and clumping of nuclear chromatin clearly visible. C, After 12 hours, a cluster of 'peripheral portal' hepatocytes showing lipid accumulation and various stages of degeneration of endoplasmic reticulum and mitochondria. D, Enlargement of a severely damaged area in (C) showing enlarged and degenerating mitochondria and dilatation of the perinuclear space (arrowed). A ×4000, B ×2400, C ×1200, D ×4800.

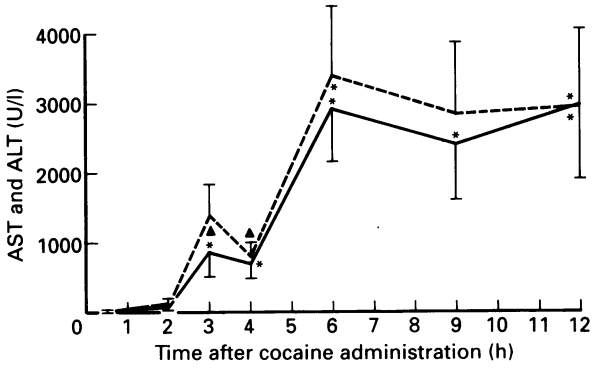

Figure 5. Response of plasma - - - -, aspartate and ————, alanine aminotransferases (AST and ALT) following i.p. administration of 60 mg/kg cocaine to phenobarbitone treated DBA/2Ha mice. Values are the means ± s.e.m. for at least 6 mice per time-point. Mean control values ± s.e.m. for AST and ALT were 44.0 ± 6.1 and 36.1 ± 11.1 U/l respectively for saline treated mice, and 49.9 ± 6.1 and 37.5 ± 4.7 U/l respectively for phenobarbitone treated mice. * And ▲, significantly different from both control groups, $P < 0.05$ and $P < 0.01$ respectively.

the portal area developed morphological signs of damage very rapidly after cocaine administration and this additive, non-uniform pattern of cell death progressed to produce a linking ribbon of confluent necrotic 'peripheral portal' hepatocytes within 12 hours.

The 'peripheral portal' areas were then examined by electron microscopy. Thirty minutes after cocaine administration, scattered hepatocytes were observed (Figure 4B), which were different from those treated with phenobarbitone alone (Figure 4A). In these cells, lipid had accumulated, mitochondria were swollen and electron-lucent with disorganized cristae, and Golgi membranes were hypertrophied. Widespread vesiculation of endoplasmic reticulum had occurred, probably of smooth endoplasmic reticulum, but possibly also of degranulated rough endoplasmic reticulum, as ribosome-associated membranes were also dilated. Nuclear changes were evident, with enlargement of the perinuclear space and clumping of heterochromatin. At 30 minutes such cells were scarce and invariably adjacent to normal hepatocytes but they became progressively more widespread up to 12 hours. By then, significant numbers of damaged cells were observed (Figure 4C), containing large electron-lucent vacuoles, more and larger lipid droplets, degenerate mitochondria and pyknotic nuclei, all indicative of cell death (Figure 4D). In an attempt to determine the sequence of organelle changes in dying hepatocytes, ultrastructural examination of organelles in the least damaged cells was performed at all time-points. Microvacuolation of the endoplasmic reticulum was conspicuous in most degenerating cells, while structurally normal mitochondria and nuclei often accompanied these changes, indicating that these organelles are less likely than the endoplasmic reticulum to be primary targets. Thus, cocaine caused limited hepatocyte damage within 30 minutes, which began with disruption of the endoplasmic reticulum and became more severe with time as the number of damaged cells increased.

Levels of plasma aspartate and alanine aminotransferases (AST and ALT) were significantly elevated ($P < 0.05$ or $P < 0.01$) within 3 hours of cocaine administration, compared to either of the control groups, and remained so for up to 12 hours, despite fluctuations in the magnitude of these elevations (Figure 5).

Total cytochrome P-450 content and FAD-mono-oxygenase activity were increased twofold and reduced by 50% respectively, by phenobarbitone treatment, but remained unaltered during the development of this type of cocaine induced hepatic necrosis (Table 1).

Table 1. Time course study: biochemical analysis

Pretreatment		Acute treatment		Post acute treatment (h)	Total cytochrome P-450 (μmol/g protein)	FAD-mono-oxygenase (μmol/g protein)
Saline	PB	Saline	Cocaine			
+	–	+	–	4	0.699 ± 0.114	1.04 ± 0.120
–	+	+	–	4	1.35** ± 0.118	0.470* ± 0.100
–	+	–	+	0.5	1.36* ± 0.162	0.350** ± 0.020
–	+	–	+	1	1.36* ± 0.092	0.380** ± 0.100
–	+	–	+	2	1.14* ± 0.055	0.450** ± 0.060
–	+	–	+	3	1.27** ± 0.065	0.530* ± 0.120
–	+	–	–	4	1.27* ± 0.124	0.420** ± 0.140

All values are mean ± s.e.m. for at least 3 sets of microsomes from pairs of homogenized livers. Significant differences from the vehicle control group are indicated: *$P < 0.05$, **$P < 0.01$.

Discussion

Hepatotoxicity resulting in mid-zonal necrosis is extremely rare. In mice pretreated with phenobarbitone, a population of periportal hepatocytes which are normally resistant to the effects of cocaine alone became damaged while, simultaneously, protection was afforded to cells in the centrilobular area, which would normally have been damaged by cocaine alone. In addition, on increasing the dose of cocaine from 60 to 90 or 120 mg/kg, the lesions which arose in the 'peripheral portal' hepatocytes progressed portally, ultimately to produce unequivocal periportal necrosis.

The hepatocytes initially damaged therefore comprise the outer edge of the periportal zone, and are thus more accurately described as 'peripheral portal' rather than mid-zonal cells, as some have called them (James *et al.* 1987; Powell *et al.* 1991). Confirmatory evidence that these cells are functionally and structurally part of the periportal area can be found in the livers of phenobarbitone treated control mice. The livers from such mice display a clear demarcation between the distinctively hypertrophied centrilobular to mid-zonal cells, associated with proliferation of smooth endoplasmic reticulum and enzyme induction, and the smaller non-hypertrophied periportal and linking portal cells. It is exactly at this junction of the two hepatocyte populations, which is usually one-third of a lobule width from the portal tract and two-thirds from the central vein in this mouse strain, that the initial lesion of cocaine toxicity develops.

Interestingly, Roth *et al.* (1992) reported cocaine damage in mid-zonal cells of non-enzyme induced mice, whereas centrilobular damage has repeatedly been reported with cocaine alone (Kloss *et al.* 1982; Powell *et al.* 1991). It is possible that the former authors observed the initial stages of the cocaine induced centrilobular lesion which commences in cells some distance from the central vein and progresses centralwards (Mehanny & Abdel-Rahman 1991), in a manner analogous to the portalwards progression we have described.

Cocaine is thought to be metabolically activated by cytochrome P-450 and FAD-mono-oxygenase enzymes to its ultimate hepatotoxin, norcocaine nitroxide, by a series of N-oxidation reactions, forming first norcocaine, then *N*-hydroxynorcocaine (Boelsterli & Göldin 1991). Normally, the cytochrome P-450 enzymes are most abundant in centrilobular cells (Wojcik *et al.* 1988), which is consistent with the development of damage in this area in previously untreated mice. The sensitivity of 'peripheral portal' hepatocytes to cocaine toxicity following phenobarbitone is likely to result from an alteration in the intralobular concentration of metabolizing enzymes. The specific location of the initial site of cocaine damage suggests that the isozyme(s) responsible are present at high concentration in only a small proportion of hepatocytes and most probably comprise a small proportion of the total cytochrome P-450, hence our inability to detect a significant change in this enzyme in homogenized whole liver preparations. In addition, as the FAD-mono-oxygenase metabolism of cocaine to its *N*-oxide is reputed to detoxify cocaine (Misra *et al.* 1979), the reduction in its activity accords with the resulting enhancement of cocaine hepatotoxicity.

In the few most sensitive 'peripheral portal' hepatocytes cocaine damage was detectable by electron microscopy within 30 minutes, and by light microscopy at 2 hours. Structural evidence of lethal cell injury from other chemical toxicants has been reported within 2 hours in lymphocytes, the epithelium of the gastrointestinal tract and neural tissue (Ijiri & Potten 1987), but compared to reports of damage from other hepatotoxins (Landon *et al.* 1986; Nakagawa *et al.* 1984), the present effect appears to be one of the most rapidly developing forms of hepatic necrosis yet observed. This in turn implies that the absorption, distribution and metabolic activation of cocaine must occur very rapidly and that the initial cellular target(s) of reactive metabolites must include functions immediately vital for survival.

Ultrastructural investigation suggests that the processes of cell degeneration and death probably differ in 'peripheral portal' and centrilobular cocaine lesions. First, the intermediate filament aggregation (Powers *et al.* 1992) and very early mitochondrial changes (Roth *et al.* 1992; Gottfried *et al.* 1986; Mehanny & Abdel-Rahman 1991) associated with centrilobular lesions, could not be found in periportal lesions. However, dilatation of rough endoplasmic reticulum (Roth *et al.* 1992; Gottfried *et al.* 1986), detachment of ribosomes (Mehanny & Abdel-Rahman 1991) and swelling or vesiculation of the smooth endoplasmic reticulum (Roth *et al.* 1992; Gottfried *et al.* 1986; Mehanny & Abdel-Rahman 1991) do appear to be present to some extent in both lesions. Secondly, in DBA mice, damage develops more rapidly in periportal cells than in centrilobular cells. In the former, the earliest observation of mitochondrial damage by electron microscopy was made after 30 minutes, with hepatocyte necrosis visible by light microscopy after 2 hours, compared to 2 hours (Gottfried *et al.* 1986) and 6 hours (Roth *et al.* 1992) respectively, for centrilobular damage in DBA or ICR mice. In addition, the release of plasma enzymes from 'peripheral portal' cells occurred within 3 hours of cocaine administration,

which is more rapid than enzyme release from centrilobular cells (Kloss *et al.* 1982). Furthermore, centrilobular damage has been associated with small but significant reductions in the activity of total P-450, glucuronyl transferase and FAD-mono-oxygenase (Kloss *et al.* 1982), but this was not observed during periportal damage. Prior exposure to phenobarbitone also appears to alter the mechanism of cocaine damage in cultured rat hepatocytes, preventing the normal elevation of intracellular calcium levels but causing a more marked depletion of reduced glutathione and subsequent lipid peroxidation (Jover *et al.* 1993).

The present study has shown that cocaine induced cell damage occurred rapidly in scattered hepatocytes and with increasing dose, or time, spread to produce confluent 'peripheral portal' necrosis. These results provide an explanation for the discrepancy between authors who have described cocaine damage in enzyme induced mice as mid-zonal (Powell *et al.* 1991), periportal (Evans & Harbison 1978; Roth *et al.* 1992; Thompson *et al.* 1984), or both (James *et al.* 1987), after equivalent treatments. As 'peripheral portal' lesions arise at the periphery of the portal area, and progress to periportal necrosis, it is likely that earlier conflicting reports of periportal and/or mid-zonal necrosis from cocaine in phenobarbitone pretreated mice were in fact descriptions of different degrees of severity of the same lesion. Illustrations and descriptions of liver damage from earlier reports explicitly support this contention. Thompson *et al.* (1984) reported that several layers of hepatocytes adjacent to portal tracts were spared, while the micrographs of James *et al.* (1987), very closely resemble the 'peripheral portal' lesions reported here.

These results, and those obtained previously (Powell *et al.* 1991), indicate that cocaine can induce one of two different types of liver damage, centrilobular *or* 'peripheral portal', but never both simultaneously. This, together with the differences in the rapidity of damage and in the underlying biochemical changes, suggests that the mechanisms of cocaine induced liver damage may be distinct in centrilobular and periportal hepatocytes.

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