

Phenobarbital Induction Does Not Potentiate Hepatotoxicity but Accelerates Liver Cell Necrosis from Acetaminophen Overdose in the Rat

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Abstract. Rats were pretreated with phenobarbital to induce hepatic cytochrome P-450. Compared to noninduced rats, a similar relation between the dose of acetaminophen and mortality, and between dose and changes in liver function (prothrombin index) and identical time courses, was found. The urinary excretion of acetaminophen mercapturate and acetaminophen cysteine was identical in induced and noninduced rats. The metabolism of acetaminophen in terms of blood levels and excreted metabolites was not influenced by phenobarbital induction. At the same dose level, hepatic necrosis was accelerated (maximum 24 h) compared to noninduced animals (maximum 72 h), but no difference in the maximum extent was found. These data cannot support the concept that induction of cytochrome P-450 leads to greater formation of the hypothetical toxic metabolite of acetaminophen, or that induction enhances its hepatotoxicity, in the rat.

Several factors may contribute to accelerate the necrotic changes which make it possible to histologically identify cell damage and death. In that case, functional studies are more relevant than morphological evaluation in quantitative assessment of liver damage.

Introduction

Acetaminophen overdoses may result in lethal hepatic necrosis in humans and animals [Prescott et al., 1971]. The toxicity is supposed to be due to the formation of a

toxic, arylating metabolite by hepatic cytochrome P-450-mediated oxidation [Mitchell et al., 1974; Mitchell et al., 1973a; Jollow et al., 1973; Potter et al., 1973; Mitchell et al., 1973b]. This is normally inactivated by conjugation to glutathione [Mitchell et al., 1974;

Mitchell et al., 1973a], but following an overdose of acetaminophen, hepatic glutathione is depleted [Mitchell et al., 1973a] and the detoxification capacity thereby reduced. The resulting covalent binding of acetaminophen is thought to initiate hepatocellular damage with subsequent necrosis [Jollow et al., 1973; Potter et al., 1973]. Only a small portion of acetaminophen is metabolized by cytochrome P-450 and conjugated to glutathione, while the major pathways are glucuronidation and sulfation [Mitchell et al., 1974]. Inducers of cytochrome P-450 are expected to increase the proportion of the dose that is converted to the reactive metabolite [Gillette, 1974], and thus the toxicity of acetaminophen, if the metabolism by other metabolic pathways are unchanged. In accordance with this it was found [Mitchell et al., 1973b] that phenobarbital induction increased the degree of hepatic necrosis 48 h after an acetaminophen overdose. The purpose of the present work was to study the effect of phenobarbital pretreatment on morphological and functional changes at different time intervals after an overdose of acetaminophen, and the functional changes in relation to the dose.

Material and Methods

Female Wistar rats weighing 162–210 g were fed Altromin® pellets and tap water ad libitum. Phenobarbital-pretreated animals received tap water with 1 g/l sodium phenobarbital for 4 days [Marshall and McLean, 1969]. Acetaminophen was given after an overnight fast by gavage as a suspension in 0.2% tragacanth gum. The dose-mortality ratio in phenobarbital-induced and noninduced animals was determined after giving 2.0, 4.25, 5.5, 7.0 and 9.0 g acetaminophen/kg body weight to groups of 10 rats.

In all experiments, except in those performed to study dose dependence, 4.25 g acetaminophen/kg

Table I. Dose mortality, 72 h after various doses of acetaminophen given, as deaths in groups of 10 animals pretreated with or without phenobarbital 1 g/l drinking water for 4 days prior to acetaminophen

	Acetaminophen, g/kg body weight					
	2.0	3.25	4.25	5.5	7.0	9.0
Phenobarbital-induced rats	0	1	2	8	10	10
Noninduced rats	0	1	2	9	10	10

body weight was given as standard dose (corresponding to LD₁₀ with this type of administration [Poulsen et al., 1981]). For time course studies each group consisted of 4–7 rats, groups for dose studies consisted of 4 or 5 rats. During ether anesthesia, aortic blood was drawn and diluted with an equal volume of isotonic saline for subsequent determination of the prothrombin index [Owren and Aas, 1951]. Separate animals were used for this analysis.

For the determination of paracetamol, undiluted plasma was extracted and measured by high-pressure liquid chromatography [Lo and Bye, 1979] using 3-acetaminophenol as internal standard. The urinary excretion of acetaminophen and its metabolites was investigated in animals kept in metabolic cages. Urine was collected over dry ice during 4 periods of 24 h after administration of the standard dose of acetaminophen. A group of 6 noninduced and a group of 10 induced animals were investigated. Acetaminophen and its metabolites were estimated in urine [Knox and Jurand, 1977] from standard reference metabolites (Sterling Winthrop, Sweden).

After exsanguination, the liver was excised, a slice was removed for microscopic investigation (see below), and the rest blotted on filter paper, sliced with scissors in iced isotonic KCl and homogenized in a Potter-Elvehjem teflon glass homogenizer with 10 ml of isotonic KCl. Liver homogenate was assayed for cytochrome P-450 [Omura and Sato, 1964], total glutathione [Tietze, 1969] and protein [Groves et al., 1968]. Microsomes [Andreassen et al., 1974] were assayed for protein [Groves et al., 1968], cytochrome P-450 [Omura and Sato, 1964] and *p*-nitroanisole demethylase activity [Schoene et al., 1972].

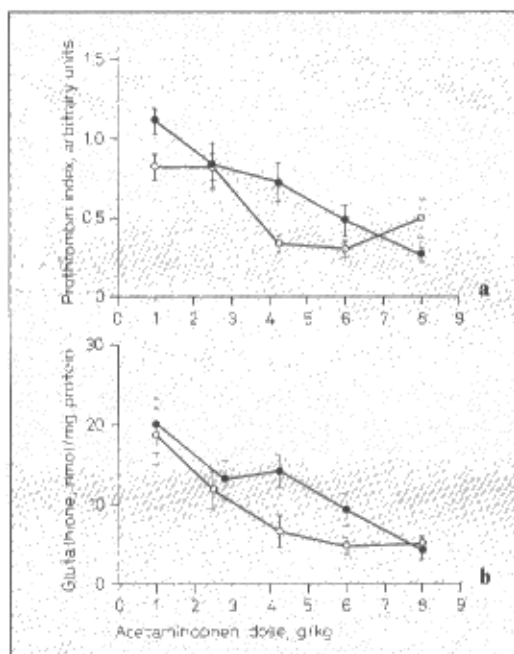
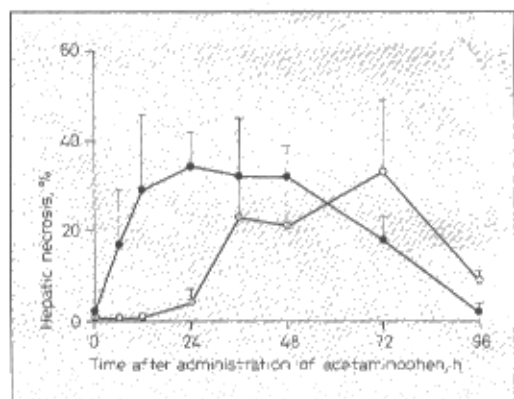


Fig. 1. a Relation of prothrombin index to acetaminophen dose given 12 h earlier. Symbols indicate mean of 4 or 5 animals, bars \pm SEM. \circ = Noninduced animals; \bullet = animals induced with phenobarbital



1 mg/ml drinking water for 4 days prior to acetaminophen administration. **b** Relation of total hepatic glutathione to acetaminophen dose given 12 h earlier. Symbols are as in figure 1a.

Fig. 2. Time course of hepatic necrosis in percent of lobule radius after an oral dose of acetaminophen 4.25 g/kg body weight in animals induced with phenobarbital 1 mg/ml in the drinking water for 4 days (\bullet) or noninduced animals (\circ). Necrosis was read (randomly and blindly, i.e. without knowledge of treatment or group) with the use of a micrometer ocular in 5 lobules of a section from each animal. Symbols indicate means \pm SEM of 4-7 rats.

Table II. Time courses of cytochrome P-450 and *p*-nitroanisole demethylation

	Control	Time after acetaminophen (4.25 g/kg body weight)			
		6 h	12 h	24 h	36 h
<i>Cytochrome P-450, nmol/mg microsomal protein</i>					
Phenobarbital pretreated	1.40 \pm 0.04	1.34 \pm 0.12	1.05 \pm 0.05	0.91 \pm 0.16	0.62 \pm 0.10
Saline pretreated	0.60 \pm 0.02*	0.78 \pm 0.03**	0.77 \pm 0.02	0.69 \pm 0.07	0.52 \pm 0.02
<i>p-N-anisole demethylase activity, nmol/mg protein/min</i>					
Phenobarbital pretreated	23.9 \pm 1.2	23.5 \pm 1.9	17.2 \pm 1.0	15.1 \pm 3.1	9.5 \pm 2.7
Saline pretreated	6.7 \pm 0.7*	10.4 \pm 0.4**	13.8 \pm 1.3	13.9 \pm 2.6	6.4 \pm 0.8

Microsomal cytochrome P-450 concentration and *p*-nitroanisole demethylase activity in phenobarbital-induced and noninduced animals before and various periods after acetaminophen 4.25 g/kg body weight. Results are means \pm SEM of 4-7 animals. Animals were induced for 4 days with phenobarbital in the drinking water (1 g/liter) prior to acetaminophen.

For histological examination, liver tissue was fixed in 10% formaldehyde and embedded in paraffin. 5- μ m sections were stained with hematoxylin and eosin (HE) and were examined in random order, without knowledge of treatment group or interval. 5 lobules were selected at random, the radius of the lobule and of the centrilobular necrotic area was measured by a micrometer ocular. Necrosis was expressed as percent, calculated as radius of the necrosis divided by radius of lobulus times 100. Necrotic area was identified by nuclear pyknosis or karyorrhexis, blurring of the cellular membrane, strongly acidophilic cytoplasm, and inflammatory infiltration. Areas with hydropic degeneration were not included.

Calculations

The influence of time on the various measures from 1 of the 2 pretreatments was tested with one-way analysis of variance [Armitage, 1977]. The difference between 2 time courses or a dose relationship was tested with multiple t tests with correction for mass significance [Duncan, 1955]. This method was also used to analyze the metabolite excretion data. p values of less than 0.05 (after correction when appropriate) were considered statistically significant.

48 h	72 h	96 h
0.63 \pm 0.05	0.7 \pm 0.07	0.73 \pm 0.05
0.55 \pm 0.05	0.57 \pm 0.06	0.63 \pm 0.03
7.4 \pm 1.2	9.4 \pm 1.2	9.6 \pm 1.2
5.5 \pm 0.8	5.5 \pm 1.1	6.4 \pm 0.7

*p < 0.01 and **p < 0.05 for comparison of induced and noninduced animals.

Results

The dose-mortality relation was not significantly different between phenobarbital-induced and noninduced rats (table I; p > 0.05, χ^2 test). All deaths occurred within 18 h. Figure 1a, b demonstrates that liver injury, as reflected by the fall in prothrombin index in the blood and glutathione content in the liver, 12 h after acetaminophen, shows the same relation to dose in phenobarbital-induced and untreated animals.

Figure 2 shows the extent of hepatocellular necrosis at different intervals after acetaminophen in phenobarbital-induced and noninduced animals. The curves are significantly different (p < 0.01) with maximum necrosis after 24 h in phenobarbital-induced and after 72 h in noninduced rats. The maximum extent of necrosis, corresponding to $\frac{1}{3}$ of the liver lobule radius, was identical.

Table II gives the hepatic content of cytochrome P-450 and p-nitroanisole demethylation activity at the same intervals. The inducing effect of phenobarbital pretreatment is statistically significant after 6 and 12 h, after longer intervals, phenobarbital-induced rats have higher values, but not statistically significant.

Figure 3a, b shows that the prothrombin index is at minimum after 12 h and hepatic glutathione after 6 h, with no significant difference between phenobarbital induced and untreated animals.

From table III it is seen that acetaminophen levels at all intervals are similar in phenobarbital-induced and untreated animals. The excretion of acetaminophen, acetaminophen-glucuronide, acetaminophen-sulfate and acetaminophen-mercapturate, given as cumulative percentage of the dose during 4 periods of 24 h (table IV), was not signifi-

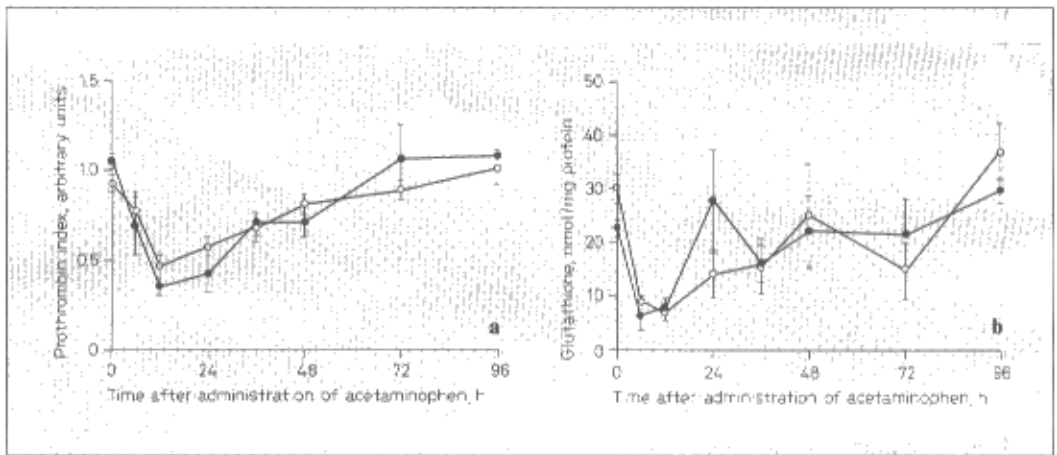


Fig. 3. a Time course of prothrombin index after acetaminophen 4.25 g/kg body weight. Symbols are as in figure 2. **b** Time course of hepatic glutathione after acetaminophen 4.25 g/kg body weight. Symbols are as in figure 2.

cantly different in phenobarbital-treated and untreated animals. The total excretion during the period accounted for about 60% of the amount given.

Discussion

In the present study, the only difference between induced and noninduced rats regarding reactions to an acetaminophen overdose was the acceleration of liver cell necrosis in induced animals. The relation of mortality and functional impairment to dose and the time course of functional impairment after fixed dose were not significantly different. In similarly induced rats [Mitchell et al., 1973a] more extensive hepatic necrosis was found 48 h after acetaminophen corresponding to our data after 24 h, but not after 48 h. Differences in the route of administration of acetaminophen may account for this discrepancy, but since Mitchell et al. [1973a] only

report observations after 48 h, this remains undecided.

In hamsters [Jollow et al., 1974], induction with phenobarbital has been found to protect against acetaminophen-induced liver damage. However, they found increased clearance of acetaminophen and it was suggested that increased glucuronidation reduced the amount of acetaminophen converted to the hypothetical toxic metabolite. It has been pointed out that when only a minor part of a drug is converted to a toxic metabolite, as it is assumed to be the case with acetaminophen, induction may favor the formation of a toxic metabolite formed by a minor pathway only if the other metabolic pathways are unchanged [Gillette, 1974; Jollow et al., 1974].

In our study, the rate of elimination of acetaminophen was not influenced by induction as judged from the plasma levels of acetaminophen and the urinary excretion of acetaminophen glucuronide and acetaminophen

Table III. Acetaminophen in plasma

	Controls	6 h	12 h	24 h	36 h	48 h	72 h	96 h
Phenobarbital pretreated	0	1.83±0.79	2.27±0.85	0.43±0.09	0.56±0.07	0.59±0.29	0.68±0.22	0
Saline pretreated	0	2.06±0.92	1.23±0.29	0.67±0.40	0.60±0.11	0.64±0.11	0.99±0.31	0

Plasma acetaminophen (mmol/l) after 4.25 g acetaminophen/kg body weight given by gavage. Values are means ± SEM of 4-7 animals. Phenobarbital was given for 4 days in the drinking water (1 g/liter) prior to acetaminophen.

Table IV. Acetaminophen metabolism

	0-24 h	0-48 h	0-72 h	0-96 h
Acetaminophen				
Saline	4.0±0.7	6.9±1.1	9.2±1.6	11.3±1.8
Phenobarbital	2.8±0.02	5.5±0.7	8.8±1.1	10.1±1.3
Acetaminophen-glucuronide				
Saline	8.5±1.5	16.6±1.6	20.3±2.7	26.9±3.7
Phenobarbital	8.7±1.3	18.6±2.7	24.7±3.6	28.7±3.2
Acetaminophen-sulfate				
Saline	3.6±0.5	7.4±1.6	9.0±1.3	11.7±1.7
Phenobarbital	2.4±1.2	6.2±1.1	7.3±1.3	11.0±1.3
Acetaminophen-mercapturate				
Saline	1.4±0.3	3.6±0.6	4.3±0.7	5.7±0.9
Phenobarbital	1.0±0.1	2.7±0.6	3.9±0.8	4.7±0.7

Cumulative urinary excretion of acetaminophen and its metabolites as percentage of the dose. Values are means ± SEM of 6 untreated animals (saline) and 10 animals pretreated with phenobarbital 1 mg/ml in drinking water for 4 days. All animals were given acetaminophen 4.25 g/kg body weight after an overnight fast and kept separately in metabolic cages for collection of urine over dry ice for 4 periods of 24 h.

sulfate. Whether induction influenced the fraction converted to the hypothetical toxic metabolite or not cannot be decided. It is assumed that formation of the metabolite is reflected by the depletion of glutathione [Mitchell et al., 1973a]; since the hepatic glutathione concentration in the liver after 6 h,

assumed to reflect initial glutathione depletion in the rat [Lauterburg and Mitchell, 1982], was similar in induced and noninduced animals, and since the excretion of acetaminophen mercapturate, the product of conjugation with glutathione, was also similar, there is no evidence of difference as to the

formation of the toxic metabolite. The fraction converted to the supposed toxic metabolite may also be estimated by covalent binding of acetaminophen to hepatic protein [Mitchell et al., 1974], which was not estimated in the present study. However, in a more recent study, covalent binding of acetaminophen was not increased in rats induced with phenobarbital [Pessayre et al., 1980]. A study on protection of acetaminophen-induced damage demonstrated that protection was dissociated from glutathione depletion and covalent binding suggesting that these circumstances are not sufficient causes of cell death [Devalia et al., 1982]. In a recent study on cimetidine inhibition of acetaminophen toxicity [Petersen et al., 1983], necrosis was reduced, however, without changes in glutathione depletion or covalent binding. These findings are not easily compatible with the current theory of the toxic mechanism of acetaminophen. Neither is our study on enhancement of acetaminophen hepatotoxicity from induction easily compatible with this currently believed toxic mechanism and we also suggest a careful reevaluation. Two primary challenges to the covalent binding theory have arisen. The first is that acetaminophen leads to lipid peroxidation [Wendell et al., 1979; Albano et al., 1983] and the second that rapid changes in the intracellular ionized calcium pool may account for hepatocellular injury of exposure to plasma membrane toxins or hypoxia [Trump et al., 1980], changes demonstrated to be relevant to quionoid drugs [Thor et al., 1982; Jones et al., 1983].

Despite the many challenges to the covalent binding theory of acetaminophen toxicity, the large body of research evidence for its validity requires the emergence of a novel satisfactory theory before the old is abrogated [Yamada, 1983].

It is not clear why the induction accelerated hepatic necrosis from paracetamol in the present study and why the functional impairment was not similarly accelerated. However, since phenobarbital has many effects on the liver except for increasing the hepatic cytochrome P-450 monooxygenase activity, such as increasing the hepatic blood flow [Yates et al., 1978], the glucogen content [Sato and Iwanoto, 1966], the bile flow [Klaasen, 1969], and the non-P-450-mediated demethylation [Shigematsu et al., 1976], it cannot be excluded that induction also accelerates necrotic changes and thus the histological recognition of cellular death.

In that case, functional studies are more relevant than morphological evaluations in quantitative assessment of liver damage.

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