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ADDITIVE EFFECTS OF INDUCERS AND FASTING ON ACETAMINOPHEN HEPATOTOXICITY

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Abstract—Acetaminophen is activated by cytochrome P-450 into a reactive metabolite which may bind either to glutathione and be inactivated or may bind to hepatic macromolecules; the latter binding may be involved in acetaminophen hepatotoxicity. In this study, we compared the effects of pretreatment with 3-methylcholanthrene, fasting, or the combination of both on the metabolism and the hepatotoxicity of acetaminophen (500 mg/kg i.p.)in male Sprague–Dawley rats. Pretreatment with 3-methylcholanthrene increased the depletion of hepatic glutathione, the amount of metabolite irreversibly bound to hepatic proteins, and the extent of liver cell necrosis after administration of acetaminophen. Fasting for 42 hr decreased basal and post-treatment hepatic glutathione concentration, increased the amount of metabolite irreversibly bound to hepatic proteins, and increased liver cell necrosis after administration of acetaminophen. In rats that were both pretreated with 3-methylcholanthrene and fasted, hepatic glutathione concentration fell to lower levels, the amount of bound metabolite was higher, and liver cell necrosis was more severe than in rats that were only pretreated or only fasted. These observations suggest that microsomal enzyme inducers, which increase the formation of the reactive metabolite, and fasting, which decreases the inactivation of the reactive metabolite by hepatic glutathione, may have additive effects on the hepatotoxicity of acetaminophen.

Acetaminophen is widely used as an analgesic in man. The drug is transformed into a reactive metabolite which may either bind to hepatic glutathione and be inactivated or may bind to hepatic macromolecules; the latter binding may be involved in acetaminophen hepatotoxicity [1–8]. In man, hepatitis consistently occurs after huge overdoses [9] but may also occur after therapeutic doses in a few patients [10, 11]. Occurrence of hepatitis after therapeutic doses strengthens the need for a better knowledge of those factors which may, alone or in combination, increase the hepatotoxicity of acetaminophen. In rats, the hepatotoxicity of acetaminophen may be increased by microsomal enzyme inducers [1, 7] or by fasting [12, 13]. The possibility was investigated that inducers and fasting in combination might have additive effects on the hepatotoxicity of acetaminophen in the rat.

MATERIALS AND METHODS

Animals, treatments and diets. Male Sprague–Dawley rats weighing 180–220 g were purchased from Charles River France (St-Aubin les Elbeuf, France). Some rats were pretreated with phenobarbital, 100 mg/kg i.p. daily for five days, and were used 24 hr after the last dose of phenobarbital. Other rats received 3-methylcholanthrene, 20 mg/kg i.p. in 1 ml corn oil, and were used 72 hr after the single dose of 3-methylcholanthrene. Pretreatments were administered at 9.00–10.00 a.m. All rats were allowed water *ad lib*. Some rats were fed throughout with a normal standard diet (Autoclavé 113, UAR) given *ad lib*. Other rats were fasted for 42 hr before the administration of acetaminophen; fasting was begun at 3:00–5:00 p.m.

Unlabeled acetaminophen was obtained from Sigma Chem. Co., St. Louis, MO. [^{3}H]Acetaminophen (generally labeled, sp. act. 3.4 Ci/mmole) was purchased from New England Nuclear, Boston, MA; its radiochemical purity was found by thin layer chromatography to be higher than 99 per cent. Acetaminophen, 500 mg/kg, either unlabeled or labeled (0.15 mCi/mmole) was administered i.p. as a basic solution in 0.5 ml NaOH (final pH, 11.5) at 8:00–10:00 a.m. Until killed, rats were maintained on the same diet as before the administration of acetaminophen.

Microsomal enzymes. Rats were stunned and their livers were removed. Liver fragments were minced and then homogenized with a glass–Teflon Potter–Elvehjem homogenizer in 3 vol. 0.154 M KCl–0.01 M sodium potassium phosphate buffer, pH 7.4. The liver homogenate was centrifuged at 10,000 *g* and microsomal pellets were prepared by centrifuging the 10,000 *g* supernatant fraction at 100,000 *g*. Hepatic microsomal protein concentration, hepatic microsomal cytochrome P-450 level, NADPH–cytochrome *c* reductase activity, ethylmorphine *N*-demethylase activity and benzo[*a*]pyrene hydroxylase activity were measured as previously reported [14]. Acetaminophen UDP-glucuronosyltransferase

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Table 1. Effects of pretreatments and diets on hepatic mixed function oxidase enzymes*

	Liver weight/body weight (%)	Microsomal proteins (mg/g liver)	Cytochrome P-450 (nmole/mg microsomal protein)	NADPH–cytochrome *c* reductase activity (nmoles/min/mg microsomal protein)	Ethylmorphine *N*-demethylase activity (nmoles/min/mg microsomal protein)	Benzo[*a*]pyrene hydroxylase activity (nmoles/min/mg microsomal protein)
Fed control rats	4.3 ± 0.3	41 ± 7	0.9 ± 0.1	55 ± 7	2.4 ± 0.5	0.28 ± 0.05
Fasted control rats	2.7 ± 0.2†	43 ± 6	1.0 ± 0.1	54 ± 9	1.7 ± 0.5†	0.17 ± 0.03†
Fed rats pretreated with phenobarbital	5.3 ± 0.3‡	53 ± 8‡	2.0 ± 0.3‡	97 ± 12‡	3.4 ± 0.3‡	0.31 ± 0.07
Fasted rats pretreated with phenobarbital	4.3 ± 0.3†	60 ± 11†‡	2.1 ± 0.2‡	90 ± 26‡	3.0 ± 0.4‡	0.31 ± 0.09
Fed rats pretreated with 3-methylcholanthrene	5.2 ± 0.5‡	44 ± 7	1.4 ± 0.2‡	48 ± 7	2.2 ± 0.5	0.98 ± 0.24‡
Fasted rats pretreated with 3-methylcholanthrene	3.4 ± 0.3†‡	50 ± 4†‡	1.5 ± 0.2‡	49 ± 4	1.4 ± 0.5†	0.82 ± 0.19‡

* Rats were either fed a normal standard diet or fasted for 42 hr before being killed. Some rats received phenobarbital, 100 mg/kg i.p. daily for 5 days, or 3-methylcholanthrene, 20 mg/kg i.p.; pretreated rats were killed 24 hr after the last dose of phenobarbital or 72 hr after the single dose of 3-methylcholanthrene. Results are means ± S.D. for at least eight rats.

† Different from that in fed rats, $P<0.05$.

‡ Different from that in control rats, $P<0.05$.

Table 2. Effects of inducers on acetaminophen UDP-glucuronosyl transferase activity*

	Acetaminophen UDP-glucuronosyl transferase activity (nmoles/min/mg microsomal protein)
Fed control rats	2.3 ± 0.5
Fed rats pretreated with phenobarbital	5.6 ± 0.1†
Fed rats pretreated with 3-methylcholanthrene	2.5 ± 0.5

* All rats were fed a normal standard diet. Some rats received phenobarbital, 100 mg/kg i.p.; other rats received 3-methylcholanthrene, 20 mg/kg i.p.; pretreated rats were killed 24 hr after the last dose of phenobarbital or 72 hr after the single dose of 3-methylcholanthrene. Triton X-100-activated UDP-glucuronosyl transferase activity was measured as described by Mulder and Van Doorn [15] in the presence of 5 mM UDP-glucuronic acid and 0.1 mM acetaminophen. Results are means ± S.D. for three rats.

† Different from that in control rats. $P<0.001$.

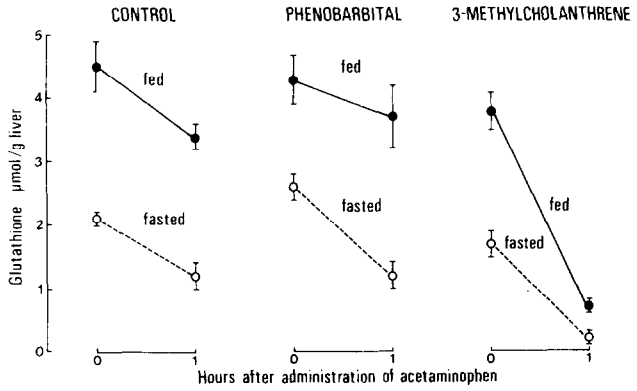

Fig. 1. Effects of pretreatments and diets on hepatic glutathione concentration before and after the administration of acetaminophen. Hepatic glutathione concentration was measured in rats that had not received acetaminophen and in rats that had received acetaminophen, 500 mg/kg i.p., 1 hr before. Rats were either fed a normal standard diet or fasted for 42 hr before the administration of acetaminophen. Some rats were pretreated with phenobarbital, 100 mg/kg i.p. daily for five days, or with 3-methylcholanthrene, 20 mg/kg i.p. Results are means ± S.E.M. for ten rats.

activity was measured by the technique of Mulder and Van Doorn [15].

Glutathione. Rats were killed at 10:00–11:00 a.m.; liver fragments were homogenized in 3 vol. of 5% trichloroacetic acid and hepatic glutathione was measured according to the technique of Ellman [16].

Irreversible binding. Animals were killed 5 hr after the administration of [^{3}H]acetaminophen (500 mg/kg, 0.5 mCi/kg). Liver fragments were homogenized in 3 vol. of 0.154 M KCl, 0.01 M Na^+ K^+ phosphate buffer, pH 7.4. Proteins in the crude liver homogenate were precipitated, washed three times with trichloroacetic acid, and repeatedly extracted with various solvents of various polarities as previously described [12]; the radioactive material that remained irreversibly bound to hepatic proteins was then counted. It was verified that pretreatment of the animals with $CoCl_2$, 6 H_2O, 35 mg/kg s.c. twice daily for three days, decreased the amount of bound material by 70 per cent.

Hepatotoxicity. Serum glutamic pyruvic transaminase (SGPT) activity was measured by the method of Reitman and Frankel [17]. Liver fragments from ten rats per group were placed in Bouin's fluid. They were embedded in paraffin 24 hr later. Paraffin sections were stained with hematoxylin and eosin. The slides were examined by a pathologist unaware of the treatments or diets. In each rat, the extent of necrosis was graded as 0, +, ++ or +++ as previously reported [1] (0, no necrosis; +, necrosis of up to 5 per cent of the hepatocytes; ++, necrosis of 6–25 per cent of the hepatocytes; +++, necrosis of more that 25 per cent of the hepatocytes). A necrosis 'score' was then calculated as the total amount of + in each group of ten rats.

RESULTS

Microsomal enzymes. Pretreatment with phenobarbital increased the liver weight/body weight ratio, hepatic microsomal protein concentration, hepatic cytochrome P-450 levels, NADPH–cytochrome *c* reductase activity and ethylmorphine *N*-demethylase activity (Table 1). Pretreatment with 3-methylcholanthrene increased the liver weight/body weight ratio, cytochrome P-448 and benzo[*a*]pyrene hydroxylase activity (Table 1).

Fasting for 42 hr decreased the liver weight/body weight ratio, tended to increase microsomal protein concentration, did not change hepatic cytochrome P-450 levels or NADPH–cytochrome *c* reductase activity, and either did not change or slightly decreased mixed function oxidase activities (Table 1).

Acetaminophen UDP-glucuronosyltransferase activity was increased by phenobarbital pretreatment but not by pretreatment with 3-methylcholanthrene (Table 2).

Hepatic glutathione. Basal hepatic glutathione concentration was lower in rats fasted for 42 hr than in fed rats (Fig. 1). Administration of acetaminophen, 500 mg/kg, decreased hepatic glutathione concentration in fed rats and further decreased hepatic glutathione concentration in fasted rats (Fig. 1). Pretreatment with phenobarbital did not modify basal or post-treatment hepatic glutathione concen-

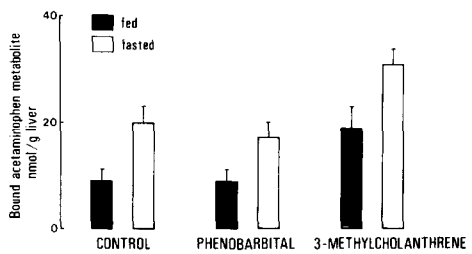

Fig. 2. Effects of pretreatments and diets on the amount of acetaminophen metabolite irreversibly bound to hepatic proteins. [^{3}H]Acetaminophen (500 mg/kg: 0.5 mCi/kg) was administered i.p.; rats were killed 5 hr later and the amount of metabolite irreversibly bound to hepatic proteins was determined as described in Materials and Methods. Results are means ± S.D for five rats.

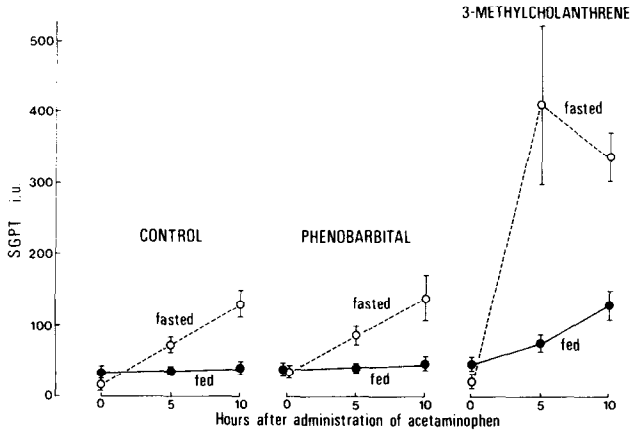

Fig. 3. Effects of pretreatments and diets on serum glutamic pyruvic transaminase (SGPT) activity before and after the administration of acetaminophen. SGPT activity was measured in rats that had not received acetaminophen and in rats that had received acetaminophen, 500 mg/kg i.p., 5 or 10 hr earlier. Results are means ± S.E.M. for ten rats.

trations in fed or in fasted rats (Fig. 1). Pretreatment with 3-methylcholanthrene decreased post-treatment hepatic glutathione concentration in fed and in fasted rats (Fig. 1). Post-treatment hepatic glutathione concentration was thus lower in rats that were both fasted and pretreated with 3-methylcholanthrene than in rats that were only fasted or only pretreated with 3-methylcholanthrene (Fig. 1).

Irreversible binding. The amount of metabolite irreversibly bound to hepatic proteins 5 hr after the administration of [^{3}H]acetaminophen was higher in fasted rats than in fed rats (Fig. 2). Pretreatment with phenobarbital did not modify the amount of bound metabolite in fed or in fasted rats (Fig. 2). Pretreatment with 3-methylcholanthrene increased the amount of bound metabolite in fed and fasted rats (Fig. 2). The amount of bound metabolite was thus higher in rats that were both fasted and pretreated with 3-methylcholanthrene than in rats that were only fasted or only pretreated with 3-methylcholanthrene (Fig. 2).

Hepatotoxicity, mortality. Fasting increased SGPT activity 5 and 10 hr after the administration of acetaminophen (Fig. 3). Pretreatment with phenobarbital had no significant effect on SGPT activity in fed or in fasted rats (Fig. 3). Pretreatment with 3-methylcholanthrene increased SGPT activity in fed and in fasted rats (Fig. 3). SGPT activity was thus higher in rats that were both fasted and pretreated with 3-methylcholanthrene than in rats that were only fasted or only pretreated with 3-methylcholanthrene (Fig. 3).

Histological examination of liver specimens obtained 5 hr after the administration of acetaminophen usually showed no necrosis in fed non-pretreated rats and in fed rats pretreated with phenobarbital (Fig. 4). Necrosis was usually present but moderate in fasted but non-pretreated rats, in fasted rats pretreated with phenobarbital, or in fed rats pretreated with 3-methylcholanthrene (Fig. 4). Necrosis was constant and more severe in rats that were both fasted and pretreated with 3-methylcholanthrene (Fig. 4). Thus, the extent of necrosis was higher in rats that were both fasted and pretreated with 3-methylcholanthrene than in rats that were only fasted or only pretreated with 3-methylcholanthrene.

No deaths occurred in the first 10 hr following the administration of acetaminophen but several deaths occurred subsequently. Mortality measured 24 hr after the administration of acetaminophen in 20 rats per group was zero in fed or fasted control rats and in fed rats pretreated with phenobarbital; it was 10 per cent in fasted rats pretreated with phenobarbital and in fed rats pretreated with 3-methylcholanthrene but was 55 per cent in rats that were both fasted and pretreated with 3-methylcholanthrene.

DISCUSSION

Acetaminophen is either conjugated with glucuronate (Table 2) or sulfate, or is activated by cytochrome P-450 into a reactive metabolite which may either bind to hepatic glutathione, the concentration of which in the liver is, in the process, decreased (Fig. 1), or may bind to hepatic macromolecules

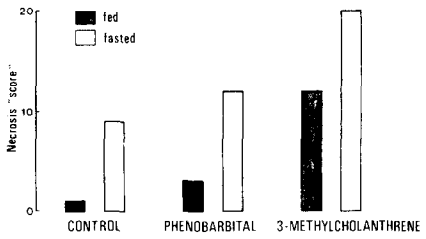

Fig. 4. Effects of pretreatments and diets on the extent of liver cell necrosis after the administration of acetaminophen. Ten rats per group received acetaminophen, 500 mg/kg i.p., and were killed 5 hr later. In each rat, the extent of liver cell necrosis was graded as 0, +, ++ or +++, as previously described [1]. The necrosis 'score' is the total number of + in each group of ten rats.

(Fig. 2); the latter binding may be involved in acetaminophen hepatotoxicity (Fig. 3 and 4). Administration of microsomal enzyme inducers alone [1, 7] or fasting alone [12, 13] may increase the hepatotoxicity of acetaminophen in rats. In this study, we tested the hypothesis that inducers and fasting in combination might have additive effects on the hepatotoxicity of acetaminophen in the rat.

Microsomal enzyme inducers alone. Pretreatment with phenobarbital induced both mixed function oxidase enzymes (Table 1) and acetaminophen UDP-glucuronosyltransferase activity (Table 2). Whereas the former effect should tend to increase the formation of the reactive metabolite of acetaminophen, the latter effect should tend to decrease it by decreasing the amount of acetaminophen available for metabolic activation by cytochrome P-450. Whether the former effect predominates or not over the latter may explain why phenobarbital pretreatment may either slightly increase [2] or not modify [7] (Figs. 1–4) acetaminophen hepatotoxicity.

Pretreatment with 3-methylcholanthrene induced mixed function oxidase enzymes (Table 1) but not acetaminophen UDP-glucuronosyltransferase activity (Table 2). Results reported in this study (Figs. 1–4) and previous findings [7] suggest that pretreatment with 3-methylcholanthrene increases the formation and thus the effects of the reactive metabolite of acetaminophen.

Fasting alone. It has been shown previously [12] that fasting does not modify metabolic activation of acetaminophen by cytochrome P-450 but instead decreases the inactivation of the reactive metabolite of acetaminophen by glutathione, therefore increasing its binding to hepatic macromolecules, and increases the hepatotoxicity of acetaminophen. Results reported in this study (Figs. 1–4) are consistent with this view.

Microsomal enzyme inducers and fasting. In fasted as well as in fed rats, pretreatment with phenobarbital had little effect on acetaminophen binding and toxicity (Figs. 1–4).

In rats that were both pretreated with 3-methylcholanthrene and fasted, hepatic glutathione concentrations fell to lower values (Fig. 1), the amount of metabolite irreversibly bound to proteins was higher (Fig. 2) and hepatic necrosis was more severe (Figs. 3 and 4) than in rats that were only pretreated with 3-methylcholanthrene or only fasted. These observations suggest that inducers which increase the formation of the reactive metabolite and fasting, which decreases its inactivation by hepatic glutathione, may have additive effects on the hepatotoxicity of acetaminophen in the rat.

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REFERENCES

1. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 185 (1973).
2. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 195 (1973).
3. W. Z. Potter, D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 203 (1973).
4. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187**, 211 (1973).
5. W. Z. Potter, S. S. Thorgeirsson, D. J. Jollow and J. R. Mitchell, *Pharmacology* **12**, 129 (1974).
6. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, M. Hashimoto and J. R. Mitchell, *Pharmacology* **12**, 251 (1974).
7. R. J. McMurtry, W. R. Snodgrass and J. R. Mitchell, *Toxic. appl. Pharmac.* **46**, 87 (1978).
8. P. Moldeus, *Biochem. Pharmac.* **27**, 2859 (1978).
9. R. Clark, R. P. H. Thompson, V. Borirakchanyavat, B. Widdop, A. R. Davidson, R. Goulding and R. Williams, *Lancet* **1**, 66 (1973).
10. H. L. Bonkowsky, G. H. Mudge and R. J. McMurtry, *Lancet* **1**, 1016 (1978).
11. G. K. Johnson and K. G. Tolman, *Ann. intern. Med.* **87**, 302 (1977).
12. D. Pessayre, A. Dolder, J. Y. Artigou, J. C. Wandscheer, V. Descatoire, C. Degott and J. P. Benhamou, *Gastroenterology* **77**, 264 (1979).
13. A. Wendel, S. Feuerstein and K. H. Konz, *Biochem. Pharmac.* **28**, 2051 (1979).
14. D. Pessayre and P. Mazel, *Biochem. Pharmac.* **25**, 943 (1976).
15. G. J. Mulder and A. B. D. Van Doorn, *Biochem. J.* **151**, 131 (1975).
16. G. L. Ellman, *Archs biochem. Biophys.* **82**, 70 (1959).
17. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).