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INHIBITION OF ACETAMINOPHEN HEPATOTOXICITY BY CHLORPROMAZINE IN FED AND FASTED MICE

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Abstract—Acetaminophen hepatotoxicity has been shown previously to be potentiated by fasting, and the mechanism of hepatotoxicity has been correlated with depletion of reduced glutathione and the resulting elevation of cytosolic calcium. Chlorpromazine inhibited the hepatotoxicity of acetaminophen in a dose-dependent manner in fed and fasted mice. A 6 mg/kg dose of chlorpromazine prevented the acetaminophen-promoted increase in SGPT levels and prevented hepatic necrosis. Chlorpromazine did not prevent the depletion of reduced glutathione by acetaminophen in fed or fasted mice, although it did decrease the extent of reduced glutathione depletion caused by acetaminophen in fed mice from 80% depletion to 67% depletion. We propose that chlorpromazine causes a negative sensitivity modulation to calcium in hepatocytes, as evidenced by chlorpromazine preventing the acetaminophen-stimulated rise in phosphorylase *a* activity. We also propose that fasting potentiates acetaminophen hepatotoxicity by causing a positive sensitivity modulation to calcium in hepatocytes via the actions of glucagon.

Acetaminophen hepatotoxicity has been investigated thoroughly [1–7]. Early steps in the sequence of events that produce hepatotoxicity include metabolism by the hepatic mixed-function oxidase system to form toxic metabolites [2–6], binding of toxic metabolites to the hepatic endoplasmic reticulum [1–3], and depletion of reduced glutathione [5–7]. The depletion of reduced glutathione is a key event in production of hepatotoxicity since sulfhydryl compounds that maintain or substitute for reduced glutathione suppress acetaminophen hepatotoxicity [4, 5, 8–10]. The mechanism that apparently couples depletion of reduced glutathione to production of hepatotoxicity is the increase in cytoplasmic calcium levels caused by release of calcium from the endoplasmic reticulum [11, 12] or the mitochondria [13] as a result of the reduced glutathione depletion. The increased calcium concentration then alters cell function to promote hepatotoxicity [12, 14]. One alteration of hepatic cell function that has been employed as a measure of increased cytoplasmic calcium is the activation of phosphorylase and the resulting glycogenolysis [15–17].

Since the elevation of cytoplasmic calcium is apparently important to the mechanism of acetaminophen-induced hepatotoxicity, drugs that interfere with calcium function in the cell should inhibit acetaminophen hepatotoxicity. The purpose of this study was to investigate the ability of chlorpromazine to inhibit acetaminophen hepatotoxicity. Chlorpromazine, a phenothiazine tranquilizer, is known to inhibit the ability of calcium to activate calmodulin [18, 19] and to inhibit the activation of phospholipase C and protein kinase C [20, 21].

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MATERIALS AND METHODS

Male Swiss mice weighing 30–40 g were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The mice were maintained on water and purina lab chow *ad lib.*, except during periods of fasting. Fasting was for 18 hr prior to administration of acetaminophen. The mice were exposed to a 12-hr light–dark cycle with the room being lit from 7:00 a.m. until 7:00 p.m.

Acetaminophen was injected subcutaneously in a dose of 250 mg/kg to fasted mice and 800 mg/kg to fed mice. The acetaminophen was dissolved in saline in a concentration of 12.5 mg/ml and heated to 37° prior to injection. Chlorpromazine was injected intraperitoneally in a dose of 0.5 to 10 mg/kg, as indicated. The chlorpromazine was dissolved in saline and injected 1 hr prior to acetaminophen.

SGPT activity was determined 24 hr after acetaminophen injection. The SGPT activity was determined by monitoring the decrease in absorbance at 340 nm as described by Wroblewski and LaDue [22]. Mice were decapitated to obtain the blood sample. Livers from these animals were then removed for histological examination, which was performed by preparing paraffin embedded sections of formalin fixed liver and staining with hematoxylin and eosin.

Covalent binding of [^{3}H]acetaminophen to microsomes was determined 3 hr after the injection of 250 mg/kg (100 μCi/kg) of [^{3}H]acetaminophen in fasted mice and 800 mg/kg (100 μCi/kg) of [^{3}H]acetaminophen in fed mice. The [^{3}H]acetaminophen was obtained from Dupont NEN. Assays for purity indicated greater than 99% purity. In these experiments the mice were decapitated and the livers were removed and homogenized in 5 vol. of cold

1.15% KCl in 0.05 M Tris buffer, pH 7.4, using a polytron. Microsomes were prepared as previously described [23], and microsomal pellets were resuspended in the 0.05 M Tris–KCl buffer, pH 7.4, using a glass hand homogenizer. Protein was determined by the method of Lowry *et al.* [24]. The amount of [^{3}H]acetaminophen bound to the microsomes was determined by liquid scintillation using a vacuum filtration technique previously described [25]. An aliquot of the microsomal preparation equal to 20 mg of microsomal protein or 800 mg of liver was filtered.

Hepatic reduced glutathione was determined 2 hr after acetaminophen injection by the method of Ellman [26] as previously described [25]. Phosphorylase *a* activity was determined by utilizing a coupled enzyme assay that measures change in absorbance at 340 nm as described by Bergmeyer *et al.* [27]. In these experiments the mice were decapitated 2 hr after the injection of acetaminophen. The livers were removed and homogenized in 10 vol. of cold 1.15% KCl in 0.05 M Tris buffer, pH 7.4, using a polytron. The homogenate was centrifuged for 20 min at 9000 *g*, and the supernatant fraction was assayed for phosphorylase *a* activity. The incubation mixture contained 45 mM potassium phosphate buffer, pH 6.8, 0.1 mM EDTA, 2 mg/ml glycogen, 0.34 mM NADP, 4 μM glucose-1,6-diphosphate, 15 mM $MgCl_2$, 0.8 units/ml phosphoglucomutase and 6 units/ml glucose-6-phosphate dehydrogenase.

RESULTS

A single injection of chlorpromazine given 1 hr prior to acetaminophen injection caused a dose-dependent decrease in acetaminophen hepatotoxicity in fed and fasted mice as measured by change in SGPT levels (Table 1). A dose of 1 mg/kg of chlorpromazine caused a significant decrease in SGPT and 6.0 mg/kg returned the SGPT level to control values. Control SGPT levels were 57.11 ± 2.71 units/ml in fed mice and 71.80 ± 6.43 units/ml in fasted mice. The inhibition of acetaminophen hepatotoxicity was confirmed by histological studies (Table 2). The chlorpromazine produced a dose-dependent decrease in hepatic necrosis in fasted mice. A dose of 3.0 mg/kg of chlorpromazine caused a significant decrease in hepatic necrosis and 6.0 mg/kg essentially prevented necrosis.

Table 2. Effect of chlorpromazine on hepatic necrosis in fasted mice treated with acetaminophen*

Chlorpromazine dose (mg/kg)	Extent of necrosis (scaled value)
0	7.00 ± 0.00
0.5	8.20 ± 0.73
1.0	7.00 ± 0.00
3.0	2.25 ± 0.75†
6.0	0.25 ± 0.25†
10.0	0.40 ± 0.24†

* Values are the mean ± SE for four to six mice. Animals were killed 24 hr after the subcutaneous injection of 250 mg/kg of acetaminophen. Chlopromazine was injected i.p. 1 hr prior to acetaminophen. Values for necrosis were determined as 10 = massive, 7 = severe (bridging), 3 = moderate, 1 = mild (swelling only).

† Significantly different from zero dose group, $P \leq 0.05$.

An investigation of the mechanism by which chloropromazine inhibits hepatic necrosis demonstrated that chlorpromazine did not prevent the depletion of reduced glutathione by acetaminophen in fed or fasted mice (Table 3). However, the amount of reduced glutathione in fed mice receiving both chlorpromazine and acetaminophen (4.11 ± 0.47) was higher than the reduced glutathione level in mice receiving only acetaminophen (2.49 ± 0.08). The percent of reduced glutathione depleted in the fed mice, as compared to the control value of 12.41 ± 0.58, was 67% in the mice administered chlorpromazine plus acetaminophen and 80% in mice administered acetaminophen alone. This may represent some protection of reduced glutathione in fed mice. However, the reduced glutathione levels in fasted mice were essentially identical in the chlorpromazine plus acetaminophen group and the acetaminophen group, indicating no protection of reduced glutathione in fasted mice. Also, the chlorpromazine did not alter the binding of [^{3}H]acetaminophen to the hepatic endoplasmic retic-

Table 1. Effect of chlorpromazine on elevated SGPT levels in fed and fasted mice treated with acetaminophen*

Chlorpromazine dose (mg/kg)	SGPT (units/ml)	
	Fed	Fasted
0	2005.87 ± 541.87	3034.58 ± 384.75
0.5	1222.18 ± 770.79	3164.40 ± 701.69
1.0	90.94 ± 14.35†	1781.71 ± 249.39†
3.0	150.33 ± 76.76†	551.24 ± 260.25†
6.0	43.67 ± 14.48†	40.09 ± 4.09†
10.0	161.14 ± 78.62†	35.27 ± 1.86†

* Values are the mean ± SE for four to six mice. The SGPT activity was determined 24 hr after the subcutaneous injection of 250 mg/kg of acetaminophen in fasted mice and 800 mg/kg in fed mice. Chlorpromazine was injected i.p. 1 hr prior to acetaminophen. A coupled enzyme assay utilizing change in absorbance at 340 nm was used to determine SGPT activity.

† Significantly different from zero dose group, $P \leq 0.05$.

Table 3. Effect of chlorpromazine on hepatic reduced glutathione levels in fed and fasted mice treated with acetaminophen*

Treatment	GSH (μmol/g liver)	
	Fed	Fasted
Control	12.41 ± 0.58	7.01 ± 0.36†
Acetaminophen	2.49 ± 0.08‡	2.69 ± 0.18‡
Chlorpromazine	11.07 ± 0.47	6.07 ± 0.20
Chlorpromazine plus acetaminophen	4.11 ± 0.47‡	2.54 ± 0.13‡

* Values are the mean ± SE for six mice. Mice were killed 2 hr after the subcutaneous injection of 250 mg/kg of acetaminophen in fasted mice and 800 mg/kg in fed mice. Chlorpromazine, 6 mg/kg, was injected i.p. 1 hr prior to acetaminophen. Livers were homogenized in 0.1 M metaphosphoric acid, and an aliquot of a 10,000 *g* supernatant fraction was assayed for GSH using Ellman's Reagent and relating the absorbance at 412 nm to the concentration of sulfhydryl groups.

† Significantly different from fed control, $P \leq 0.05$.

‡ Significantly different from the appropriate control group, $P \leq 0.05$.

ulum in fed mice, but it did cause a 50% decrease in binding in fasted mice (Table 4). Interestingly, this decrease in binding in fasted mice was not accompanied by any effect of chloropromazine pretreatment on reduced glutathione depletion.

Acetaminophen pretreatment of fed mice tripled the hepatic phosphorylase *a* activity (Table 5), which is apparently a result of increased cytosolic calcium [12] caused by acetaminophen depleting the reduced glutathione from the hepatocytes [11–13]. Chlorpromazine pretreatment prevented the elevation of phosphorylase *a* activity by acetaminophen. Interestingly, the chlorpromazine alone caused a 50% increase in phosphorylase *a* activity in fed mice. Fasting doubled the phosphorylase *a* activity as compared to the fed control, which is a result of activation of the cyclic AMP messenger system by glucagon [28, 29]. Acetaminophen pretreatment of the fasted mice increased the phosphorylase *a* activity by only 15% as compared to the fasted control. This charge was not significant using Student's *t*-test, but was significant using Dunnett's *t*-test. Chlorpromazine pretreatment prevented the elevation of phosphorylase *a* activity by acetaminophen and returned the phosphorylase *a* activity to the range of the fed control.

DISCUSSION

Chlorpromazine, a phenothiazine tranquilizer, is a potent antagonist of acetaminophen hepatotoxicity. Its protective action is apparently due to either inhibition of acetaminophen metabolism and/or alteration of cellular response to increased cytosolic calcium levels. To fully evaluate the effect of chlorpromazine pretreatment on acetaminophen metabolism, additional experiments must be performed. The possibility that chlorpromazine may inhibit the metabolism of acetaminophen is indicated by the fact that chlorpromazine pretreatment did decrease acetaminophen binding in fasted mice (Table 4). However, there may be alternate explanations of the decreased binding in fasted mice after chlorpromazine pretreatment. Interestingly, previous investigations in our laboratory have demonstrated that fasting approximately doubles the binding of acetaminophen to the hepatic endoplasmic reticulum [7]. The enhanced binding was not seen in the present experiment because the fasted mice were given a lower dose of acetaminophen than were the fed mice. This enhanced binding of acetaminophen was inhibited selectively by chlorpromazine, since chlorpromazine did not inhibit acetaminophen binding in fed mice. Experiments in our laboratory have also demonstrated that dantrolene sodium inhibited acetaminophen hepatotoxicity and selectively inhibited binding of acetaminophen in fasted mice (unpublished data). The fact that these drugs did not inhibit acetaminophen binding in fed mice indicates that they may not be inhibiting drug metabolism, but are inhibiting other processes that promote acetaminophen metabolite binding in fasted mice.

The ability of chlorpromazine to prevent acetaminophen hepatotoxicity is apparently due, at

Table 4. Effect of chlorpromazine on binding of acetaminophen metabolites to the hepatic endoplasmic reticulum in fed and fasted mice*

Treatment	[^{3}H]Acetaminophen metabolites (nmol bound/100 mg ER protein)	
	Fed	Fasted
Acetaminophen	7.87 ± 0.50	6.28 ± 1.20
Chlorpromazine plus acetaminophen	6.47 ± 0.55	3.02 ± 0.20†

* Values are the mean ± SE for six mice. The binding of [^{3}H]acetaminophen metabolites to the hepatic endoplasmic reticulum was determined 3 hr after the subcutaneous injection of 250 mg/kg of [^{3}H]acetaminophen in fasted mice and 800 mg/kg in fed mice by quantitating bound isotope using a standard vacuum filtration technique. Chlorpromazine, 6 mg/kg, was injected 1 hr prior to acetaminophen. The endoplasmic reticulum was isolated using a standard differential centrifugation technique.

† Significantly different from acetaminophen, $P \leq 0.05$.

Table 5. Effect of chlorpromazine on charges in hepatic phosphorylase *a* activity in fed and fasted mice treated with acetaminophen*

Pretreatment	Phosphorylase *a* activity (nmol/min/g liver)	
	Fed	Fasted
Control	416.00 ± 45.80	892.29 ± 55.28†
Chlorpromazine	641.32 ± 44.26‡	572.75 ± 62.24‡
Acetaminophen	1382.64 ± 79.58‡	1028.94 ± 36.21‡
Chlorpromazine plus acetaminophen	409.97 ± 30.33	361.74 ± 44.21‡

* Values are the mean ± SE of eight mice. The phosphorylase *a* activity was determined 2 hr after the injection of 250 mg/kg of acetaminophen in fasted mice and 800 mg/kg in fed mice. Chlorpromazine, 6 mg/kg, was injected 1 hr prior to acetaminophen. Phosphorylase *a* activity was determined utilizing a coupled enzyme assay that measures change in absorbance at 340 nm.

† Significantly different from fed control, $P \leq 0.05$.

‡ Significantly different from appropriate control $P \leq 0.05$.

least in part, to alteration of the cellular response to elevated cytosolic calcium levels. The ability of chlorpromazine to act as a calmodulin inhibitor is well known [18, 19], and much work has been done in the past few years investigating the ability of chlorpromazine to interfere with the protein kinase C system. Lamb *et al.* demonstrated that chlorpromazine inhibits phospholipase C activity which could be elevated by carbon tetrachloride [20] and several authors have indicated that phenothiazines, including chlorpromazine, as well as other calmodulin inhibitors also inhibit protein kinase C, apparently by inhibiting the activation of protein kinase C by phosphatidylserine [21, 30–33]. These documented actions of chlorpromazine indicate that chlorpromazine can certainly alter cellular response to elevated cytosolic calcium levels. In our present experiments, depletion of reduced glutathione by acetaminophen is the event that raises cytosolic calcium levels [11–13], and chlorpromazine did not prevent the depletion of reduced glutathione in fed or fasted mice (Table 3). Therefore, the possibility that chlorpromazine lowered phosphorylase *a* activity (Table 5) by lowering cytosolic calcium is unlikely; rather, the chlorpromazine is apparently inhibiting the ability of elevated cytosolic calcium to alter cell function. So, the chlorpromazine is causing a negative sensitivity modulation to calcium.

The importance of sensitivity modulation of hepatic cells to calcium is emphasized by the effect of fasting on acetaminophen hepatotoxicity. Fasting has been demonstrated to increase acetaminophen hepatotoxicity [7], and in this present investigation the 250 mg/kg dose of acetaminophen given to fasted mice was found to be equally hepatotoxic to the 800 mg/kg dose given to fed mice. Fasting causes a positive sensitivity modulation of hepatic cells to calcium via increased glucagon secretion. Glucagon acts on the liver to alter cell regulatory functions by simulating the cyclic AMP messenger system [28, 29]. Activation of the cyclic AMP messenger system causes the positive sensitivity modulation to calcium [28]. Activation of the hepatic cyclic AMP system by glucagon has also been shown to enhance calcium mobilization by activators of the hepatic alpha-1 adrenergic receptors [29]. Thus, fasting increases the hepatic response to elevation of cytosolic calcium levels and can even promote calcium mobilization.

In conclusion, we propose that the ability of chlorpromazine to cause a negative sensitivity modulation to calcium in hepatocytes is partially responsible for its ability to decrease the hepatotoxic potency of acetaminophen. Elucidation of the relative importance of inhibition of the calmodulin pathway and the protein kinase C pathway requires further studies. Also, further studies are needed to determine the effect of chlorpromazine on acetaminophen metabolism.

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