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SHORT COMMUNICATIONS

Effect of piperonyl butoxide post-treatment on acetaminophen hepatotoxicity*

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Acetaminophen (APAP) is a relatively safe antipyretic/analgesic when administered at therapeutic doses. However, toxic doses of APAP cause hepatic centrilobular necrosis [1, 2]. The hepatic mixed-function oxidase system (MFO) generates a toxic, electrophilic metabolite of APAP which is normally detoxified by conjugation with reduced glutathione (GSH) [3]. At toxic doses of APAP, hepatic GSH becomes depleted, and the toxic metabolite is free to bind covalently to hepatic macromolecules and to initiate a sequence of events which ultimately leads to cell death [3, 4]).

In previous studies with mice, covalent binding reached a maximum about 2 hr after a hepatotoxic dose of APAP [4, 5]. Similarly, maximal GSH depletion also occurred at 2 hr and remained at very low levels through 4 hr with no further detectable increase in covalent binding [4–6]. Since other studies with antidotes [7, 8] suggested that GSH synthesis capabilities are not impaired during this time, the extended GSH depletion may reflect continued production of APAP electrophile beyond achievement of maximal covalent binding. If such continued electrophile production were important to the progression of APAP-induced hepatotoxicity, then interference with electrophile production beyond 2 hr after APAP administration should diminish the toxicity. To test this, piperonyl butoxide, an MFO inhibitor, was administered at selected times during APAP exposure.

Methods

APAP, corn oil, 5,5′-dithiobis-(2-nitrobenzoic acid), fructose, NADH, propylene glycol, tetrasodium EDTA, trichloroacetic acid and triethanolamine were all purchased from the Sigma Chemical Co. (St. Louis, MO). Mono- and dibasic sodium phosphate were obtained from Fisher Scientific (Fair Lawn, NJ), glutathione (GSH) was from Calbiochem (San Diego, CA), and piperonyl butoxide (80%) was from ICN Biochemicals, Inc. (Plainview, NY).

Fasted (18–20 hr), 3-month-old, male mice [Charles River, Crl: CD-1 (ICR)BR, Wilmington, MA] were used for all experiments. Animals were housed in stainless steel cages in a humidity- and temperature-controlled room with a 12-hr light/dark cycle and provided with food and water *ad lib.* prior to fasting. APAP (600 mg/kg, 10 ml/kg, p.o.) was dissolved in 1 : 1 propylene glycol : redistilled, deionized water (37°). Piperonyl butoxide (600 mg/kg, 5 ml/kg, i.p.) was administered in corn oil 1 hr before, or 2 or 4 hr after APAP. Mice were killed by decapitation at selected times after dosing. Appropriate vehicle-injected controls were included in each experiment, and all treatment groups included at least three mice. Previously, we characterized the dose and time dependency of APAP-induced hepatotoxicity in fasted CD-1 mice [9]. Biochemical and morphological examinations revealed that 600 mg of APAP/kg was slightly above the threshold for hepatotoxicity, causing overt hepatic necrosis with minimal mortality over 24 hr. Based upon these earlier observations, 600 mg/kg was selected for the present studies.

Hepatic GSH was estimated by measuring non-protein sulfhydryls in liver homogenates according to the method of Ellman *et al.* [10] as described previously [9]. Hepatic GSH was normalized per kg of body weight to correct for changes in liver weight associated with hepatic damage.

Trunk blood was collected into heparinized test tubes. Following centrifugation, plasma was collected and stored frozen (−20°) for no more than 2 days for determination of plasma sorbitol dehydrogenase (SDH) activity as described [11].

Liver sections for histopathologic evaluation by light microscopy were prepared as described previously [9]. APAP-induced hepatic centrilobular necrosis was graded on a scale of 0 to (+5), where (0) = no lesion; (+1) = minimal: <5% necrosis of the liver lobule; (+2) = mild: 5–10% necrosis of the liver lobule; (+3) moderate: 10–15% necrosis; (+4) = marked: 25–30% necrosis; and (+5) = severe; ≥50% necrosis.

Student's *t*-test was used for statistical analysis with $P \leq 0.05$ regarded as significant.

Results and discussion

Piperonyl butoxide, a well established MFO inhibitor [12, 13], prevents APAP-induced GSH depletion, covalent binding and hepatotoxicity through reduction of APAP reactive metabolite formation [3, 4, 6, 14–16]. In the present studies, initial experiments were directed at selecting a pretreatment dose of piperonyl butoxide that effectively blocked APAP hepatotoxicity in the CD-1 mouse. As shown in Fig. 1, A and B, administration of APAP (600 mg/kg) to mice given a vehicle pretreatment resulted in rapid depletion of hepatic GSH to 11% of control within 2 hr and elevated plasma SDH by approximately 96-fold by 12 hr after APAP. Pretreatment with piperonyl butoxide 1 hr prior to APAP administration significantly diminished hepatic GSH depletion from 0.5 to 4 hr after APAP compared to vehicle-pretreated controls and completely blocked APAP-induced increases in plasma SDH activity (Fig. 1, A and B). Vehicle alone had no effect on either plasma SDH or hepatic GSH levels (not shown). These data, which confirm earlier studies [3, 4, 6, 14–16], show that the dose of piperonyl butoxide employed was effective in preventing APAP hepatotoxicity when given prior to APAP.

If continued generation of electrophile beyond that which resulted in maximal covalent binding were important to the outcome of APAP overdose, then intervention with an MFO blocker such as piperonyl butoxide at critical times after APAP administration should diminish the toxicity. Furthermore, it should also be possible to define a "window in time" beyond which such intervention would no longer alter the outcome of APAP overdose. To test this, piperonyl butoxide was given at either 2 or 4 hr post APAP, and hepatic damage was assessed at selected times thereafter. Administration of piperonyl butoxide 2 hr after APAP reduced APAP-induced elevations in plasma SDH activity compared to the elevations observed in mice given APAP alone (Fig. 2A). In agreement with this, liver GSH levels recovered more rapidly in mice given piperonyl butoxide 2 hr post APAP (Fig. 2B). For instance, hepatic GSH levels were 71, 43 and 32% higher in piperonyl butoxide post-

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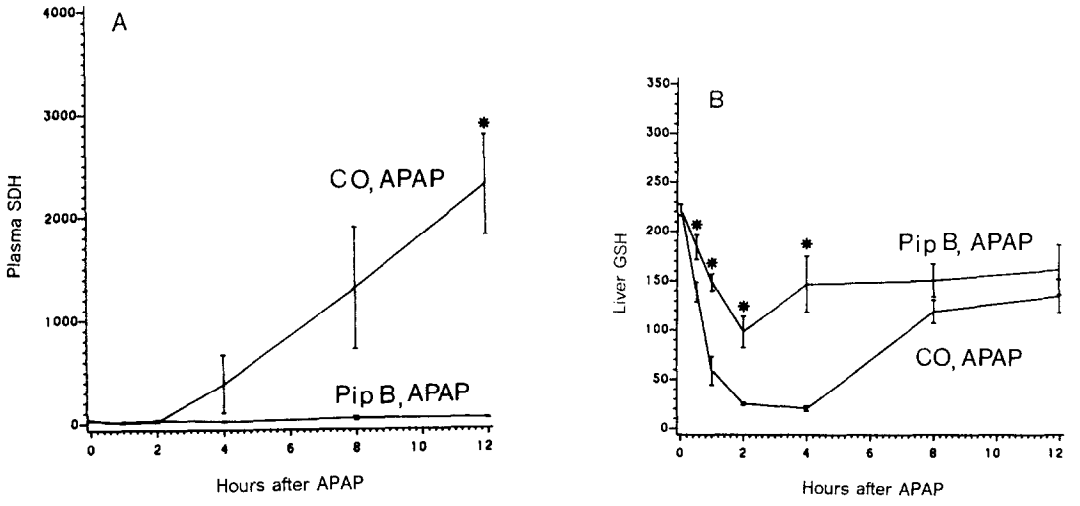

Fig. 1. Effect of a 1-hr piperonyl butoxide pretreatment on acetaminophen-induced alterations in (A) plasma sorbitol dehydrogenase (SDH) activity and (B) hepatic glutathione (GSH). Mice were pretreated with piperonyl butoxide (Pip B, 600 mg/kg, i.p.) or corn oil (CO) 1 hr before acetaminophen (APAP, 600 mg/kg, p.o.) administration. Plasma SDH = units of sorbitol dehydrogenase activity/ml plasma. Liver GSH = nmol liver non-protein sulfhydryls/kg body weight. All values are mean ± SE ($N \geq 3$). Statistical comparisons were made between treatment groups at corresponding times. An asterisk indicates $P \leq 0.05$ (Student's *t*-test).

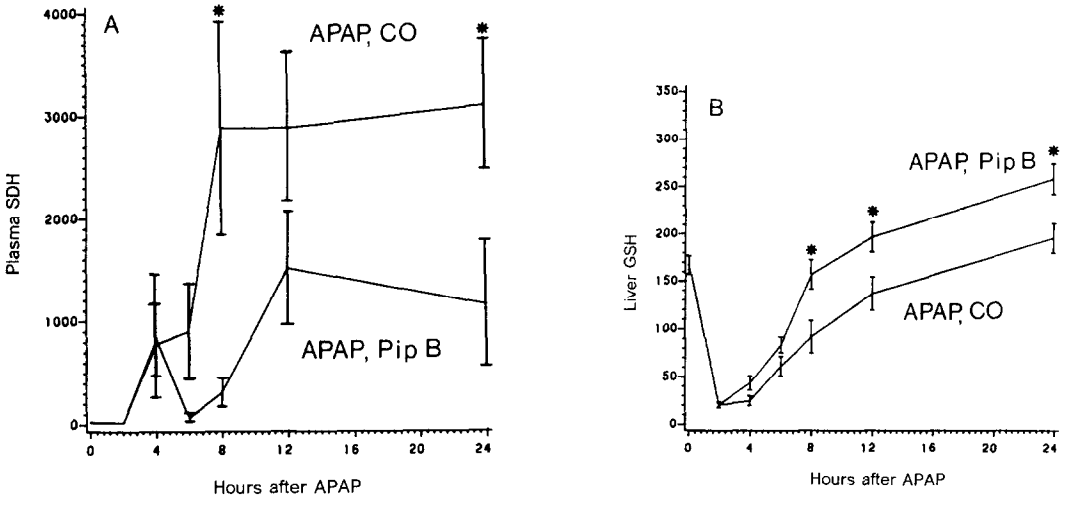

Fig. 2. Effect of a 2-hr piperonyl butoxide post-treatment on acetaminophen-induced alterations in (A) plasma sorbitol dehydrogenase (SDH) activity and (B) hepatic glutathione (GSH). All mice were dosed with acetaminophen (APAP, 600 mg/kg, p.o.) at 0 hr and given piperonyl butoxide (Pip B, 600 mg/kg, i.p.) or corn oil (CO) vehicle 2 hr later. Plasma SDH and liver GSH are expressed as in Fig. 1. All values are mean ± SE ($N \geq 3$). Statistical comparisons were made between treatment groups at corresponding times. An asterisk indicates $P \leq 0.05$ (Student's *t*-test).

treated mice at 8, 12 and 24 hr after APAP, respectively, compared to APAP-dosed controls. Liver histopathologic evaluation 24 hr after APAP showed that only 40% of mice treated with piperonyl butoxide 2 hr after APAP had a grade of necrosis greater than +4 compared to 87% in control mice given APAP (Table 1). In contrast, when piperonyl butoxide was given 4 hr after APAP, there was no protection against hepatic damage as assessed biochemically (data not shown) or morphologically (Table 1). Thus, a 2-hr, but not 4-hr, piperonyl butoxide post-treatment effectively reduced APAP hepatotoxicity. These results are in agreement with those of Letteron *et al.* [17] who showed that treatment with an MFO inhibiting dose of methoxsalen 2 hr after an hepatotoxic dose of APAP also diminished APAP hepatotoxicity.

Previous work in our laboratory [5] and others [4] has

Table 1. Effect of a 2- or 4-hr piperonyl butoxide post-treatment on APAP-induced hepatic centrilobular necrosis

Time (hr)	Treatment	Frequency of grade: Grade 0	+1	+2	+3	+4	+5
2	Corn oil	0	1	0	0	3	4
	Pip B	3	1	1	1	2	2
4	Corn oil	0	0	0	2	4	4
	Pip B	1	0	0	1	2	6

Fasted (18–20 hr) mice were dosed with acetaminophen (APAP, 600 mg/kg, p.o.) and given piperonyl butoxide (Pip B, 600 mg/kg, i.p.) or corn oil vehicle 2 or 4 hr later. All mice were killed 24 hr after APAP. Livers were examined histologically by light microscopy and graded for the severity of hepatic centrilobular necrosis on the following scale: no lesion (0); minimal (+1); mild (+2); moderate (+3); marked (+4); and severe (+5).

shown that maximal covalent binding is attained 2 hr after administration of APAP to fasted mice. That a 2-hr post-treatment with the MFO inhibitor, piperonyl butoxide, reduced APAP hepatotoxicity in the same animal model suggests that continued production of APAP electrophile occurred beyond attainment of maximal covalent binding and played a significant role in determining the severity of hepatic damage. In support of this, *N*-acetylcysteine, a GSH precursor, is also an effective antidote of APAP hepatotoxicity when given after maximal covalent binding [7, 8]. Toxicologically significant electrophile production was probably complete by 4 hr after APAP as treatment with piperonyl butoxide at this time had no protective effect.

Covalent binding of APAP metabolite to cell macromolecules may be mechanistically involved in the toxicity or it may merely reflect the existence of the electrophilic metabolite [18, 19]. In the former case, our findings would suggest that the binding which occurs beyond 2 hr after APAP played a significant role in the toxicity. Alternatively, in the latter case, our findings would suggest that the continued generation of electrophile within the hepatocyte beyond 2 hr was important in and of itself.

The present findings raise the question of why covalent binding reaches an apparent maximum before toxicologically relevant electrophile production ceases. Several explanations are possible: (1) covalent binding sites are saturated, (2) as "new" macromolecule sites become covalently bound, "old" ones are degraded or secreted, (3) covalent binding after the apparent maximum is quantitatively very small, but qualitatively very selective for "critical" sites [20], and (4) other electrophile-dependent mechanisms (e.g. protein sulfhydryl oxidation and/or redox cycling) play a role in initiating APAP hepatotoxicity [21–25].

This study has demonstrated that delayed administration of piperonyl butoxide (at 2 hr) diminished the severity of APAP hepatotoxicity. This indicates the toxicologic importance of electrophile production beyond the time of apparent maximal covalent binding. Studying the effects of electrophile produced between 2 and 4 hr after APAP dosing will provide additional insight into mechanisms involved in APAP hepatotoxicity.

§ Address reprint requests to: Steven D. Cohen, D.Sc., University of Connecticut, School of Pharmacy, Toxicology Program, Box U-92, 372 Fairfield Rd., Storrs, CT 06268.

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Toxicology Program
‡*Department of Molecular and Cell Biology,*
†*Department of Pathobiology, and*
**Department of Pharmacology and Toxicology*
University of Connecticut
Storrs, CT 06268, U.S.A.

JOSEPH T. BRADY*
DORIS A. MONTELIUS*
WILLIAM P. BEIERSCHMITT*
D. STUART WYAND†
EDWARD A. KHAIRALLAH‡
STEVEN D. COHEN*§

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