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INTERACTION OF CAFFEINE WITH ACETAMINOPHEN

1. CORRELATION OF THE EFFECT OF CAFFEINE ON ACETAMINOPHEN HEPATOTOXICITY AND ACETAMINOPHEN BIOACTIVATION FOLLOWING TREATMENT OF MICE WITH VARIOUS CYTOCHROME P450 INDUCING AGENTS

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Abstract—The combination of caffeine with acetaminophen (APAP) is used widely in the treatment of headache. The effects of caffeine on APAP-induced hepatotoxicity and APAP bioactivation by liver microsomes from uninduced mice and from mice pretreated with various agents that induce cytochrome P450 were studied. When 1 mM caffeine was included, the rate of glutathione–APAP conjugate (GS–APAP) formation was increased significantly by 33 and 39% in microsomes from phenobarbital (PB)- and dexamethasone (DEX)-treated mice, respectively, whereas this parameter was decreased 39 and 12% by caffeine in microsomes from β-naphthoflavone (βNF)- and acetone-treated mice, respectively. A 5 mM concentration of caffeine increased GS–APAP formation by 47, 107 and 117% in microsomes from control, PB-, and DEX-treated mice, respectively, and decreased it 39 and 25% in microsomes from βNF- and acetone-treated mice, respectively. Caffeine was a competitive inhibitor of APAP bioactivation in microsomes from βNF- and acetone-treated mice. While caffeine increased APAP bioactivation in microsomes from uninduced, PB-, and DEX-treated mice, the apparent K_m values for APAP were increased by caffeine, indicating that this enhancement was not due to a direct effect of caffeine on APAP binding to cytochrome P450 but may be due to an effect of caffeine on the substrate–enzyme complex. The variable effect of caffeine on APAP hepatotoxicity correlated with the effect of caffeine on APAP bioactivation by liver microsomes, regardless of pretreatment. Lack of correlation of aminopyrine *N*-demethylase, but good correlation of erythromycin *N*-demethylase activity with the extent of caffeine enhancement of APAP bioactivation following PB or DEX treatment suggests that a murine P450 subfamily similar to the rat P450 3A subfamily may be the candidate in mediating the stimulatory effect of caffeine on APAP bioactivation and APAP-induced hepatotoxicity.

Acetaminophen (APAP§), on a mg for mg basis, has just about the same analgesic and antipyretic effect as aspirin. Its advantage over aspirin is that it does not cause gastric bleeding or irritation, nor does it depress thromboxane synthesis at therapeutic doses. When taken in therapeutic doses (325–600 mg, every 4 hr), APAP is a safe analgesic. The most important serious adverse effect of APAP is a potential fatal hepatic necrosis, which is evident following the ingestion of 10–15 g of APAP in humans [1]. Acetaminophen is metabolized in the liver primarily by conjugation to the glucuronide and sulfate. These polar metabolites are relatively non-toxic and are eliminated by the kidney. A minor metabolic pathway involves oxidation by cytochrome P450 (P450), forming an electrophilic intermediate, *N*-acetyl-*p*-benzoquinone imine (NAPQI) [2], which binds to glutathione (GSH) and this glutathione–APAP conjugate (GS–APAP) may then be excreted in bile or undergo further metabolism, forming cysteine and mercapturic acid conjugates, which are excreted in the urine [3]. As the APAP dosage increases, the glucuronidation and sulfation pathways become saturated and more APAP metabolism is diverted through P450. At sufficiently high doses, GSH becomes depleted, leaving NAPQI free to bind to possibly critical cellular proteins and to cause hepatic necrosis. The toxicity of APAP is therefore a function of the amount of NAPQI formed and the availability of hepatic GSH for nontoxic elimination [2, 4].

The combination of caffeine with an analgesic, such as aspirin or APAP, is widely used in the treatment of headache (e.g. Excedrin). Results of some studies have suggested that caffeine may increase the analgesic effect of aspirin, APAP [5] or ibuprofen [6] without changing the pharmacokinetics of these analgesics. However, other studies have been unable to substantiate such enhancement [7]. It has been reported [8] that in Japan, where APAP is not available except compounded with caffeine, that APAP-induced hepatic injury occurs with relatively small doses of APAP (2.3 to 8.8 g). The reason for this increased sensitivity is not known. The metabolism of caffeine is a complex process and

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§ Abbreviations: APAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinone imine; ALT, alanine aminotransferase; DEX, dexamethasone; GSH, glutathione; GS–APAP, glutathione–acetaminophen conjugate; βNF, β-naphthoflavone; NED, *N*-(1-naphthyl)ethylenediamine dihydrochloride; and PB, phenobarbital.

not fully understood. Caffeine may be demethylated at N-1, N-3, and N-7, and hydroxylated at C-8 by hepatic P450s [9–11]. It has been shown that in rats caffeine demethylation at N-3 is selectively catalyzed by P450 1A2 [12]. Although the isozyme(s) of P450 involved in other metabolic pathways of caffeine is not as well defined, theophylline, the metabolic product of caffeine N-7 demethylation, is reportedly hydroxylated at C-8 by P450 2E1 [13].

The interaction between caffeine and APAP has become the topic of several studies over the last decade. Sato and co-workers reported that the simultaneous administration of APAP and caffeine potentiates APAP-induced hepatotoxicity in rats [14] by enhancing the production of NAPQI by mixed-function oxidases [8]. In contrast, Gale and co-workers showed that caffeine, when given concomitantly with APAP, protects mice from APAP-induced hepatotoxicity [15], and further suggested that caffeine competes with APAP for biotransformation by P450 [16], although the effect of caffeine on microsomal NAPQI formation was not studied directly. Earlier, species difference had been identified in the effect of acetone on APAP bioactivation using hepatocytes from rats and mice [17]. Based on this, Gale and co-workers suggested that the discrepancies between their results and those of Sato and co-workers were due to a difference in the relative affinities of APAP and caffeine for hepatic P450s in the two species studied, mouse and rat [15]. However, differences in the composition and proportion of the various P450 isozymes in rat and mouse may also be important factors to consider.

A number of isozymes of rat P450 (1A1, 1A2, 2C11, 2E1, and 3A) have been suggested to play a role in NAPQI formation [18–21], their relative importance varying with induction state and therefore relative amount of each isozyme present. A study in rats has shown that the effect of caffeine on APAP hepatotoxicity is dependent on the induction state [22]. We interpret this to mean that the effect of caffeine may be isozyme specific. For example, in 3-methylcholanthrene-induced rats, caffeine protects against hepatotoxicity, but in uninduced and in phenobarbital (PB)-induced rats, caffeine potentiates hepatotoxicity [22]. Thus, in the present study, we evaluated the involvement of several subfamilies of P450 in mediating the effect of caffeine on NAPQI formation by mouse liver microsomes by using different inducers known to increase the levels of specific subpopulations of P450. The following inducers have been characterized in the rat*: β-naphthoflavone (βNF) which induces P450 1A2, although P450 1A1 is also induced somewhat [24], phenobarbital which mainly increases P450 2B1 and 2B2 [25], although P450 3A is induced as well [26], acetone which increases P450 2E1 [27] and dexamethasone (DEX) which induces P450 3A1 [26]. In addition, we evaluated kinetic differences among isozymes *in situ* in the microsomal membrane with the hope that the results might reveal something of the mechanism of the effect of caffeine on APAP bioactivation. Furthermore, an *in vivo* study of the effect of caffeine on APAP-induced hepatotoxicity was conducted to identify any relationship between *in vitro* and *in vivo* effects of caffeine on NAPQI formation and APAP hepatotoxicity, respectively.

* Because the isozymes of murine cytochrome P450 have not been fully characterized, this paper will refer to microsomes from βNF-, PB-, acetone- and DEX-treated mice, rather than using the nomenclature for rat P450s recommended by Nebert *et al.* [23].

MATERIALS AND METHODS

Materials. β-Naphthoflavone was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Sodium phenobarbital and ethyl acetate were obtained from J. T. Baker Inc. (Phillipsburg, NJ). Sodium dithionite, methanol, and acetone were purchased from Fisher Scientific (Pittsburgh, PA). Glucose-6-phosphate dehydrogenase was obtained from Boehringer-Mannheim (Indianapolis, IN). Caffeine, APAP, aminopyrine, DEX, erythromycin, sulfosalicylic acid, NADP, NADPH, glucose-6-phosphate, GSH, GSH-*S*-transferase, *o*-dinitrobenzene, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED), α-ketoglutarate, *dl*-alanine, and 2,4-dinitrophenylhydrazine were from Sigma (St. Louis, MO).

Animals. Adult female Swiss–Webster mice (20–25 g), obtained from Harlan Industries (Indianapolis, IN), were housed in a temperature-controlled environment with a 12-hr light–dark cycle. Mice were allowed water and food (Purina Lab Chow) *ad lib.* After at least 4 days of acclimation, they were treated with βNF (80 mg/kg, i.p., in corn oil) for 3 days, PB (80 mg/kg, i.p., in saline) for 3 days, acetone (1% in drinking water) for 10 days, or DEX (100 mg/kg, i.p., in corn oil) for 4 days. Untreated mice were used as the control.

Preparation of microsomes and cytosol. Mice were killed by cervical dislocation 24 hr after the last injection, gall bladders were removed, and the livers were excised after perfusion *in situ* with 1.15% ice-cold KCl. Liver homogenates (25%, w/v) were prepared in ice-cold isotonic KCl and centrifuged at 9,000 *g* for 20 min at 4°. The resulting post-mitochondrial supernatant was centrifuged at 104,000 *g* for 60 min. The pellet of microsomes was resuspended in cold isotonic KCl and used the same day. The supernatant was saved as cytosol. Proteins were measured according to the procedure of Lowry *et al.* [28], using bovine serum albumin as standard. The content of P450 was estimated from the dithionite-reduced CO difference spectrum, using an extinction coefficient of $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$ [29].

Microsomal incubation. The reaction mixtures contained 60 mM Tris–HCl buffer, pH 7.4, 1 mM GSH, 3 mM $MgCl_2$, an NADPH-generating system (0.4 mM NADP, 4 mM glucose-6-phosphate, 0.4 units glucose-6-phosphate dehydrogenase), various concentrations of APAP, various concentrations of caffeine, cytosol (0.1 mg protein) and microsomes (0.5 mg protein) in a final volume of 1 mL. The reaction mixtures were preincubated at 37° for 1 min prior to the initiation of reaction by the addition of microsomes. The reaction mixtures were incubated at 37° for 20 min in a shaking water bath, and terminated by the addition of 1 mL of cold methanol.

The mixture was centrifuged at 2,666 *g* for 10 min (Labofuge B, American Scientific), and the supernatant transferred for drying under a stream of N_2 gas. Methanol (2 mL) was added to the dried sample and the supernatant transferred for drying under a stream of N_2 gas. The final dried samples were stored at −20° for HPLC analysis.

GS–APAP estimation. A Beckman HPLC equipped with a 110B pump, a 210 2A sample injector, a 166 UV-Visible detector, and a 406 analog interface was used to isolate and quantify GS–APAP, essentially by the method of Moldéus [30]. Briefly, dried samples were dissolved in mobile phase (1% acetic acid:methanol:ethyl acetate = 100:10:0.111, by vol.), and an aliquot (20 μL) was injected onto a reverse phase PartiSphere 5 μm C_{18} column (4.7 × 125 mm, Whatman). The eluent was monitored at 254 nm, and the GS–APAP was quantified using a standard curve derived from injection of GS–APAP standards, given to us by J. Sinclaire, VA Medical Center, White River Junction, VT.

Erythromycin or aminopyrine N-demethylation. Microsomal incubations were essentially as described for APAP metabolism except that erythromycin (0.03125 to 2.0 mM) or aminopyrine (0.5 mM) replaced APAP, and no caffeine, cytosol, or GSH was added. Incubations were carried out for 20 min at 37° and stopped by the addition of zinc and barium [31]. Demethylation was evaluated by measuring formaldehyde formed [32].

Kinetic analysis. Enzyme kinetic experiments were performed using APAP concentrations ranging from 0.05 to 10 mM. Lineweaver–Burk plots were used to determine apparent K_m and V_{max}. When the test for lack of fit in linear regression was significant, two *Km* values were reported.

Evaluation of APAP-induced hepatotoxicity. Eight-week-old female Swiss–Webster mice, pretreated with the various P450 inducers mentioned above, were divided into four groups. The first group received premixed caffeine (100 mg/kg, i.p.) and APAP (300 mg/kg for DEX-pretreated mice or 500 mg/kg for others, i.p.). The second and third group received caffeine and APAP, respectively. The fourth group received saline only and served as control. All injections were given at around 10:00 a.m. Six hours after dosing, mice were killed, trunk blood was collected for serum alanine aminotransferase (ALT) estimation, and livers were excised for GSH determination.

Serum ALT assay. A colorimetric method was used to determine the serum ALT level [33]. An aliquot of serum was mixed with prewarmed ALT substrates (1.8 mM α-ketoglutarate and 200 mM *dl*-alanine) and incubated at 37° for 30 min. The reaction was stopped by the addition of the color reagent (2,4-dinitrophenylhydrazine, 1 mM in 1 N HCl); and allowed to stand at room temperature for 20 min. Sodium hydroxide (0.4 N) was added and absorbance read at 505 nm after 10 min.

GSH determination. Liver homogenates (20%, w/v) were prepared in ice-cold 2% sulfosalicylic acid and centrifuged at 9000 *g* for 15 min at 4°. The supernatant was neutralized with 1 N NaOH, and an aliquot was used for GSH analysis [34]. A 0.025-mL sample or GSH standard was added to 0.5 mL of 0.5 M phosphate buffer, pH 7.0, 0.355 mL water, 0.1 mL of 10 mM *o*-dinitrobenzene dissolved in ethanol, and 0.02 mL GSH *S*-transferase. After incubation at 37° for 30 min, a mixture of 1 mL of 1% sulfanilamide and 1 mL of 0.02% NED solution was added to stop the reaction. The absorbance was read at 540 nm after the reaction mixture was left for 20 min at room temperature.

Statistical analysis. Two-way analysis of variance for a 3 × 4 (APAP concentrations × caffeine concentrations) factorial experiment was used to determine the effect of caffeine treatment and the interaction between caffeine and APAP. When the effect of caffeine on a single APAP concentration was to be evaluated, two-way analysis of variance followed by the Student–Newman–Keuls test was used.

RESULTS

Effect of caffeine on APAP bioactivation. The effect of caffeine on the *in vitro* oxidation of APAP by liver microsomes from mice treated with various P450 inducing agents is shown in Table 1. The rate of APAP oxidation was not changed to any significant extent by 0.1 mM caffeine. When 1 mM caffeine was used, the rate of GS–APAP formation was not altered in microsomes from control mice, but was increased significantly by 33 and 39% in microsomes from PB- and DEX-treated mice, respectively. The addition of 5 mM caffeine significantly increased the rate of GS–APAP formation by 47, 107, and 117% in microsomes from control, PB-, and DEX-treated mice, respectively. Thus, the greatest stimulation was seen with high APAP and high caffeine concentrations.

In contrast, an inhibitory effect of caffeine on the rate of GS–APAP conjugate formation was observed using microsomes from βNF- or acetone-treated mice. When 1 mM caffeine was used, the rate of GS–APAP formation was decreased significantly by 39 and 12% in microsomes from βNF- and acetone-treated mice, respectively (Table 1). Furthermore, the addition of 5 mM caffeine decreased the rate of GS–APAP formation by 39 and 25% in microsomes from βNF- and acetone-treated mice, respectively.

Kinetics of APAP bioactivation by microsomes. A kinetic study was conducted in microsomes to determine if either the affinity of the enzyme for APAP or the maximal velocity of formation of GS–APAP was altered by either induction of certain P450 isozymes or the presence of 5 mM caffeine. As shown in Table 2, in the absence of caffeine, there were no significant differences in apparent K_m between treatments except that DEX-treated microsomes exhibited a second activity with lower affinity and lower capacity than the major activity, and that acetone pretreatment caused an increase in V_{max}. The addition of 5 mM caffeine increased the apparent K_m in microsomes, regardless of the pretreatment of mice. However, while this was accompanied by an increase in V_{max} in microsomes from PB- and DEX-treated mice, there was no change in V_{max} in microsomes from βNF- and acetone-treated mice. Caffeine caused an increase in the apparent K_m, with no change in V_{max} in

Table 1. Effect of caffeine on acetaminophen bioactivation by mouse liver microsomes

Treatment	APAP (mM)	GS–APAP*† (nmol/min/mg protein)	GS–APAP†‡ (%) Caffeine (mM)		
		No caffeine	0.1	1	5
Control	0.5	0.95 ± 0.05[a]	97[a]	104[a]	126[b]
(N = 4)	2	1.40 ± 0.03[a]	97[a]	109[a]	143[b]
	10	1.83 ± 0.11[a]	98[a]	112[a]	147[b]
PB	0.5	1.18 ± 0.09[a]	100[a]	103[a]	139[b]
(N = 4)	2	1.85 ± 0.17[a]	103[a]	117[a]	193[b]
	10	2.25 ± 0.21[a]	106[a]	133[b]	207[c]
DEX	0.5	1.26 ± 0.08[a]	104[a]	134[b]	186[c]
(N = 4)	2	1.94 ± 0.10[a]	99[a]	132[b]	216[c]
	10	2.47 ± 0.11[a]	103[a]	139[b]	217[c]
βNF	0.5	1.73 ± 0.36[a]	76[a,b]	61[b]	61[b]
(N = 3)	2	2.70 ± 0.59[a]	82[a,b]	64[b]	73[b]
	10	2.84 ± 0.61[a]	96[a]	85[a]	93[a]
Acetone	0.5	2.23 ± 0.31[a]	98[a]	88[b]	75[c]
(N = 4)	2	3.32 ± 0.48[a]	97[a]	92[a]	89[a]
	10	3.62 ± 0.51[a]	99[a]	102[a]	99[a]

* Values are means ± SEM.
† Values in the same row not sharing a common superscript differ significantly ($P < 0.05$).
‡ GS–APAP formation is expressed as a percentage of the rate of GS–APAP conjugate formation when no caffeine was added.

Table 2. Kinetic parameters of mouse hepatic microsomes for acetaminophen bioactivation

Treatment	Caffeine (mM)	K_m* (mM)	V_{max}* (nmol GS–APAP/min/mg)
Control	0	0.30 ± 0.01	1.72 ± 0.14[a]
	5	0.71 ± 0.13	2.88 ± 0.30[b]
PB	0	0.29 ± 0.03[a]	2.27 ± 0.27[a]
	5	1.11 ± 0.07[b]	5.49 ± 0.72[b]
DEX	0	0.24 ± 0.02[a]	1.87 ± 0.24[a]
		8.06 ± 0.52[b]	1.42 ± 0.26[a]
	5	0.69 ± 0.05[a]	6.41 ± 0.13[b]
βNF	0	0.28 ± 0.02[a]	2.15 ± 0.16
	1	0.42 ± 0.04[a,b]	1.78 ± 0.15
	3	0.56 ± 0.08[b]	1.96 ± 0.16
	5	0.77 ± 0.08[c]	2.31 ± 0.27
Acetone	0	0.29 ± 0.01[a]	3.89 ± 0.49
	5	0.69 ± 0.07[b]	4.09 ± 0.26

Values are means ± SEM, N = 4.
* Data in the same column and within the same treatment group not sharing the same superscript are significantly different ($P < 0.05$).

microsomes from βNF- or acetone-treated mice, indicative of competitive inhibition. Since βNF is known to induce P450 1A2 in several species, and since caffeine and APAP have been reported to be metabolized in the liver by P450 1A2 [12, 18, 20] in humans and rabbits, the possibility of caffeine serving as a competitive inhibitor of APAP oxidation in microsomes from βNF-treated mice was examined further. Results are shown in Fig. 1. The addition of increasing concentrations of caffeine exhibited classical competitive kinetics, increasing the apparent K_m, but not changing the V_{max} in microsomes from

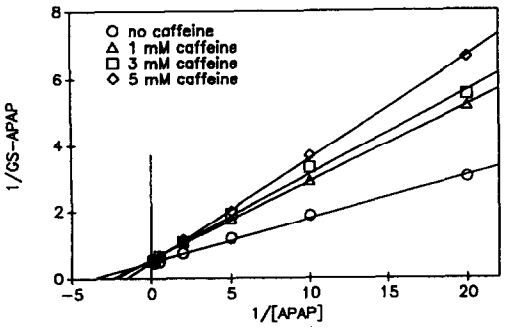

Fig. 1. Lineweaver–Burk plot of the effect of caffeine on acetaminophen bioactivation by hepatic microsomes from βNF-treated mice. Each data point represents the mean of four experiments.

βNF-treated mice. This confirmed the competitive nature of the inhibitory effect of caffeine on APAP oxidation by microsomes from βNF-treated mice. In contrast, caffeine increased V_{max} from 1.72 to 2.88 nmol GS–APAP formed/min/mg protein in microsomes from control mice, and increased V_{max} to a far greater extent, from 2.27 to 5.49 and 1.87 to 6.41 nmol GS–APAP formed/min/mg protein in microsomes from PB- and DEX-treated mice, respectively. In the absence of caffeine, at least two enzyme activities for bioactivation of APAP were found in microsomes from DEX-treated mice. The major had a low K_m (0.24 mM) and a higher V_{max} (1.87), while the other had a much higher K_m (8.06 mM), but lower V_{max} (1.42). Addition of caffeine caused a new single K_m (0.69 mM) for APAP bioactivation, and a dramatic increase in V_{max} (6.41) in microsomes from DEX-treated mice.

Erythromycin and aminopyrine N-demethylation. The effects of PB and DEX treatments on hepatic erythromycin and aminopyrine *N*-demethylase activities are shown in Table 3. Erythromycin *N*-demethylase in livers of untreated female Swiss–Webster mice had an apparent K_m of 0.23 mM and a V_{max} of 13 nmol HCHO formed/min/mg protein. When mice were treated with PB, the V_{max} of the erythromycin *N*-demethylase was increased more than 3-fold, while no change was observed in apparent K_m, indicating that the constitutive erythromycin *N*-demethylase was induced by PB treatment. Dexamethasone treatment produced a greater than 7-fold increase in the V_{max} of the enzyme, and significantly changed the affinity of the enzyme for erythromycin, indicating the induction of a different P450 isozyme(s) by DEX treatment which also catalyzes erythromycin N-demethylation. Microsomal aminopyrine *N*-demethylase activity was increased 2.5- and 4-fold by DEX and PB treatment, respectively.

Effect of caffeine on in vivo *APAP hepatotoxicity.* As shown in Table 4, caffeine significantly protected βNF-pretreated mice from APAP-induced hepatotoxicity as assessed by serum ALT activity and hepatic GSH level. In contrast, caffeine significantly potentiated APAP-induced hepatotoxicity in DEX-pretreated mice as determined by serum ALT activity and hepatic GSH level. In PB- and acetone-pretreated mice, the activity of serum ALT was elevated after the administration of APAP, and caffeine did not alter this significantly. The dose of APAP administered (0.5 g/kg) did not cause any hepatotoxicity, and caffeine did not show any significant effects, in control mice.

DISCUSSION

The present results demonstrate that caffeine, depending on the induction state of the mouse, has both stimulatory and inhibitory effects on APAP bioactivation. The results agree well with the study in rats which showed that in 3-methylcholanthrene-treated rats, caffeine protects against hepatotoxicity, while in uninduced or PB-treated rats, caffeine potentiates hepatotoxicity [22]. Our results show that caffeine increased the rate of APAP activation by liver microsomes from uninduced, PB-, and DEX-treated mice, but decreased the rate of APAP activation by liver microsomes from βNF- and acetone-treated mice. A study reported by Gale and co-workers [15] showed that caffeine protects against APAP-induced hepatotoxicity in uninduced mice. One difference between our study and that of Gale and co-workers is that Price and Gale determined the urinary excretion of GS–APAP and related conjugates, and proposed that caffeine might inhibit APAP bioactivation [35], while we estimated APAP bioactivation directly. Another difference is that we

Table 3. Effects of phenobarbital and dexamethasone treatments on hepatic erythromycin and aminopyrine *N*-demethylase activities in female mice

Treatment	Erythromycin *N*-demethylase*		Aminopyrine *N*-demethylase* (nmol HCHO formed/min/mg protein)
	K_m (mM)	V_{max} (nmol HCHO formed/min/mg protein)	
Control	0.23 ± 0.03[a]	13.15 ± 1.30[a]	24.77 ± 4.16[a]
PB	0.31 ± 0.05[a]	41.12 ± 5.92[b]	96.80 ± 12.77[b]
DEX	0.57 ± 0.02[b]	95.61 ± 2.14[c]	60.89 ± 2.38[c]

Values are means ± SEM, N = 4.
* Data in the same column not sharing the same superscript are significantly different ($P < 0.05$).

Table 4. Effect of caffeine on acetaminophen-induced hepatotoxicity *in vivo*

Pretreatment	Treatment	Serum ALT* (SF units/mL)	Hepatic GSH* (mM)
Control	Saline	21 ± 1	7.1 ± 0.3
	Caffeine (0.1 g/kg)	33 ± 3	7.0 ± 0.4
	APAP (0.5 g/kg)	71 ± 23	9.0 ± 0.8
	APAP + caffeine	65 ± 15	9.0 ± 0.5
PB	Saline	26 ± 1	5.6 ± 0.6
	Caffeine (0.1 g/kg)	24 ± 2	6.4 ± 0.4
	APAP (0.5 g/kg)	599 ± 206	6.7 ± 0.6
	APAP + caffeine	1245 ± 999	8.5 ± 0.6
DEX	Saline	34 ± 2[a]	6.4 ± 0.7[a]
	Caffeine (0.1 g/kg)	60 ± 9[a]	7.4 ± 0.6[a]
	APAP (0.3 g/kg)†	83 ± 12[a]	7.0 ± 0.6[a]
	APAP + caffeine	2086 ± 766	3.5 ± 0.9[b]
βNF	Saline	16 ± 1[a]	8.2 ± 0.8[a]
	Caffeine (0.1 g/kg)	16 ± 2[a]	7.7 ± 0.5[a]
	APAP (0.5 g/kg)	9445 ± 3040[b]	0.1 ± 0.02[b]
	APAP + caffeine	292 ± 115[a]	6.3 ± 0.6[a]
Acetone	Saline	18 ± 1	6.7 ± 0.6
	Caffeine (0.1 g/kg)	30 ± 7	7.4 ± 0.3
	APAP (0.5 g/kg)	228± 127	7.8 ± 0.9
	APAP + caffeine	523 ± 347	8.4 ± 1.1

Values are means ± SEM of four to eight mice.

* Data within the same column and same treatment group not sharing the same superscript are significantly different ($P < 0.05$).

† DEX-pretreated mice were given 0.3 g APAP/kg because those receiving a combination of 0.5 g APAP/kg and caffeine died prior to the 6-hr termination of the experiment.

used female Swiss–Webster mice, whereas Gale and co-workers used male BDF1 mice. However, in another study where liver microsomes from male BDF1 mice were used to investigate the effect of caffeine on APAP activation in microsomes, Liu and co-workers observed no effect [36]. Thus, any evidence for decreased NAPQI formation by caffeine in microsomes from uninduced mice to support the *in vivo* data reported by Gale and co-workers is still lacking.

That the stimulatory effect of caffeine on APAP bioactivation in microsomes from control mice was increased somewhat in microsomes from PB-treated mice and increased even more in microsomes from DEX-treated mice suggests that the specific P450 isozyme(s) mediating this effect is constitutive, modestly induced by PB, and induced well by DEX. Because both PB and DEX are known to increase P450 2B-catalyzed activities in rats [37], we evaluated aminopyrine *N*-demethylase activity in microsomes from control, PB-, and DEX-treated mice. Results showed that aminopyrine *N*-demethylase activity was increased in the order of control < DEX < PB, and therefore did not correlate with the stimulatory effect of caffeine on GS–APAP formation observed in microsomes from control, PB, and DEX-treated mice ($r = 0.21$; Fig. 2B). Dexamethasone pretreatment had been shown to increase P450 3A-catalyzed activities 6-fold and PB pretreatment increased P450 3A catalyzed erythromycin N-demethylation 4-fold in mice [38]. Here we also report greater increases in rates of erythromycin N-demethylation following DEX treatment than following PB treatment (Table 3). In rat liver at least two isozymes have been identified in the P450 3A subfamily. Cytochrome P450 3A1 is steroid inducible, and its corresponding mRNA is not found constitutively in either male or female rat liver [26]. This is therefore an unlikely candidate for caffeine stimulation in rats. Cytochrome P450 3A2, which is 90% homologous to P450 3A1, is PB inducible, and is constitutively present in adult male, but not female, rats [26, 39]. Immunoblot analyses of electrophoretically separated microsomal proteins have indicated the absence of P450 3A in female adult rats [39]. While two members of the 3A subfamily have been identified in mouse [38], their relative preponderance in treated vs untreated and in male vs female mice has not been well characterized. Not only did we find erythromycin *N*-demethylase activity in untreated female Swiss–Webster mice in this study, but erythromycin *N*-demethylase has been reported in both male and female CF-1 mice [38], and in female but not male DBA/2 mice [39]. The presence of P450 3A in female but not male DBA/2 mice was further confirmed by immunoblot analysis of microsomal proteins [39]. Therefore, there seems reasonable

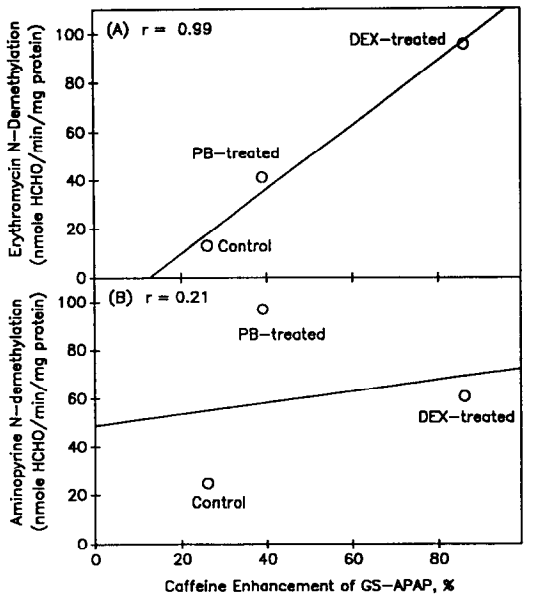

Fig. 2. Correlations between the extent of caffeine enhancement of APAP oxidation and erythromycin or aminopyrine N-demethylation. The percentage increase of the rate of GS–APAP formation from APAP (0.5 mM) by caffeine (5 mM) in microsomes from control, PB-, and DEX-treated mice was plotted against (A) the V_{max} of the microsomal erythromycin *N*-demethylase activity or (B) microsomal aminopyrine *N*-demethylase activity from control, PB-, and DEX-treated mice.

evidence that the P450 3A subfamily is present in our control mice, and that it is induced by PB and DEX treatment. Furthermore, the rate of erythromycin N-demethylation was found to correlate well with the stimulatory effect of caffeine on GS–APAP formation in microsomes from control, PB-, and DEX-treated mice, $r = 0.99$ (Fig. 2A). This suggests that P450 3A may be the candidate in mediating the stimulatory effect of caffeine on APAP bioactivation in microsomes from control, PB-, and DEX-treated female mice in this study. The lack of stimulatory effect of caffeine on APAP activation in the study by Liu and co-workers [36] could be explained if there were a strain and/or sex-specific lack of hepatic P450 3A content in BDF1 male mice, similar to that reported for DBA/2 mice [39].

The kinetic study reported here supports the proposal that caffeine acts as a competitive inhibitor (increase apparent K_m for APAP, but no change in V_{max} for GS–APAP formation) of APAP bioactivation in microsomes from βNF- and acetone-treated mice. In rats, β-naphthoflavone is known to induce P450 1A2 (and 1A1 to a lesser extent) and acetone is known to induce P450 2E1. In βNF-treated hamsters and in humans, APAP bioactivation has been reported to be catalyzed by P450s 1A2 and 2E1 [20]. It therefore seems likely that the same may be true for mice. Although the metabolism of caffeine is not fully understood, N-3 demethylation of caffeine is reported to be P450 1A2 dependent [11]. Thus, this could explain why caffeine acts as a competitive inhibitor of APAP bioactivation in microsomes from βNF-treated mice. In human liver microsomes, it has been reported that microsomal content of P450 2E1 correlated with 8-hydroxylation of theophylline [13]. Since theophylline is the product of caffeine N-7 demethylation, one can speculate that the inhibitory effect of caffeine on APAP bioactivation in microsomes from acetone-treated mice may be due to the 8-hydroxylation of caffeine and/or theophylline competing with bioactivation of APAP by P450 2E1. However, P450 2E1 may also be involved in one or more of the demethylation steps during caffeine metabolism.

Our *in vitro* data correlate very well with the *in vivo* study (Table 4), where the simultaneous administration of caffeine and APAP protected βNF-treated mice from APAP-induced hepatotoxicity, although potentiating APAP-induced hepatotoxicity in DEX-treated mice. Although caffeine increased the V_{max} for APAP bioactivation in microsomes from PB-treated mice, and coadministration of caffeine doubled the serum ALT, this increase in APAP-induced hepatotoxicity in PB-treated mice was not statistically significant. One possible explanation for this minimal toxicity following PB induction may be that APAP was metabolized to the less hepatotoxic catechol, 3-hydroxyacetaminophen, as well as NAPQI [19]. When uninduced or acetone-treated mice were used to study the effect of caffeine on APAP toxicity in the whole mouse, we were not able to correlate the results with data from *in vitro* experiments. The lack of any effect on APAP hepatotoxicity in uninduced mice may be due to the very modest increase in V_{max} for APAP activation in the presence of caffeine. The acetone-treated mice showed no hepatotoxicity with or without caffeine administration. Had the dose of APAP administered been greater, so that APAP-induced hepatotoxictiy was more evident in the absence of caffeine, then caffeine protection might have been more evident in acetone-treated mice.

While there are many isozymes of P450, a single flavoprotein, NADPH–cytochrome P450 reductase, donates electrons to all isozymes. If certain characteristics of this flavoprotein, such as the rate of reduction by NADPH, were altered by caffeine, then this should affect all P450-dependent activities. In contrast, an effect that alters the interaction between the flavoprotein and P450 may well be isozyme specific. That βNF microsomes showed very clean competitive inhibition argues against there being any, even masked, stimulatory effect following βNF treatment. Furthermore, if caffeine were affecting enzyme activity by a generalized effect on all P450s, then one should expect a similar percentage increase in NAPQI formation following caffeine addition. However, our results showed that NAPQI formation by microsomes from control mice was increased 26% by the addition of 5 mM caffeine, whereas NAPQI formation by microsomes from PB- and DEX-treated mice showed 39 and 86% increases, respectively. Thus, the effect of caffeine on APAP bioactivation appears to be P450 isozyme selective. Results of the kinetic study show that caffeine apparently altered the K_m of microsomes from

control, PB-, and DEX-treated mice for APAP. All K_m values were increased, indicating that caffeine lowers the affinity of enzyme for APAP. Therefore, a direct effect of caffeine on APAP binding does not account for enhancement of activity. An increase in both K_m and V_{max} by caffeine, but keeping the ratio of these two parameters approximately unchanged (Table 2, control, PB and low K_m DEX) is indicative of an effect on the complex of P450 with APAP and/or the reduced flavoprotein [40], similar to, but in the opposite direction to, a modifier that causes uncompetitive inhibition. However, the exact nature of this effect cannot be determined from these results.

Activation, unlike induction, does not require *de novo* protein synthesis. Examples of activators of P450-catalyzed reactions include acetone, which stimulates the oxidation of aniline and APAP [17, 40], and flavonoids which stimulate benzo[*a*]-pyrene [41] and zoxazolamine [42] hydroxylation. The most extensively studied mechanism of activation is the activation of P450 by flavonoids [41]. Huang and co-workers [41] demonstrated that 7,8-benzoflavone enhanced benzo[*a*]pyrene metabolism by lowering the K_m for NADPH–cytochrome P450 reductase and therefore enhancing the electron transfer form NADPH–cytochrome P450 reductase to P450. Since caffeine undergoes extensive metabolism by microsomal enzymes, Lee and co-workers [43] hypothesized that a metabolite of caffeine rather than, or in addition to, caffeine might stimulate APAP bioactivation and investigated the effect of monomethylxanthine and dimethylxanthine metabolites of caffeine on APAP oxidation *in vitro*. These researchers found that, in contrast to caffeine, none of these metabolites enhances APAP bioactivation. They concluded that caffeine exerts its stimulatory effect directly. However, they were unable to define the mechanism of activation. As with other activators of P450, the mechanism of the stimulatory effect of caffeine on APAP oxidation remains to be elucidated.

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