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EFFECT OF CAFFEINE ON HEPATIC MICROSOMAL CYTOCHROME P-450

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Abstract—Previously we reported that pretreatment of rats with caffeine did not result in any observable increase in the P-450 hemoprotein content of the liver microsomes. However, the measurement of ethyl isocyanide difference spectra of dithionite reduced microsomes showed a small but definite increase in the 455-nm peak in the caffeine-treated group. There was no significant increase in the 430-nm peak. In this respect, caffeine action resembled that of 3-methylcholanthrene. Caffeine treatment also increased the rate of NADPH-cytochrome P-450 reductase activity. Microsomal enyzme induction by caffeine was not obliterated by adrenalectomy. The preferential increase in the 455-nm peak was more pronounced in the microsomes of the adrenalectomized animal after caffeine pretreatment.

WE PREVIOUSLY reported that the administration of caffeine to rats resulted in an increase in the enzymatic activity of the hepatic drug-metabolizing system.[1,2] However we were unable to demonstrate an increase in the liver weight or stimulation of amino acid incorporation as a result of this treatment. Moreover, the cytochrome P-450 content of the microsomes was not significantly increased by caffeine administration.[2]

In the present study we have investigated whether the predominant forms of the microsomal cytochrome have been altered by caffeine treatment. The effect of caffeine treatment on adrenalectomized rats was also examined. The classical inducers, phenobarbital and 3-methylcholanthrene, have been included in these studies as guideposts to which we can relate our findings.

EXPERIMENTAL

Treatment of animals. Male Sprague–Dawley rats, purchased from Simonsen Laboratories, Inc., Gilroy, Calif., were used throughout. They were kept in cages (four to six rats per group) suspended over Absorb-Dri or San-i-cel and were maintained on a stock diet and water *ad lib.* The weight of the rats ranged from 170 to 210 g.

The rats were pretreated with saline, caffeine, phenobarbital or 3-methylcholanthrene for 3 or 4 days. The dosage and duration of administration for each experiment are described in the tables. The first dose of caffeine or phenobarbital usually was administered in the afternoon of the first day. The animals were given two doses of caffeine or phenobarbital in the morning and afternoon during the next 2 or 3 days and a final dose on the morning of the last day. The rats were sacrificed in the afternoon of the last day by cervical dislocation. The livers were removed and frozen intact immediately or the 12,000 *g* supernatant fraction was prepared and then the latter was frozen for subsequent assays.

The livers were homogenized in 3 ml of cold potassium phosphate buffer (0·1 M) pH 7·4, for each gram of liver. A postmitochondrial supernatant fraction containing the microsomes was prepared by centrifuging the homogenate in a Sorval refrigerated centrifuge for 10 min at 12,000 *g*.

Incubation. To assay the drug-metabolizing activity, 1 ml of the supernatant fraction (25–35 mg protein) was mixed with a solution containing Tris buffer, pH 8·0 (200 μmoles), NADP (0·25 μmole), NAD (5 μmoles) and various substrates in a final volume of 3·1 ml. The incubation was carried out in a Dubnoff metabolic shaker for 30 min at 37° in air. All reactions were approximately linear during this period. The amounts of substrates added per incubation beaker were 10 μmoles for acetanilide and 7 μmoles for *o*-nitroanisole. The assay techniques for drug-metabolizing activities for acetanilide and *o*-nitroanisole have been described previously.[3] The results are expressed as the number of micromoles of product formed ± standard error per gram of 12,000 *g* supernatant protein. Comparisons of the mean enzymic activities between the control and experimental rats were made by the Student *t*-test.

Measurement of microsomal cytochrome P-450 levels. The measurement of the microsomal cytochrome P-450 levels, using ethyl isocyanide as a ligand, was performed essentially as described by Sladek and Mannering.[4,5] The livers were removed, washed in two rinses of 1·15% KCl, and then perfused with KCl to remove blood. The livers were then homogenized in 3 vol. of 1·15% KCl, and centrifuged for 5 min at 3000 *g* and for 10 min at 12,000 *g* in the same tube. Ten ml of the supernatant was centrifuged at 30,000 rpm in a no. 30 Spinco rotor for 60 min. The microsomal pellet was resuspended in 10 ml of 1·15% KCl.

The microsomal suspension was diluted with three parts (v/v) of potassium phosphate buffers (1·0 M, pH 6·0, 6·5, 7·0, 7·5 and 8·0) for the assay of saline-, caffeine- and 3-methylcholanthrene-treated rat liver microsomes and with seven parts of the buffers for the assay of phenobarbital-treated microsomes. The absorbance was determined in sealed cells in a Gilford model 2000 spectrophotometer at 430, 455 and 500 nm using water as the blank. The first reading was taken 5 min after adding a few milligrams of solid sodium dithionite and the second reading at 1 min after adding 20 μl of 20-fold diluted ethyl isocyanide. The absorption at 500 nm was subtracte dfirst from those at 430 and 455 nm after each reading. The corrected values from the reading were subtracted from those from the second reading to obtain the Δ430-nm and Δ455-nmole values.

Cytochrome P-450 reductase assay. The rate of NADPH-cytochrome P-450 reduction was measured according to the method of Gigon *et al.*[6] The livers were homogenized in 3 ml of potassium phosphate buffer (0·1 M, pH 7·4) for each gram of liver and stored frozen. The samples were thawed at room temperature, and centrifuged for 5 min at 3000 *g* and for 10 min at 12,000 *g* in the same tube. Four-ml aliquots were centrifuged in the no. 30 Spinco rotor at 30,000 rpm for 70 min, and the microsomal pellet was suspended in 12 ml Tris-KCl buffer (pH 7·4, 0·02 M Tris in 1·15% KCl). A 2·5-ml aliquot of the microsome suspension, representing 3–6 mg protein per ml, was used per cell that was CO gassed, temperature equilibrated (37°) and capped tightly with a silicon rubber stopper. The reaction was initiated by the injection of 50 μl of NADPH-generating system,[6] which caused a momentary drop in absorbance. The change in absorbance at 450 nm was recorded on a chart issuing at 12 in./min using O.D. of 0·1 as the full scale. The rate of the reaction was determined from the

slope of the increasing absorbance curve during the first few seconds after the initial disturbance.

RESULTS

Ethyl isocyanide difference spectra. Data have been presented to show that caffeine pretreatment did not result in an increased level of the CO-binding pigment in the liver, although it stimulated the rate of drug metabolism.[2] In the present study, the difference spectra of dithionite-reduced microsomes complexed with ethyl isocyanide were examined at 430 and 455 nm. First, the pH-intercept was determined, i.e. the pH at at which the peak heights of the two interconvertible forms (430- and 455-nm peaks) of the microsomal hemoproteins are equal. The microsomes from untreated rats and those pretreated with caffeine, phenobarbital (PB) and 3-methylcholanthrene (3-MC) were compared. In agreement with the data reported in the literature,[5] PB treatment had little effect on the pH-intercept, whereas the microsomes from 3-MC-treated rats showed an intercept at a lower pH than did the microsomes from untreated or PB-treated rat liver (Table 1). This was interpreted by Sladek and Mannering[4] as evidence for a new hemoprotein (P_1-450) inducible by 3-MC. The pH-intercept of the microsomes from caffeine-treated rats was between those of the untreated (or PB-treated) and 3-MC-treated liver microsomes.

The shift in the pH-intercept observed above is explainable on the basis of the

TABLE 1. EFFECT OF INDUCERS ON ETHYL ISOCYANIDE DIFFERENCE SPECTRA OF DITHIONITE-REDUCED MICROSOMES*

Group	$\frac{\Delta\ 455\ nm\dagger}{\Delta\ 430\ nm\ddagger}$	pH-intercept
Saline	0·50 ± 0·15	7·7 ± 0·2
PB	0·50 ± 0·04	7·4 ± 0·1 NS
Caffeine	0·87 ± 0·09	7·1 ± 0·1 P < 0·05
3-MC	1·33 ± 0·18	6·6 ± 0·1 P < 0·01
PB + Caffeine	0·44 ± ·04	7·6 ± 0·1 NS

*Rats (210 g, four per group) were injected i.p. for 4 days with saline (0·5 ml), phenobarbital (40 mg/kg, once daily), or 3-methylcholanthrene (20 mg/kg in corn oil, once daily) and were sacrificed 20 hr after the last medication. Caffeine (75 mg/kg, twice daily) was injected i.p. eight times and rats were sacrificed 4 hr after the last medication. The mixture of caffeine (75 mg/kg) and phenobarbital (37·5 mg/kg) was administered twice daily for 3 days and the rats were sacrificed 4 hr after the last medication. Values represent means ± standard error. NS = not significant.

†$(A_{455-500})$Dithionite + ethyl isocyanide — $(A_{455-500})$ dithionite at pH 8·0.

‡$(A_{430-500})$Dithionite + ethyl isocyanide — $(A_{430-500})$ dithionite at pH 6·0. Both of these values are per microsomal preparation equivalent to 156 mg of wet liver.

change in the relative heights of the 430- and 455-nm peaks. In our experiments, the peak heights were measured at the pH extremes of 6·0 and 8·0, where the maximal absorptions of the 430- and 455-nm peaks, respectively, are observed with a minimal absorption of the other. As can be seen from Fig. 1, the 430-nm peak was significantly elevated only with PB, whereas the 455-nm peak was elevated with caffeine, 3-MC and PB. The simultaneous administration of caffeine and PB to the rat produced no change in the two peak heights that could not be attributed to PB alone Fig 1.

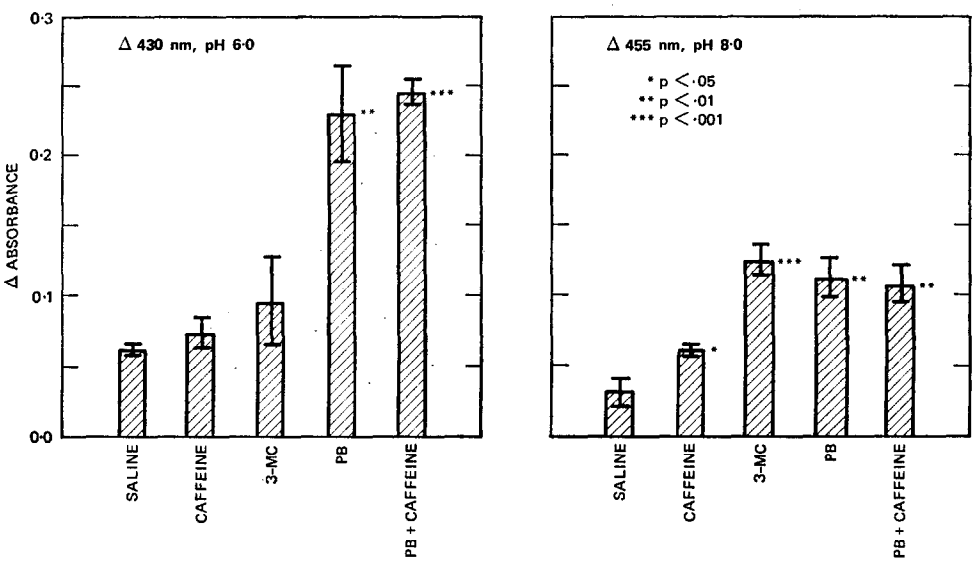

FIG. 1. Effect of inducers on absorption maxima of ethyl isocyanide difference spectra of dithionite-reduced microsomes. See Table 1 for explanation of various treatments. Values represent means + standard error.

The ratio Δ455 nm/Δ430 nm in Table 1 numerically expresses the increase observed in the 455-nm peak after caffeine and 3-MC treatment. The increase in the 455-nm peak by 3-MC treatment results in the ratio increasing to 1·33 as compared with 0·50 for the control (or PB-treated) microsomes. The smaller increase in the 455-nm peak by caffeine induction results in an intermediate increase in the ratio.

Rate of reduction of cytochrome P-450 by NADPH. The changes in the rates of reduction of cytochrome P-450 by NADPH were measured in relation to induction of the microsomal enzymes by PB, caffeine and 3-MC. PB treatment resulted in a marked increase in the reductase activity, as reported by Flynn *et al.*[7] Caffeine treatment resulted in a moderate increase in the reductase activity (Table 2). Although 3-MC produced the greatest increase in the acetanilide hydroxylase activity, it produced no change in the reductase activity. A similar finding with 3-MC has been reported by Gigon *et al.*[8]

TABLE 2. EFFECT OF INDUCTION ON RATE OF REDUCTION OF CYTOCHROME P-450 BY NADPH IN RAT LIVER MICROSOMES*

Group	*p*-Hydroxyacetanilide formed (μmoles/g protein†)	Relative activity	Cytochome P-450 reduced (nmoles/min/mg protein‡)	Relative activity
Saline	7·77 ± 0·43	100	0·76 ± 0·05	100
PB	12·54 ± 1·08 P < 0·01	160	4·19 ± 0·50 P < 0·001	552
Caffeine	11·33 ± 0·75 P < 0·01	146	1·45 ± 0·13 P < 0·01	191
3-MC	23·23 ± 1·63 P < 0·001	299	0·76 ± 0·11 NS	100

*Four rats (170 g) were used per group. Three groups were injected i.p. for 4 days with saline (0·5 ml), phenobarbital (37·5 mg/kg, twice daily) or caffeine (75 mg/kg, twice daily). The fourth group received a single i.p. injection of 3-methylcholanthrene (50 mg/kg). The rats were sacrificed 4 hr after the last injection of saline, phenobarbital or caffeine, and 93 hr after the 3-methylcholanthrene injection. Values are expressed as means ± standard error. NS = not significant.
†Supernatant (12,000 *g*) protein.
‡Protein in microsomes prepared from above supernatant.

Studies on adrenalectomized rats. Data presented in Table 3 show that the stimulatory effect of caffeine on drug metabolism is similar to that of phenobarbital[9,10] in that its effect is not mediated through the adrenal glands. The metabolism of acetanilide and *o*-nitroanisole was significantly increased by administering caffeine to adrenalectomized rats. Figure 2 shows that the microsomes from adrenalectomized rats pretreated with saline or caffeine have lower 455-nm peaks relative to 430-nm peaks than with the microsomes from intact rats (Fig. 1) pretreated with saline or caffeine respectively. The net result after adrenalectomy is a decreased Δ455-nm/Δ430-nm ratio and a shift of the pH-intercept of the two peaks to a higher pH (Table 3).

TABLE 3. EFFECT OF CAFFEINE AND PHENOBARBITAL ON THE ENZYMATIC ACTIVITY AND ETHYL ISOCYANIDE DIFFERENCE SPECTRA OF ADRENALECTOMIZED RATS*

Group	Substrates		$\frac{\Delta\ 455\ nm}{\Delta\ 430\ nm}$ ‡	pH-intercept
	Acetanilide†	*o*-Nitroanisole†		
Saline (4)	3·9 ± 0·2	7·9 ± 0·3	0·19 ± 0·04	> 8·0
Caffeine (3)	13·8 ± 1·8 P < 0·001	13·1 ± 0·6 P < 0·001	0·42 ± 0·09	> 8·0
PB (4)	9·1 ± 1·5 P < 0·02	16·9 ± 1·0 P < 0·001	0·42 ± 0·02	7·8 ± 0·1

*Saline (0·5 ml), caffeine (75 mg/kg, twice daily) or phenobarbital (37·5 mg/kg, twice daily) was injected i.p. to 200-g rats for 4 days, beginning 1 week after adrenalectomy. The rats were sacrificed 4 hr after the last administration. Values are expressed as means ± standard error. Figures in parentheses refer to number of animals.
†Values are expressed as micromoles of *p*-hydroxyacetanilide or *o*-nitrophenol formed/gram of protein.
‡See footnotes of Table 1.

The relative peak heights of the PB-pretreated rats were similar with or without adrenalectomy. Thus, the Δ455-nm/Δ430-nm ratio and the pH-intercept of the microsomes from these adrenalectomized rats were similar to those from the PB- treated intact rats.

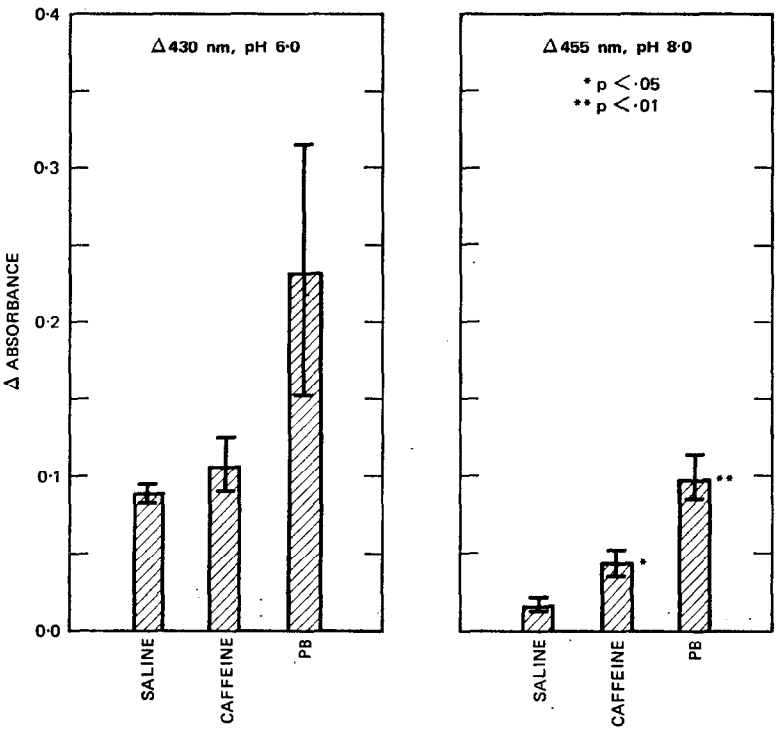

FIG. 2. Effect of caffeine and phenobarbital on absorption maxima of ethyl isocyanide difference spectra of dithionite-reduced microsomes from adrenalectomized rats. See Table 3 for explanation of various groups. Values represent means ± standard error.

DISCUSSION

In a previous communication,[2] we presented indirect evidence that caffeine acts as an inducer of the hepatic drug-metabolizing enzyme system. The increases presently observed in the 455-nm peak of the dithionite-reduced microsomes reacted with ethyl isocyanide indicate a change in the quality of the P-450 hemoprotein. A small but significant increase in the Δ455-nm /Δ430-nm ratio or decrease in the pH-intercept observed after caffeine administration is more characteristic of induction by 3-MC than by PB. There seems to be some parallelism between the observed changes in the P-450 hemoprotein after caffeine and 3-MC treatment and the resulting increased activity in acetanilide hydroxylation. However, caffeine differed from 3-MC in slightly increasing the rate of reduction of cytochrome P-450. Moreover, caffeine does not increase the urinary excretion of ascorbic acid in the rat,* unlike other microsome inducers, including 3-MC.[11]

Studies on adrenalectomized rats indicated that the caffeine effect is not mediated by the adrenal gland, thus eliminating stress as a possible cause for enzyme induction.[10,12] A more definitive proof for this must await studies with isolated perfused liver, as was done with benzpyrene by Juchau *et al.*[13]

The present studies indicate that induction of hepatic enzymes may not always be accompanied by an increase in the P-450 content of the liver, but may be explained by a change in the quality of the hemoprotein. Indeed, it has been reported[14,15] that species and sex differences in drug oxidase activity are not related to the P-450 content.

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