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A study of the nature of pilocarpine inhibition of hepatic drug-metabolizing enzymes

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Recently we have reported that pilocarpine possesses an inhibitory action on the hepatic microsomal drug metabolizing enzyme activities and the drug was a potent inhibitor of nicotine and aniline metabolism in comparison with DPEA (2,4-dichloro-6-phenylphenoxyethylamine hydrobromide), imipramine and SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride), though a less potent inhibitor of hexobarbital metabolism [1], and that the inhibition of hepatic drug metabolism was involved in the mechanisms of the pilocarpine potentiation of nicotine-induced convulsions [2] and of hexobarbital hypnosis [3].

It has been reported that pilocarpine inhibited the activity of glucose-6-phosphate (G-6-P) dehydrogenase in the calf eye [4] and pure yeast enzyme [5]. However, the G-6-P dehydrogenase of rabbit liver was not affected by pilocarpine [1]. The present experiment was undertaken to elucidate the nature of the inhibition of drug metabolizing enzyme activities by pilocarpine.

Rabbits were sacrificed by immediate exsanguination via the carotid artery. The liver was homogenized with 3 vol. of 1.15% KCl solution in a Potter–Elvehjem's teflon–glass homogenizer. A liver supernatant fraction was prepared by centrifugation of the homogenate at 10,700 *g* for 40 min. This supernatant fraction was centrifuged at 105,000 *g* for 1 hr and the firmly packed pellet of microsomes was re-suspended in 1.15% KCl solution and again centrifuged as above. Finally, the washed microsomal pellet was suspended to a concentration of 2 mg protein per ml in 1.15% KCl solution containing 50 mM Tris–HCl buffer, pH 7.4. The supernatant fraction, 2.5 ml, was mixed with a solution containing 1.5 μmoles NADP, 25 μmoles G-6-P, 25 μmoles $MgCl_2$ and 3 μmoles substrates. The final volume made a total of 5.0 ml with 0.1 M sodium, potassium phosphate buffer, pH 7.4. Incubation was carried out at 37° for 10 min under air in a Dubnoff metabolic shaker.

Metabolism of nicotine was determined by measuring the radioactivity of [^{3}H]nicotine remaining, in a liquid scintillation spectrometer (Packard Model 3320) by the method reported by Hug [6] and described in detail by Tsujimoto *et al.* [7]. The rate of hydroxylation of hexobarbital was determined by the method of Cooper and Brodie [8]. The changes in optical density were determined from the difference spectra caused by the addition of drugs to the microsomal suspension according to the method of Schemkman *et al.* [9]. The NADPH oxidase activity was determined by following the disappearance of NADPH at 340 nm according to the method described by Peters and Fouts [10]. The NADPH-cytochrome *c* reductase activity was determined by measuring the appearance of reduced cytochrome *c* at 550 nm according to the method described by Peters and Fouts [10]. NADPH-cytochrome P-450 reductase activity was determined in a Hitachi two-wavelength double beam spectrophotometer (Model 356) in the dual wavelength mode, by measuring the difference in absorption between 450 and 465 nm according to the method described by Schenkman and Cinti [11]. 2.75 milliliters of a microsomal suspension (2 mg protein per milliliter in 50 mM Tris–HCl buffer, pH 7.4) was bubbled in a Thunburg-type cuvette for 5 min with oxygen-free carbon monoxide (prepared by passing through a solution of 0.5% sodium dithionite plus 0.05% sodium anthraquinone-2-sulfonate in 0.1 N NaOH) and was equilibrated at 30°. After the sidearm was set, two polyethylene tubes with stopcocks were inserted into the cuvette through a rubber stopper fitted to the hole and one was put into the sample. Air in the cuvette was aspirated-off from one tube and then oxygen-free carbon monoxide was bubbled from the other tube until it filled the cuvette. This ventilation procedure was repeated 3 times and the tubes were sealed. From the gassing tube, 0.25 ml of NADPH-generating system (1.5 μmoles NADP, 30 μmoles isocitrate, isocitric dehydrogenase (0.3 mg/ml, Sigma Type I) and 10 μmoles $MgCl_2$ in 50 mM Tris–HCl buffer, pH 7.4) was injected into the sample. The rate of cytochrome P-450 reduction was determined using the initial linear rate of increase in absorbance. Protein concentrations of the liver supernatant fraction and the microsomal suspension were estimated by the method of Lowry *et al.* [12] using bovine serum albumin as the standard.

Pilocarpine did not inhibit the G-6-P dehydrogenase activity of the rabbit liver [1]. Effects of pilocarpine on the activity of NADPH oxidase, NADPH-cytochrome *c* reductase and NADPH-cytochrome P-450 reductase of rabbit liver microsomes were examined (Table 1). Pilocarpine did not affect the NADPH oxidase activity and the NADPH-cytochrome *c* reductase activity at the concentrations up to 5×10^{-3} M. Pilocarpine reduced cytochrome P-450 reduction with higher concentrations than those causing 50 per cent inhibition of the metabolism of nicotine ($3.6 \pm 1.6 \times 10^{-5}$ M), aniline ($1.1 \pm 0.1 \times 10^{-4}$ M) and hexobarbital ($1.8 \pm 0.4 \times 10^{-4}$ M).

Effect of pilocarpine on kinetics on nicotine and hexobarbital metabolism was studied. Pilocarpine was added to the incubation systems at different concentrations. A double-reciprocal plots of nicotine and hexobarbital meta-

Table 1. Effect of pilocarpine on NADPH-cytochrome *c* reductase, NADPH oxidase and NADPH-cytochrome P-450 reductase activities in hepatic microsomes from rabbit

Pilocarpine ($\times 10^{-4}$ M)	Cytochrome *c* reductase	%	NADPH oxidase	%	Cytochrome P-450 reductase	%
—	226 ± 15*		20.1 ± 1.1*		62.5 ± 6.9†	
1					48.0 ± 5.8‡	77
5					40.5 ± 5.9‡	65
10					36.9 ± 4.2‡	59
50	215 ± 18	95	19.0 ± 1.0	95	26.9 ± 4.8‡	43

Values represent mean ± S.E.
* nmoles/mg protein/min.
† ΔOD ($\times 10^{-3}$) 450–465 nm/mg protein/min.
‡ Significantly different from control; $P < 0.05$.

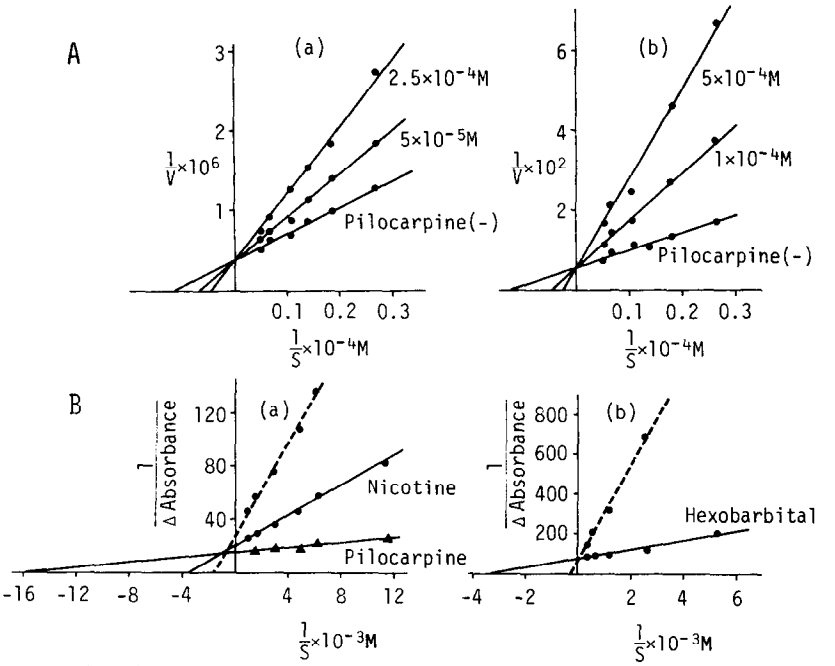

Fig. 1. A: Double-reciprocal plots of nicotine (a) and hexobarbital (b) metabolizing enzyme activities with varying concentrations of pilocarpine. B: Double-reciprocal plots of the spectral changes induced by the drugs. Spectral changes were obtained by the addition of various concentrations of the drugs to normal microsomal suspension (solid line). Nicotine (a) and hexobarbital (b) were added to pilocarpine-containing microsomal suspension (dotted line). The concentrations of pilocarpine causing 50 per cent inhibition of nicotine metabolism (a: 3.6×10^{-5} M) and hexobarbital metabolism (b: 1.8×10^{-4} M) were used respectively.

bolizing enzyme activities revealed the inhibition by pilocarpine (a type II substance) to be the competitive type for the both metabolism of nicotine, a type II substrate, and hexobarbital, a type I substrate (Fig. 1).

The interaction of pilocarpine with cytochrome P-450 is indicated by the appearance of an absorption peak at about 430 nm and a trough at about 395 nm in the difference spectrum and the magnitude at the peak and the trough of spectral changes caused by pilocarpine was much greater than that by nicotine [1]. Double-reciprocal plots of the spectral changes induced by drugs against the drugs concentrations were shown in Fig. 1. The apparent dissociation constant, K_s, of nicotine and hexobarbital could be determined to be 0.32 and 0.41 mM respectively for rabbit liver microsomes. K_s of pilocarpine could be determined to be 0.06 mM. K_s of nicotine and of hexobarbital were increased to 0.59 and 6.7 mM respectively by the addition of pilocarpine.

The mechanisms by which numerous substances inhibit the metabolism of drugs by liver microsomes are not well understood. It has been known that the mechanism of inhibition by type II substances varies with inhibitor, substrate and species [13]. This phenomenon is explained by the view that a type II inhibitor might act by interacting with more than one kind of cytochrome P-450 and by slowing the rate of cytochrome P-450 reduction and by interacting with various intermediate enzyme forms [14]. In accordance with this view, it seems from the present results that pilocapine may interact with the microsomal cytochrome P-450 with a greater affinity than nicotine and hexobarbital and it might act by slowing the reduction of cytochrome P-450 by a competitive mechanism.

Pilocarpine contains a tetrahydrofuran and imidazole ring in its structure. Muscarine, a quaternary ammonium compound containing a tetrahydrofuran ring. We examined the effects of muscarine, tetrahydrofuran and imidazole on the nicotine metabolsim in rabbit liver microsomes in comparison with pilocarpine. Pilocarpine produced a marked inhibition (57 per cent inhibition) at a concentration of 5×10^{-5} M but the other compounds showed a slight inhibition (10–20 per cent inhibition) even at a higher concentration of 10^{-3} M.

It was reported that tetrahydrofuran inhibited specifically the *O*-dealkylation activity for 7-ethoxycoumarin in microsomes from ethanol-pretreated rats [15]. The presence of multiple forms of hepatic cytochrome P-450 have been reported [16–22] and several forms of cytochrome P-450 have recently been purified [23–28]. We obtained the data that the potency of the pilocarpine inhibition on drug metabolism varied greatly with species (unpublished data). It remains to examine the inhibitory action of pilocarpine on the catalytic activities of different forms of cytochrome P-450 and the interaction with them.

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