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Ryukyu Med. J., 9(3•4) : 151~159, 1986.

ENHANCEMENT OF HEPATIC MICROSOMAL AMINOPYRINE N-DEMETHYLATION BY NEUTRAL SALTS

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Key Words: aminopyrine N-demethylation, neutral salt, cytochrome P-450

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Abstract

Enhancement of aminopyrine N-demethylation by neutral salts was kinetically studied using hepatic microsomes from nontreated, phenobarbital- or 3-methylcholanthrene-treated rats. Aminopyrine N-demethylation was increased 3-fold by 0.4 M sodium sulfate in nontreated or 3-methylcholanthrene-treated microsomes. On the other hand, the enhancement was 1.7-fold in phenobarbital-treated microsomes. Lineweaver-Burk plots of aminopyrine N-demethylase were not linear in nontreated microsomes and two Km values (2×10^{-3} M, 5×10^{-4}M) were obtained. However, the addition of neutral salts to the microsomes brought on a linear Lineweaver-Burk plots of the demethylation and only one km, which was less than 2×10^{-3}M. Aminopyrine-induced difference spectra of the microsomes were increased by the addition of neutral salts, and double reciprocal plots of the spectrum vs aminopyrine concentration were linear, though the plots were not linear in absence of the salts. Dissociation constant (Ks) values were also decreased by the addition of neutral salts corresponding enhancement of the demethylation, as were the km values. In phenobarbital or 3-methylcholanthrene-treated microsomes one Km was obtained regardless of the presence or absence of sodium sulfate, though Km of the demethylase in the former microsomes was higher than that in the latter after addition of the salt. These results suggest that there are multiple forms of aminopyrine N-demethylase in nontreated and 3-methylcholanthrene -treated microsomes and some of them slightly contribute to the demethylation in normal condition but exhibite the demethylase activity by addition of the salts. Phenobarbital may induce the aminopyrine N-demethylase capable of demethylating aminopyrine in normal condition resulted in less enhancement of the demethylation by the salts.

Introduction

Many drugs are oxidized by hepatic microsomal monooxygenase system which contains cytochrome P-450 as a terminal oxidase.[1)2)] There are multiple forms of cytochrome P-450 which have an overlapping activity for various substrates.[3)4)] Previous reports show that neutral salts, especially sulfate, enhance hepatic microsomal aminopyrine N-demethylation of rats, but

not aniline hydroxylation, and that the effect of neutral salts on changing aminopyrine or aniline oxidation follows Hofmeister's lyotropic series of ions.[5)6)] Aminopyrine and aniline are classified as type I and tipe II substrates, respectively, for cytochrome P-450 according to substrate-induced difference spectrum.[7)] Type I substrate-induced difference spectrum is due to binding of the substrate with the hydrophobic region of the cytochrome P-450 while type II substrate causes the spectral change by reaction with the heme iron of the cytochrome P-450.[8)] Thus it was suggested that neutral salts alter the hydrophobic environment around cytochrome P-450 associated with aminopyrine N-demethylation resulting in enhancement of the enzyme activity. In order to clarify the mechanism of enhancing aminopyrine N-demethylation by salts, the kinetic aspect of the N-demethylation was investigated and the effect of cytochrome P-450 induction on the enhancement was also discussed.

Materials and Methods

Chemicals: Nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate were purchased from the Sigma Chemical Co., (St. Louis MO); aminopyrine from Daiichi-Seiyaku Ltd., (Tokyo); glucose-6-phosphate dehydrogenase and reduced nicotinamide adenine dinucleotide phosphate from Oriental Yeast Ltd., (Tokyo). Phenobarbital sodium and 3-methylcholanthrene were obtained from Kanto Chemicals Ltd., (Tokyo). Acetylacetone was purified by distillation. All other chemicals were of analytical reagent grade.

Male Wistar rats weighing 150 to 300g (from Nihon Rat Ltd., Saitama) were used. Phenobarbital and 3-methylcholanthrene were given intraperitoneally once a day for three days at a dose of 60mg/kg(in isotonic sodium chloride solution) and 40 mg/kg(in olive oil), respectively. Nontreated rats were given only isotonic sodium chloride solution. Liver microsomes were prepared 24 hrs after the final injection of reagents as described in the previous report.[5)] The microsomal pellets were stored at −20°C in nitrogen gas and were used within one week. Aminopyrine N-demethylase activity was determined by measuring the formation of formaldehyde using the method of Nash under the same conditions as described in the previous report.[5)9)] Aminopyrine-induced difference spectrum was measured by the method of Schenkman et al. with a Shimazu (MPS-50L) spectrophotometer.[7)] Briefly, microsomes of 2.1ml (6-8 mg protein in 0.1M phosphate buffer, pH 7.4) in the sample cuvette with 1 cm lightpath were mixed with the salt solution of 0.6ml followed by addition of 0.3ml of aminopyrine. Water was added in the reference cuvette instead of the salt. A difference spectrum was recorded from 350nm to 500nm of wavelength. Difference in absorbance between 420nm and 500nm was used for calculation of dissociation constant. Protein concentration in microsomes was measured by the method of Lowry et al. [10)]

Results

Figure 1 shows aminopyrine-induced difference spectra of hepatic microsomes from nontreated rats in presence or absence of lithium sulfate. Difference in absorbance between peak and trough was increased by treatment with the salt. Other salts, which enhance the

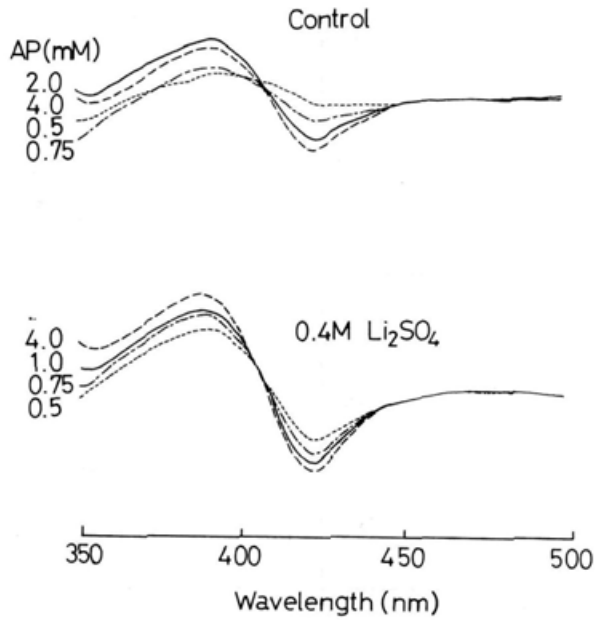

Fig. 1. Effect of lithium sulfate on aminopyrine-induced difference spectra of microsomes. Microsomes from nontreated rats were resuspended with 0.1M phosphate buffer, pH 7.4 (3.5 mg protein/ml) and were divided into sample and reference cuvettes. Aminopyrine-induced spectral changes were recorded in presence or absence of 0.4M lithium sulfate as described in Materials and Methods.

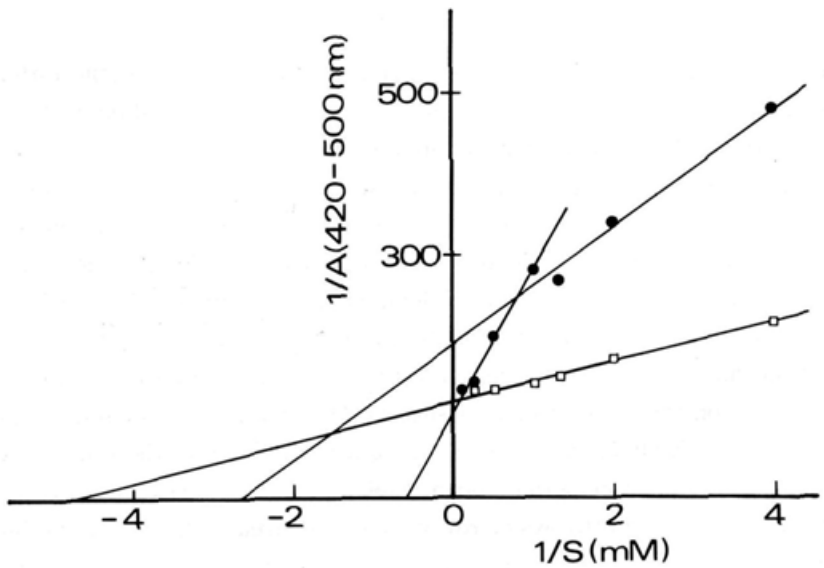

Fig. 2. Double reciprocal plots of aminopyrine binding to nontreated microsomes. Microsomes were resuspended with 0.1M phosphate buffer, pH 7.4 (2.4 mg protein/ml) and the difference spectrum was measured as described in Materials and Methods. ●:control, □:0.4M Na_2SO_4, S:aminopyrine.

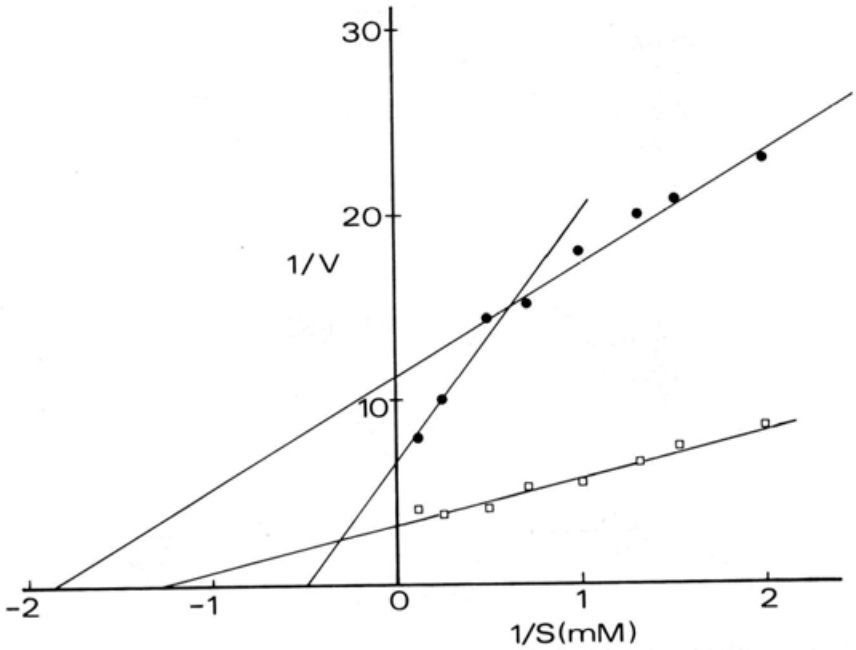

Fig. 3. Lineweaver-Burk plots for the N-demethylation of aminopyrine in nontreated microsomes. Aminopyrine N-demethylase activity was measured as described in Materials and Methods. Protein concentration in these assay was 0.49 mg/ml. ●:control, □: 0.4M Na_2SO_4. V:formaldehyde (nmol/mg/20min)

aminopyrine N-demethylase activity, also increased the intensity of the difference spectra corresponding to potentiation of the demethylase activity. Reciprocal plots of the difference in absorbance between 420nm and 500nm vs aminopyrine concentration were presented in Fig. 2. In absence of sodium sulfate the reciprocal plots were not linear and two dissociation constants, 0.377mM and 1.724mM, were obtained. On the contrary the plots were linear and only one dissociation constant (0.208mM) was observed after addition of the sulfate. Figure 3 shows Lineweaver-Burk plots of aminopyrine N-demethylase activity in nontreated microsomes in presence or absence of sodium sulfate. Nonlinear and linear plots were observed in absence and presence of the sulfate, respectively, as similar to shown in difference spectra. Corresponding two dissociation constants, two Km values(0.540mM, 2.0mM) were obtained in absence of the sulfate and only one Km(0.714mM) was observed after addition of the sulfate. Kinetic aspect of the effect of salts on aminopyrine N-demethylase was summarized in Table 1. These data show that the high values in two Ks or Km values in nontreated microsomes (absence of salts) are altered to one low Ks or Km value by salts corresponding the degree of enhancement of aminopyrine N-demethylase activity.

Table 1. Kinetic parameters for N-demethylation and binding reaction of aminopyrine.

	N-demethylation* (%)	Km(mM)	Ks(mM)
Control	100	2.051±0.46	1.811±0.18
		0.491±0.15	0.413±0.04
		(n=4)	(n=4)
0.4M Li_2SO_4	305	0.479±0.11	0.431±0.06
		(n=2)	(n=2)
0.4M Na_2SO_4	313	0.585±0.16	0.248±0.06
		(n=4)	(n=2)
0.4M LiCl	161	1.149	0.914
0.4M KCl	137	1.428	1.388

* Aminopyrine N-demethylase activity (1 mM substrate) in control microsomes was 52.6±6.8 HCHO nmole/mg/20min.

Table 2. Effect of phenobarbital or 3-methylcholanthrene treatment on enhancement of aminopyrine N-demethylation by sodium sulfate.

Microsomes	HCHO (nmole/mg/20min)			
	0.5mM AP		4mM AP	
	Salt(−)	Salt(+)	Salt(−)	Salt(+)
Nontreated	28.8±8.3	90.2±21.6**	53.4±8.2	143.1 ±21.7**
	(100±21.2)	(312.9±74.9)	(100±15.2)	(267.7±40.6)
PB-treated	59.6±7.5*	102.9±16.0**	118.8±17.3*	204.3±27.1**
	(100±12.6)	(172.3±26.8)	(100±14.5)	(171.9±22.7)
MC-treated	33.0±3.0	99.8±18.0**	55.6±7.0	138.4±11.1**
	(100±9.0)	(302.0±54.6)	(100±12.5)	(248.5±20.0)

Sodium sulfate was added in the concentration at 0.4M. AP, PB and MC are aminopyrine, phenobarbital and 3-methylcholanthrene, respectively. Values are shown mean±SD. (*); nontreated vs treated, $P<0.05$ and (**); salt (−) vs salt (+), $P<0.05$.

Effect of giving phenobarbital or 3-methylcholanthrene on enhancement of aminopyrine N-demethylation by sulfate was studied (Table 2). The demethylase activity associated with low Km (aminopyrine 0.5mM) was increased 2-fold by phenobarbital treatment but 3-methylcholanthrene did not affect the demethylase activity. However, the demethylase activity was altered by addition of sodium sulfate and reached almost same degree(99～102nmol/20min) in all cases. The demethylase activity was also increased 2.2-fold by phenobarbital but there were no increase of the activity by 3-methylcholanthrene when the high concentration (4mM) of aminopyrine was used. In these conditions, the degree of enhancement of the demethylase by sulfate was same in nontreated and 3-methylcholanthrene treated rats, and was small in phenobarbital treatment. Kinetic study of aminopyrine N-demethylase in phenobarbital or 3-methylcholanthrene treated microsomes was presented in Figs. 4 and 5. In contrast to nontreated microsomes Lineweaver-Burk plots of the demethylase activity were linear in both phenobarbital and 3-methylcholanthrene treated microsomes : Km value of 1.515mM in 3-methylcholanthrene treated microsomes was altered to 0.520mM by addition of sodium sulfate, whereas the Km of 1.070 mM in phenobarbital treated microsomes was little changed by the same salt.

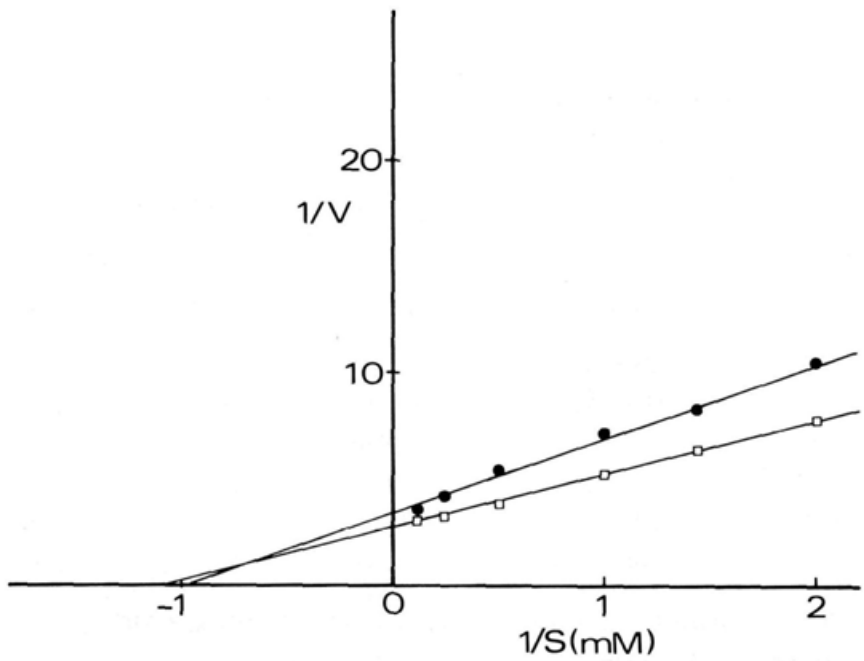

Fig. 4. Lineweaver-Burk plots for the N-demethylation of aminopyrine in phenobarbital treated microsomes. Aminophyrine N-demethylase activity was measured as described in Materials and Methods. Microsomal protein concentration was 0.40 mg/ml. ●:control, □:0.4M Na_2SO_4. V:formaldehyde (nmol/mg/20min)

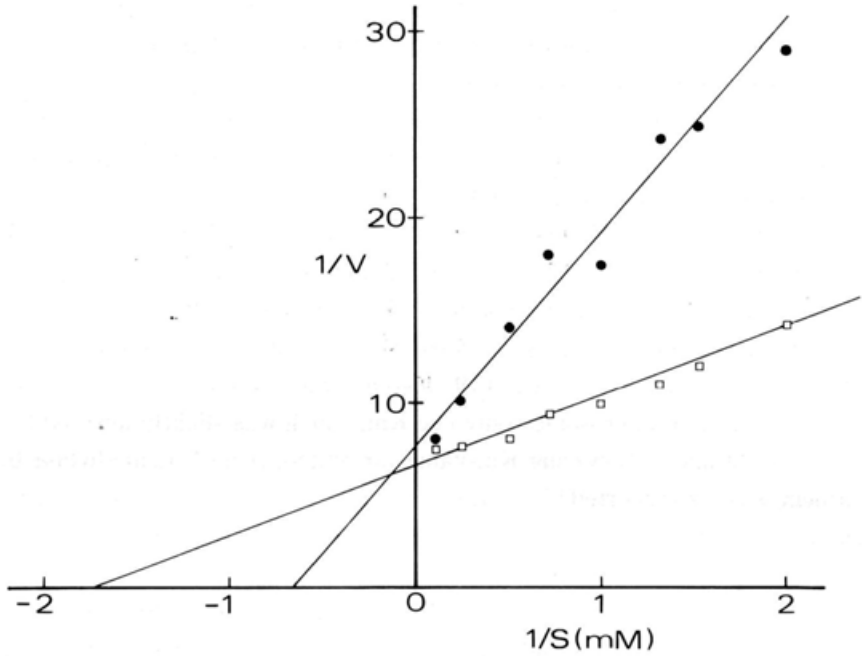

Fig. 5. Lineweaver-Burk plots for the N-demethylation of aminopyrine in 3-methylcholanthrene treated microsomes. Aminopyrine N-demethylase activity was measured as described in Materials and Methods. Microsomal protein concentration was 0.57 mg/ml. ●: control, □:0.4M Na_2SO_4. V:formaldehyde (nmol/mg/20min)

Discussion

Nonlinear Lineweaver-Burk plots of aminopyrine N-demethylase indicating multiplicity of the enzyme were obtained in the present study in nontreated microsomes as shown in other reports.[11)-13)] Two Km values for aminopyrine N-demethylase were observed in absence of the salts but only one low Km was obtained after addition of the salts (Table 1). Moreover, the kinetic aspect of aminopyrine binding to the demethylase (Ks value) also showed a similar behavior to that of the demethylase activity in absence or presence of the salts. These results suggest that there are at least two groups of aminopyrine N-demethylase in nontreated microsomes : one has a high affinity for aminopyrine which reacts at low concetrations of aminopyrine and the other is a high Km component which is not primarily responsible for demethylation but is capable of doing at high substrate concentration. After addition of salts to the microsomes these demethylases behaved like one enzyme with one Km value. Remmer and Coon proposed that there is an oily, hydrophobic pocket of cytochrome P-450 which provides a non-specific substrate binding site.[14)] Considering that neutral salts enhance the demethylase activity in relation to lyotropic series of ions,[5)] it is likely to assume that the enhancement of aminopyrine N-demethylase activity by the salts is due to alteration of the hydrophobic environment existing around the active site of the demethylase. In other words,

considerable amounts of aminopyrine N-demethylase are embedded into microsomal membrane and the salts alter the hydrophobic environment of the demethylase resulting in easy entrance of the aminopyrine to the active site. Since aminopyrine causes spectral change by binding to the hydrophobic region of the cytochrome P-450,[8] it is reasonable that positive correlation of aminopyrine N-demethylase activity with the affinity for aminopyrine binding to the enzyme has been observed in microsomes in absence or presence of salts. As shown in Table 2, aminopyrine N-demethylation in nontreated microsomes was enhanced at low or high concentrations of aminopyrine by addition of the sulfate, it is, therefore, suggested that both demethylases with high or low Km value are enhanced by the salt.

Enhancement of aminopyrine N-demethylation by salts was observed in phenobarbital treated microsomes though the degree of the increase was low compared with that of nontreated microsomes. In these microsomes only one Km, which was slightly lowered by addition of the sulfate, was obtained. Lowering Km value for aminopyrine N-demethylase by phenobarbital treatment was also reported.[11][12] Vainio showed that phenobarbital increased the amounts of protein in the outer membrane layer in microsomes.[15] It is, therefore, suggested that aminopyrine N-demethylase induced by phenobarbital is little affected by the sulfate because salts act mainly on the demethylase which locates in the inner layer of microsomes rather than the outer layer. Thus, it is considered that phenobarbital enhances the aminopyrine N-demethylase activity by increasing cytochrome P-450 contents whereas salts do by altering microsomal membrane conditions.

3-Methylcholanthrene did not stimulate the aminopyrine N-demethylase acitivity and the same degree of enhancement of the demethylase as seen in nontreated microsomes was observed after addition of the sulfate (Table 2). However, considering linear Lineweaver-Burk plots of the demethylase a qualitative change of the enzyme may occur in 3-methylcholanthrene treated microsomoes. This is supported by the view that 3-methylcholanthrene modifies mainly lipid areas of microsomal membranes. [15]

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