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Position selective sex difference in imipramine metabolism in rat liver microsomes*

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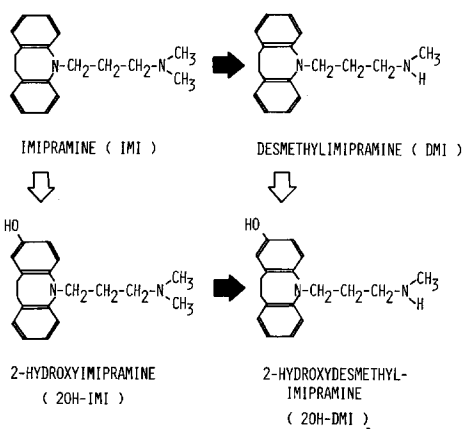
The subject of sex differences in drug metabolism in rats has been reviewed extensively [1, 2]. The extent of the sex difference depends on the substrate used. For example, aminopyrine *N*-demethylase and hexobarbital aliphatic hydroxylase activities are about three and eight times higher in male than in female rats, respectively, while aniline *p*-hydroxylation shows no sex difference [3, 4].

It is currently proposed that sex difference is caused by the presence of sex specific forms of cytochrome P-450†. Fujita *et al.* [5] showed the presence of male and female specific P-450 by EPR spectroscopy, and this observation was supported by the purification study by Kamataki *et al.* [6]. Therefore, substrate selectivity of the sex difference most likely depends on the substrate specificity of the P-450 which is responsible for the sex difference.

It has been known also that P-450-dependent reactions are not only substrate specific, but also position specific, or P-450 species involved in the metabolism at one position of a single substrate are different from the species involved in the metabolism at the other position of the same substrate [7–10]. If indeed the substrate selectivity of the sex difference is due to the substrate specificity of the P-450 species involved, there is a possibility of position selectivity in sex difference. Since imipramine is either *N*-demethylated or 2-hydroxylated to form desipramine or 2-hydroxyimipramine, respectively (Scheme 1) [11], and these metabolic pathways have been suggested to be mediated by different P-450 isozymes [12], there is a possibility of position selective sex difference in imipramine metabolism. Therefore, in this report, we studied the metabolism of imipramine in liver microsomes from male and female rats.



Scheme 1. Major pathways of imipramine metabolism mediated by cytochrome P-450 isozymes in rat liver microsomes. Open arrows show 2-hydroxylation and closed arrows, *N*-demethylation.

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† For the sake of brevity, cytochrome P-450 is referred to as P-450 for the rest of this communication.

Materials and methods

Chemicals. Imipramine (IMI), desmethylimipramine (DMI), 2-hydroxyimipramine (2OH-IMI), and 2-hydroxydesipramine (2OH-DMI) were donated by Geigy (Basel), and nortriptyline was donated by the Dainippon Pharmaceutical Co., Ltd. (Osaka). NADP, glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were obtained from the Oriental Yeast Co., Ltd. (Tokyo). All other chemicals and solvents were of analytical grade.

Preparation of microsomes. Hepatic microsomes were prepared according to the method described by Omura and Sato [13] from the livers of 3-month-old male (N = 4, 364–390 g) and female rats (N = 3, 293–315 g) of the Wistar strain. Microsomal protein was determined by the method of Lowry *et al.* [14].

Enzyme assay. The rates of oxidation of IMI were measured in a 1-ml assay mixture containing microsomes (1.0 mg protein/ml incubation mixture), 20 mM $MgCl_2$, 10 mM G-6-P, 1.2 mM NADP, 2.0 I.U. G-6-P dehydrogenase, 0.26 mM EDTA, and imipramine, concentrations ranging from 0.9 to 17.5 μM, in 0.15 M Tris–HCl buffer (pH 7.4). After a 5-min preincubation under air at 37°, the reaction was started by the addition of NADP. The formation of DMI and 2OH-IMI from IMI was linear up to 45 sec at the substrate concentration range used in this study. The linearity of the reaction was lost after this time period due to the formation of a secondary metabolite, 2OH-DMI. Within the first 45 sec, 2OH-DMI was not detected. The incubation was therefore performed for 30 sec and stopped by the addition of 1.0 ml of 1.0 M carbonate buffer (pH 10.0). The formation of the primary metabolites of IMI in this incubation period was well within the detectable range with our high-performance liquid chromatographic (HPLC) methods described below.

Analysis of imipramine metabolites. Metabolites formed from imipramine were measured by the high-performance liquid chromatography described by Sutfin and Jusko [15] with the modifications described below. To 1.0 ml of the stopped-reaction mixture, 125 ng of nortripyline as internal standard and 5.0 ml of ethyl acetate were added. After extraction by vigorous mixing for 1.0 min and centrifugation for 10 min at 1200 *g*, the organic layer was transferred to another glass vial containing 500 μl of 0.1 N hydrochloric acid. The mixture was vigorously mixed for 1.0 min with a vortex mixer and centrifuged for 10 min. After discarding the organic layer, 500 μl of 1.0 M carbonate buffer (pH 10.0) and 150 μl of chloroform were added. After mixing and centrifugation as described above, 50–100 μl of the chloroform phase was injected into a Lichrosorb SI-60 column of a high-performance liquid chromatograph (model TWINCLE, Japan Spectroscopic Co., Tokyo) and the IMI metabolites, DMI, 2OH-IMI, and 2OH-DMI, were detected at 254 nm with an ultraviolet detector (model UVIDEC 100-III, Japan Spectroscopic Co.). The mobile phase consisted of methanol, acetonitrile, and ammonium hydroxide (5:35:1, by vol.), and the flow rate was 1.5 ml/min. This procedure allows simultaneous quantitative determinations of the IMI metabolites.

Calculations of kinetic parameters. Kinetic parameters V_{max} and K_m were calculated using a SIMPLEX method incorporated in an iterative nonlinear least-squares program, MULTI [16]. Each observed datum was weighted by the reciprocal of the square of its value. The initial values were estimated from the Lineweaver–Burk plots of the obtained data.

Results and discussion

Figure 1A shows the effect of IMI concentrations on the formations of DMI and 2OH-IMI in male liver microsomes. At concentrations less than approximately 4 μM, the mean rates of 2OH-IMI formation were higher than those of DMI. The rate of 2OH-IMI approached a plateau at this IMI concentration whereas that of IMI demethylation did not approach a plateau in the concentration range used in this study. The rate of formation of DMI exceeded the rate of formation of 2OH-IMI at higher concentrations of IMI. In the case of female liver microsomes (Fig. 1B), however, the rate of formation of 2OH-IMI was higher than that of DMI over the entire range of concentrations of IMI in this study.

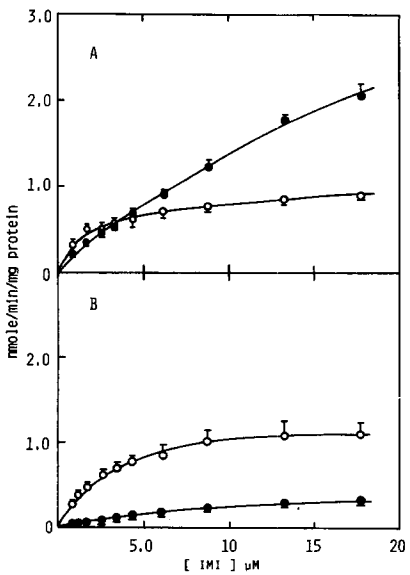

Fig. 1. Rate of formation of desipramine (DMI) and 2-hydroxyimipramine (2OH-IMI) from imipramine at different substrate concentrations in male (A) and female (B) rat liver microsomes. The rate of formation of DMI (●) and 2OH-IMI (○) at IMI concentrations of 0.9 to 17.5 μM in liver microsomes prepared from 3-month-old male (A) and female (B) rats was determined according to the method described in the text. Values are means ± S.E. for four male and three female rats.

Apparent Michaelis constants (K_m) and maximum velocities (V_{max}) for these reactions were summarized in Table 1. In male liver microsomes, K_m and V_{max} of IMI *N*-demethylation were about ten and six times higher than those of IMI hydroxylation respectively. In contrast, K_m of *N*-demethylation in female liver microsomes was about five times higher than that of 2-hydroxylation, but no significant difference in V_{max} between *N*-demethylation and 2-hydroxylation was observed. The values of K_m and V_{max} of *N*-demethylation in liver microsomes obtained from male rats were about two and nine times higher than those from female rats, respectively. In contrast, V_{max} and K_m of 2OH-IMI showed no sex difference. In this study, it was found that IMI *N*-demethylase activity in males was much higher than that in females, especially in high IMI concentrations, while IMI hydroxylase activity in males almost equaled that in females. Therefore, the presence and the absence of the sex difference in the metabolism of a single substrate depend on the position of the substrate to be metabolized. This phenomenon can be easily explained by the fact that P-450 species can be position selective in the metabolism of certain substrates [7, 8, 12, 17–19]. Our present observation strongly suggests that *N*-demethylation and 2-hydroxylation of the single substate, IMI, depends on different species of P-450.

In summary, the rate of *N*-demethylation of imipramine by liver microsomes from male rats was much higher than that from female rats. In contrast, the rate of 2-hydroxylation of imipramine did not show much sex difference. It is concluded that the sex difference in imipramine metabolism is position selective, and this selectivity is most likely to be caused by the difference in P-450 species involved in these metabolic pathways.

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Table 1. Kinetic parameters for 2-hydroxylation and *N*-demethylation of imipramine*

Sex	2-Hydroxylation K_m (μM)	2-Hydroxylation V_{max} (nmoles/min/mg protein)	*N*-Demethylation K_m (μM)	*N*-Demethylation V_{max} (nmoles/min/mg protein)
M (4)†	3.62 ± 1.25	1.06 ± 0.0565	34.9 ± 4.38‡	6.17 ± 0.875‡
F (3)†	3.17 ± 0.482	1.36 ± 0.223	16.2 ± 2.07§‖	0.684 ± 0.127¶

* Values represent means ± S.E. of V_{max} and K_m determined as described in Materials and Methods. The range of substrate concentrations employed was 0.9 to 17.5 μM.

† Values in parentheses indicate the number of male (M) and female (F) rats. Liver microsomes prepared from each rat were used for the determination of kinetic parameters.

‡ Significantly different from K_m or V_{max} for 2-hydroxylation in male liver microsomes, $P < 0.01$.

§ Significantly different from K_m for 2-hydroxylation in female liver microsomes, $P < 0.01$.

‖ Significantly different from K_m for demethylation in male liver microsomes, $P < 0.05$.

¶ Significantly different from V_{max} for demethylation in male liver microsomes, $P < 0.01$.

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Salicylate-induced loose coupling: protonmotive force measurements

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The Surgeon General's report on salicylate and Reye Syndrome [1] emphasizes the concern about the role of aspirin in Reye Syndrome. A prospective study [2] found a 100% correlation between aspirin and Reye Syndrome in patients compared to 45% in carefully matched controls. A recent paper pointed out the histological similarity between salicylate hepatotoxicity and Reye Syndrome [3]. Salicylate has been known for many years to have effects on intact mitochondria. Early work [4] showed an uncoupling effect, and other early researchers [5] reported that salicylate stimulates mitochondrial adenosine triphosphatase (ATPase) activity. Jeffrey and Smith [6] found an inhibition of mitochondrial swelling with salicylates similar to that seen with 2,4-dinitrophenol. However, more recently You [7] found that salicylate produces swelling of mitochondria similar to that seen in patients with Reye Syndrome. Miyahara and Karler [8], using a Warburg technique, demonstrated an uncoupling effect of salicylate in mitochondrial fragments isolated from rat liver and brain. However, their paper emphasized the confusion in interpretation of salicylate effects. Different results were seen with alternate substrates, and the results of their study, as from many earlier studies, could have been explained by an ATPase-stimulating effect of salicylate which would produce the decreased ADP:O ratios seen. More recent studies in rat liver mitochondria demonstrated an effect on the adenine nucleotide exchange across the inner mitochondrial membrane as well as an uncoupling effect in the presence of salicylate [9]. Both the effects were half-maximal at about 3 mM extra matrix concentration. Oligomycin, an F_1 ATPase inhibitor, does not block the uncoupling effect of salicylate. It has been suggested that this uncoupling is closely related to that of dinitrophenol in spite of the other effects of salicylate [6].

One approach to resolving the mechanism of apparent uncoupling is to measure directly the protonmotive force across the inner mitochondrial membrane in the presence of salicylate. This paper describes the results of these experiments.

Rat liver mitochondria were prepared and polarographic assay was performed as previously described with the following modifications [10]. Polarographic assays were performed in a 384 μl thermostatically controlled glass microchamber of the authors' own design with a Teflon-coated Microflex stir bar. A Clark oxygen electrode (Radiometer E5046-0) was used with a Radiometer model PM72 pO_2 module as a polarographic system. Chamber volume was calculated by tritiated water dilution after scintillation counting of an aliquot. Control and salicylate-treated mitochondria were preincubated for 3 min before ADP was added. Final mitochondrial protein concentrations were in the range of 0.5 to 1.5 mg/ml. Liver mitochondrial protonmotive force and matrix volumes were measured with glutamate as substrate using a double-label equilibration technique with [^{14}C]triphenylmethyl phosphonium iodide, [^{14}C]acetate, [^{3}H]water and [^{3}H] mannitol. Our method is described in detail elsewhere [11]. The amounts of ADP added to the protonmotive force assay to achieve State 3 and State 4 rates were selected by polarographic assay of equivalent concentrations of mitochondria in protonmotive force assay mixture.

Salicylate (1 mM) produced an acceleration of state 4 rates of oxygen consumption with glutamate, succinate and α-ketoglutarate as substrates (Table 1). The modest state 3 rate inhibition reached significance only with α-ketoglutarate. Respiratory control ratios were reduced markedly for all substrates, reflecting both the acceleration of state 4 and the modest reduction of state 3. An increase in glutamate and α-ketoglutarate ADP/O ratios was seen although this is not thought to be of biological significance.

The protonmotive force in state 4 was reduced by 15% in salicylate (1 mM) treated mitochondria using glutamate as substrate (Table 2). There was also a small reduction in the transmembrane potential. The membrane proton conductance increased more than 4-fold from 0.595 to 2.669 nmoles of $H^+ \cdot min^{-1} \cdot$(mg mitochondrial protein)$^{-1} \cdot mV^{-1}$. No significant changes were seen in state 3.

Matrix volumes did not differ ($P > 0.05$, paired t-test) in control and salicylate-treated groups suggesting that salicylate effects are not the result of mitochondrial breakage. Matrix volumes (μl/mg protein, N = 6) were: control state 3, 1.40 ± 0.5 (means ± S.E.); salicylate-treated state 3, 1.47 ± 0.47; control state 4, 1.68 ± 0.46; and salicylate-treated state 4, 1.25 ± 0.39.

The acceleration of state 4 rates of oxygen consumption by salicylate which we observed in these polarographic studies was similar to results obtained with tissue slices and Warburg techniques by previous investigators [4, 6, 8]. This uncoupling effect was considered to be similar to that of 2,4-dinitrophenol [6]. Aprille [9] found in rat liver that the uncoupling effect is insensitive to oligomycin, suggesting that stimulation of the F_1 ATPase coupling enzyme is not the major mechanism of accelerated oxygen consumption.