These may include sites on the receptor-activated Ca^{2+} inflow system as well as sites through which verapamil exerts other effects on hepatocytes.

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Impairment of bunitrolol 4-hydroxylase activity in liver microsomes of Dark Agouti rats

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Bunitrolol (BTL*), a β-adrenoceptor blocking agent, is predominantly biotransformed to 4-OH BTL in rat liver microsomes [1, 2]. We have shown that this microsomal oxidation reaction is mediated by the mixed function oxidase system including P450 in a previous study using various enzyme inhibitors [3]. Genetic polymorphisms in the oxidation of many drugs represented by DB and mephenytoin in man have been described [4]. Poor metabolism of DB is closely associated with various degrees of impairment in the oxidations of other, but not all, drugs

* Abbreviations: BTL, bunitrolol; 4-OH BTL, 4-hydroxybunitrolol; P450, cytochrome P450; DB, debrisoquine; DA, Dark Agouti; G-6-P, glucose-6-phosphate.

[5]. Extensive studies in the rat and human have indicated that hydroxylations of other β-adrenoceptor blocking agents, for example propranolol 4-hydroxylation and bufuralol 1'-hydroxylation, are catalysed by DB 4-hydroxylase [4, 6–10]. These findings suggest the involvement of DB 4-hydroxylase in BTL metabolism. To examine this possibility, we investigated BTL 4-hydroxylase activities in liver microsomes of adult Wistar and DA rats of both sexes.

Materials and Methods

General. Adult male and female Wistar rats (7–9-weeks-old) were purchased from Takasugi Experimental Animals Co. (Kasukabe, Japan); adult male and female DA rats were from Shizuoka Laboratory Co. (Shizuoka, Japan). The animals were obtained at least 3 days before experiments and housed in an air-conditioned room (22–24°) under a 12 hr light–dark cycle. BTL and 4-OH BTL as hydrochloride were gifts from Nippon C.H. Boehringer Sohn Co. (Osaka, Japan); DB and 4-OH DB as hemisulfate were from Hoffmann-La Roche Ltd (Basel, Switzerland). G-6-P, G-6-P dehydrogenase and NADPH were obtained from Oriental Chemicals (Tokyo, Japan). Other chemicals and solvents used were of analytical grade.

Assay of enzymatic activities. Rat liver microsomes were prepared according to the method of Omura and Sato [11]. 4-OH BTL was quantitated by an HPLC procedure reported previously [3] with a slight modification. Namely, the reaction medium consisted of microsomal protein (0.5 mg), G-6-P (10 μmol), $MgCl_2$ (8 μmol), G-6-P dehydrogenase (2 units) and BTL (0.1–3000 nmol) and 154 mM Tris–HCl buffer (pH 7.4) to make a final volume of 1.0 mL. Reaction was started at 37° by adding NADPH (0.5 μmol) and continued for 1 min. It was terminated by adding 1 mL of 1 M carbonate buffer (pH 9.8). After adding 0.5 nmol of propranolol as an internal standard, 4-OH BTL was extracted into ethyl acetate (4 mL). The organic layer was evaporated *in vacuo*, and the residue was dissolved in 100 μL of the mobile phase described below. 4-OH BTL was then determined by the HPLC method under the conditions as follows: instrument, a Hitachi 655-type liquid chromatograph equipped with a Hitachi 650-10S-type fluorescence spectrophotometer and a Hitachi 561-type recorder; column, a Gaskuro-Kogyo's Inertsil ODS column (4.6 mm × 25 cm); mobile phase, acetonitrile/methanol/water/acetic acid (30:12.25:57.75:0.225, by vol.); flow rate, 0.7 mL/min; detection, fluorescence (excitation 310 nm, emission 380 nm). Amounts of 4-OH BTL formed were calculated on the basis of calibration curves made by adding known amounts of synthetic 4-OH BTL and propranolol (0.5 nmol) instead of the substrate to the reaction medium cooled in ice. Kinetic parameters were obtained using a nonlinear least-squares regression technique based on a simplex method [12]. DB 4-hydroxylase activity was determined by the method of Masubuchi *et al.* [13]. Protein concentrations were measured by the method of Lowry *et al.* [14]. Statistical significance was calculated by Student's *t*-test.

Results and Discussion

The initial rates of BTL 4-hydroxylation were measured over a substrate concentration range of 100 nM to 3 mM in liver microsomes of male Wistar and DA rats. The microsomal reaction rates in both strains were represented by a single Michaelis–Menten equation (Fig. 1). Table 1 summarizes the kinetic parameters calculated. A substrate concentration of 10 μM was employed in the following experiments, since at this concentration the BTL 4-hydroxylase activity determined corresponds to approximately 90% of its maximum rates (V_{max}) in Wistar and DA rats.

As described above, DB 4-hydroxylase may be involved also in microsomal BTL 4-hydroxylation, since other β-adrenoceptor blockers such as propranonol and bufuralol were oxidized by this P450 enzyme. To explore this possibility, BTL 4-hydroxylase activities were assessed in liver microsomes of adult DA rats of both sexes and compared with those of adult Wistar rats of both sexes. The adult female rat of the DA strain is known to be an animal model defective in a P450 isozyme(s) belonging to the IID gene subfamily in liver microsomes [15, 16]. As shown in Fig. 2, microsomal BTL 4-hydroxylase activities in male and female DA rats were significantly lower than those in male and female Wistar rats, respectively. Furthermore, significant sex differences in BTL 4-hydroxylase activity were seen in microsomes of both Wistar and DA rats.

As a comparison, DB 4-hydroxylase activities were assessed in the same microsomal preparation as those in the experiments of Fig. 2. Using Fisher and DA rats, Kahn *et al.* [17] have obtained the kinetic data for DB 4-hydroxylase activity which could be analysed as a two-

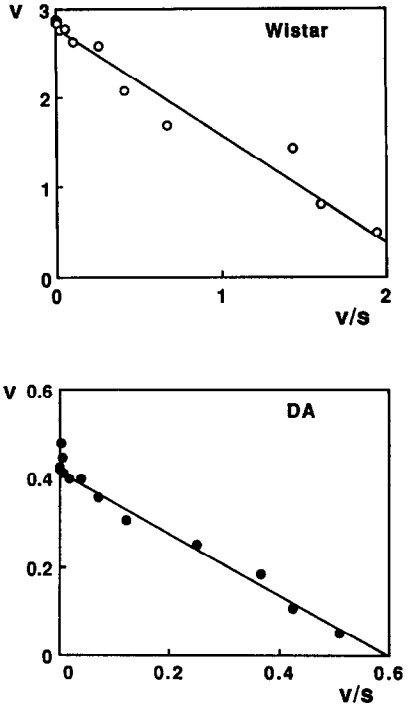

Fig. 1. Eadie–Hofstee plots for BTL 4-hydroxylation in liver microsomes of male Wistar and DA rats. BTL 4-hydroxylation was determined using three samples of different liver microsomes obtained from male Wistar and DA rats. Each plot represents a typical result of the three determinations. Ordinate; *V*, nmol/min/mg protein. Abscissa; *V*/*S*, nmol/min/mg protein/μM.

Table 1. Kinetic parameters of BTL 4-hydroxylation in liver microsomes of male Wistar and DA rats

Strain	Parameters	
	K_m (μM)	V_{max} (nmol/min/mg protein)
Wistar	1.36 ± 0.21	2.73 ± 0.14
DA	0.71 ± 0.05	0.50 ± 0.06

Each value represents the mean ± SE (N = 3).

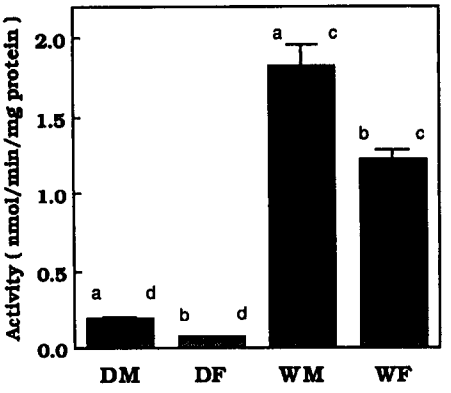

Fig. 2 Strain and sex differences in BTL 4-hydroxylase activity in rat liver microsomes. BTL 4-hydroxylase was assayed at a substrate concentration of 10 μM under the conditions given in Materials and Methods. D, W, M and F represent DA, Wistar, male and female, respectively. Each bar represents the mean ± SE of four determinations. a–d Show significant differences between the values marked with the same letters ($P < 0.01$).

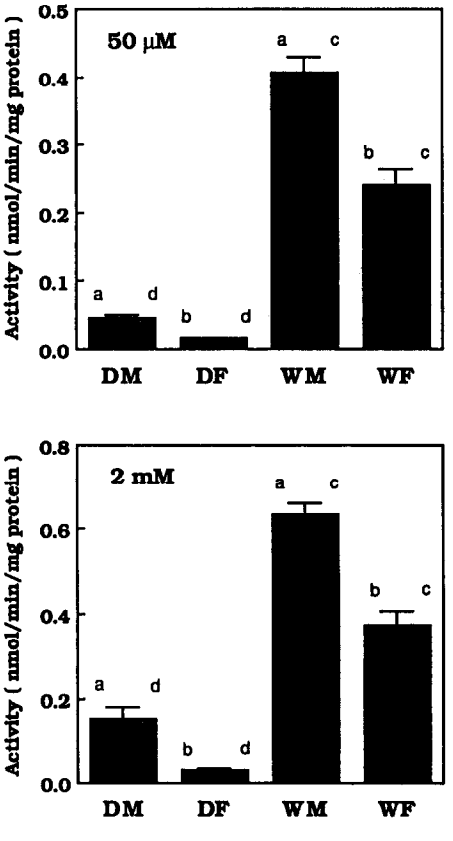

Fig. 3 Strain and sex differences in DB 4-hydroxylase activity in rat liver microsomes. DB 4-hydroxylase was assayed at substrate concentrations of 50 μM and 2 mM under the conditions given in Materials and Methods. D, W, M and F represent DA, Wistar, male and female, respectively. Each bar represents the mean ± SE of four determinations. a–d Show significant differences between the values marked with the same letters ($P < 0.01$).

enzyme system (K_m1 and K_m2 ranging between 9 to 19 and 770 to 1300 μM, respectively) [17]. Two substrate concentrations (50 μM and 2 mM) were thus employed for the assay of DB 4-hydroxylation. At both substrate concentrations, DB 4-hydroxylase activity was significantly higher in Wistar rats than in DA rats of the same sex and the activity in Wistar rats was significantly higher in male than in female rats (Fig. 3). Similarly, significant sex differences (male > female) were observed in the microsomal DB 4-hydroxylation at both substrate concentrations of 50 μM and 2 mM in DA rats (Fig. 3). Sex and strain differences of DB 4-hydroxylation in both strains of rats are very similar to those of the BTL oxidation in Fig. 2. The sex difference observed indicates that isozyme(s) catalysing BTL 4-hydroxylation may be controlled, at least in part, by sex hormones as in the case of male- and female-specific P450 isozymes [18, 19]. Alternatively, some male-specific P450 isozyme(s) may participate partially in microsomal BTL 4-hydroxylation.

In summary, the results obtained here suggest that P450 isozyme(s) catalysing DB 4-hydroxylation mediates also BTL 4-hydroxylation in rat liver microsomes. However, enzymatic studies using purified P450 isozyme(s), namely, BTL 4-hydroxylase, are necessary to prove this possibility. We are now performing experiments along this line.

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Scavenging of hypochlorous acid by lipoic acid

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Upon stimulation, neutrophils are able to produce the reactive oxygen species, superoxide ($O_2^{\cdot -}$) and hydrogen peroxide. Moreover, neutrophils release the enzyme myeloperoxidase. This enzyme catalyses the conversion of chloride (Cl^-) to the powerful oxidant hypochlorous acid (HOCl*) [1, 2]. The reactive oxygen species and HOCl contribute to the bactericidal action of neutrophils. However, the damaging effect of these products is not limited to bacteria, also the surrounding tissue is vulnerable. An important target for HOCl is the α_1-antiproteinase (α_1-AP). α_1-AP is the most important inhibitor of elastase [1, 2]. HOCl oxidizes a critical methionine residue of α_1-AP to a sulphoxide derivative with the consecutive loss of activity of the inhibitory protein [3]. In addition, activated neutrophils excrete elastase. A resulting imbalance between elastase and anti-elastase activity in the respiratory tract may cause the enzymatic destruction of the elastic fibers in the lung, a process believed to be central in the development of certain types of emphysema [1–3].

Lipoate is an 8-carbon fatty acid with, in its reduced form, two thiol groups on the 6th and 8th carbon atom. Oxidized lipoate contains an intramolecular disulfide bridge in a 5-membered ring. Lipoate has been shown to be a potent antioxidant [4, 5]. In the present study the ability of lipoate to scavenge HOCl was determined. The scavenging activity is compared to that of the potent scavengers *N*-acetylcysteine and GSH.

* Abbreviations: HOCl, hypochlorous acid; α_1-AP, α_1-antiproteinase; GSH, glutathione; GSMe, *S*-methyl glutathione; GSSG, oxidized glutathione.

Materials and Methods

N-Acetylcysteine, GSH, GSSG, *S*-methyl glutathione (GSMe), *N-t*-BOC-L-alanine *p*-nitrophenol ester, α_1-antiproteinase (α_1-AP, code A 9024) and elastase (code E 0258) were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Reduced lipoate (dihydrolipoate) and oxidized lipoate were gifts from Asta Pharma A.G. (Frankfurt am Main, Germany).

Elastase activity was determined according to the assay described by Wasil *et al.* [2], with minor modifications. All reagents were dissolved in phosphate buffer used (19 mM KH_2PO_4-KOH) pH 7.4, containing 140 mM NaCl, unless otherwise noted. Twenty micrograms of α_1-AP (unless otherwise noted) were mixed with the compound under investigation (0–100 μM) and preincubated at 25°. After 5 min, HOCl was added (50 μM unless otherwise noted). The final volume was 100 μL. The concentration of the test compounds (0–100 μM) and HOCl (usually 50 μM) indicated in the text and the figures, refers to the concentration in the 100 μL incubation mixture. After an additional 5 min, 200 μL buffer containing 5 μg elastase was added. Again after 5 min, 700 μL buffer and 50 μL of