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Involvement of a Cytochrome P450 System in Microsomal Debromination of α-(Bromisovaleryl)urea

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Abstract

The reductive debromination of a hypnotic, (α-bromisovaleryl)urea to (3-methylbutyryl)urea by rat liver microsomes was studied.

Pretreatment of rats with cytochrome P450 inducers such as phenobarbitone, 3-methylcholanthrene, acetone and pregnenolone-16α-carbonitrile enhanced the debromination of (α-bromisovaleryl)urea by liver microsomes. Microsomal debromination was inhibited by cytochrome P450 inhibitors such as metyrapone, α-naphthoflavone, SKF 525-A and carbon monoxide. Microsomal debromination was enhanced by addition of NADPH-cytochrome P450 reductase and inhibited by addition of an antibody against the flavo enzyme to the liver microsomes. A reconstituted cytochrome P450 system containing NADPH-cytochrome P450 reductase, and cytochrome P450 1A1 or P450 2B1 exhibited debrominating activity toward the hypnotic.

These results indicated that a cytochrome P450 system plays an essential role in the microsomal debromination of (α-bromisovaleryl)urea.

(α-Bromisovaleryl)urea is widely used as a mild sedative or hypnotic. Early studies showed that the drug was metabolized to (3-methylbutyryl)urea, [2-(2-amino-2-carboxyethylthio)-3-methylbutyryl]urea and [2-(methylthio)-3-methylbutyryl]urea in rabbits, and α-(cystein-S-yl)isovalerylurea, α-(*N*-acetyl-cystein-S-yl)isovalerylurea and α-(cysteamin-S-yl)isovaleric acid in man. In addition, the reductive debromination of the drug to its main metabolite, (3-methylbutyryl)urea, was also demonstrated in-vitro using rabbit liver microsomes.

The present study was undertaken to show that a liver microsomal cytochrome P450 system is responsible for the reductive debromination of (α-bromisovaleryl)urea.

Materials and Methods

Chemicals

Materials used were obtained from the following sources: (α-bromisovaleryl)urea from Nippon Shinyaku Co., Ltd.; phenobarbitone sodium and α-naphthoflavone from Tokyo Chemical Industry Co., Ltd.; 3-methylcholanthrene from Nacalai Tesque, Inc.; metyrapone and pregnenolone-16α-carbonitrile from Aldrich Chemical Co.; NADPH and NADH from Oriental Yeast Co.; dilauroyl phosphatidylcholine from Wako Pure Chemical Industry; bovine serum albumin from Armour Pharmaceutical Co. SKF 525-A was a gift from Smith Kline-Beecham Laboratories. (3-Methylbutyryl)urea was synthesized as described by Narafu (1967).

Preparation of microsomes

Male Wistar rats, 130–150 g, were fed a standard pellet diet MM-3 which was withdrawn 15 or 24 h before they were killed. The animals were stunned by a blow on the head and exsanguinated. The liver was immediately perfused with 1·15% KCl and homogenized in 4 vol KCl solution, using a Potter–Elvehjem homogenizer. The microsomal fraction was obtained from the homogenate by successive centrifugation at 9000 *g* for 20 min and 105 000 *g* for 60 min. The fraction was washed once with the KCl solution and resuspended in the solution to make 1 mL equivalent to 1 g liver.

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Pretreatment of animals

Phenobarbitone sodium was dissolved in saline solution and then given to rats intraperitoneally in doses of 80 mg kg^{-1} day^{-1} for 3 days. 3-Methylcholanthrene and pregnenolone-16α-carbonitrile were dissolved in corn oil and then given to rats intraperitoneally in a dose of 25 and 50 mg kg^{-1} day^{-1} for 3 days, respectively. After the final medication, the animals were starved for 24 h and then killed. Sixty percent aqueous acetone was given to rats orally in a dose of 4·8 g kg^{-1}. After medication, the animals were starved for 15 h and then killed.

Purification of enzymes

Cytochrome P450 1A1 was purified from livers of 3-methylcholanthrene-pretreated rats (Nagata et al (1985). Cytochrome P450 2B1 (Guengerich et al 1982) and NADPH-cytochrome P450 reductase (Yasukochi & Masters 1976) were purified from livers of phenobarbitone-pretreated rats.

Preparation of an antibody

Rabbit antisera against NADPH-cytochrome P450 reductase (anti-fp_2 1G) was prepared as described previously (Noshiro & Omura 1978).

Assay methods

Assays of cytochrome P450 and NADPH-cytochrome P450 reductase were performed as described by Omura & Sato (1964), and by Yasukochi & Masters (1976), respectively. Debrominating activity toward (α-bromisovaleryl)urea was

assayed using a Thunberg tube under anaerobic conditions. In the debromination by liver microsomes, a typical incubation mixture consisted of the substrate (1 μmol), NADPH or NADH (4 μmol), and the liver preparation (equivalent to 0·2 g liver) in a final volume of 2 mL of Clark Lubs buffer (0·2 M KH_2PO_4 – 0·2 M NaOH, pH 8.5). In the debromination by a reconstituted cytochrome P450 system, a typical incubation mixture consisted of the substrate (2 μmol), NADPH (1 μmol), NADPH-cytochrome P450 reductase (2 units), cytochrome P450 (1 nmol) and dilauroyl phosphatidylcholine in a final volume of 1 mL of the Clark Lubs buffer described above. In either case, the side arm of a Thunberg tube contained the substrate and its body contained all other components. The tube was gassed for 5 min with nitrogen, which was passed through a deoxygenizing solution consisting of 0·5% sodium dithionite and 0·05% sodium 2-anthraquinonesulphonate in 0·4% NaOH, evacuated with an aspirator for 5 min and again gassed with nitrogen. After the tube was tightly closed, the reaction was started by mixing the components of the side arm and the body together, and continued for 40 min at 37°C. After incubation, the reaction was stopped by adding 0·2 mL 1 M HCl to the mixture. 2,4-Dinitrochlorobenzene (100 μg) was added as an internal standard, and the mixture was extracted once with 5 mL ethyl acetate and the extract was evaporated to dryness in vacuo. The residue was dissolved in 200 μL methanol and then subjected to HPLC, which was performed in a Hitachi L-6000 chromatograph fitted with a 4 × 125 mm LiChrospher 100 RP-18 column. The mobile phase was methanol : water (7 : 3). The chromatograph was operated at a flow rate of 0·2 mL min^{-1} and at a wavelength of 210 nm. The elution times of (α-bromisovaleryl)urea and its debrominated product, (3-methylbutyryl)urea, were 8·3 and 6·9 min, respectively. The product was determined from its peak area.

Determination of protein

Protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as a standard protein.

Statistics

Results are expressed as the mean ± s.d. Statistical significance was determined by Student's *t*-test.

Results

Pretreatment of rats with phenobarbitone, 3-methylcholanthrene, acetone and pregnenolone-16α-carbonitrile, which are different types of cytochrome P450 inducers, increased liver microsomal debromination of (α-bromisovaleryl)urea; phenobarbitone was the most effective (Table 1).

The effects of cytochrome P450 inhibitors on the liver microsomal debromination of the drug are shown in Table 2. Metyrapone and α-naphthoflavone preferentially inhibited debromination by liver microsomes from rats pretreated with phenobarbitone and acetone, and 3-methylcholanthrene, respectively. The inhibition was also observed with SKF 525-A. In all cases, the liver microsomal debromination was almost completely inhibited by carbon monoxide.

When NADPH-cytochrome P450 reductase was added to the liver microsomes from phenobarbitone-pretreated rats, the microsomal debromination of the drug was enhanced; the microsomal debromination was inhibited partially by an anti-

Table 1. Effects of pretreatment with phenobarbitone, 3-methylcholanthrene, acetone and pregnenolone-16α-carbonitrile on debromination of (α-bromisovaleryl)urea by rat liver microsomes.

Pretreatment	(3-Methylbutyryl)urea formed (nmol / 40 min (g liver)$^{-1}$)
Control	415 ± 61
Phenobarbitone	3065 ± 147*
3-Methylcholanthrene	1160 ± 191*
Acetone	990 ± 145*
Pregnenolone 16α-carbonitrile	1258 ± 28*

Each value represents the mean ± s.d. of three rats. *$P < 0.001$ compared with control.

Table 2. Effects of some chemicals on debromination of (α-bromisovaleryl)urea by liver microsomes from rats pretreated with phenobarbitone, 3-methylcholanthrene or acetone.

Inhibitor	Inhibition (%)		
	Phenobarbitone	3-Methylcholanthrene	Acetone
Metyrapone 10^{-5} M	80	20	77
10^{-4} M	98	66	85
SKF 525-A 10^{-5} M	22	10	36
10^{-4} M	56	25	70
α-Naphthoflavone 10^{-5} M	13	87	24
10^{-4} M	53	93	64
Carbon monoxide —	100	100	100

Each value represents the mean of three experiments.

Table 3. Effects of addition of NADPH-cytochrome P450 reductase and the antibody against the flavoenzyme on debromination of (α-bromisovaleryl)urea by liver microsomes from phenobarbitone-pretreated rats.

Addition	(3-Methylbutyryl)urea formed (nmol / 40 min (g liver)$^{-1}$)
Control	3090 ± 177
fp_2	3718 ± 268*
Anti-fp_2 IG	2261 ± 314*

Each value represents the mean ± s.d. of three experiments. fp_2 = NADPH-cytochrome P450 reductase; anti-fp_2 IG = antibody against NADPH-cytochrome P450 reductase. *$P < 0.05$ compared with control.

body against NADPH-cytochrome P450 reductase (Table 3). These results strongly suggested the involvement of NADPH-cytochrome P450 reductase and cytochrome P450 in the debromination of the drug by liver microsomes.

To confirm the essential role of a cytochrome P450 system in the microsomal debromination of the drug, we carried out reconstitution experiments. The complete system containing NADPH, NADPH-cytochrome P450 reductase, cytochrome P450 (cytochrome P450 1A1 or P450 2B1) and phospholipid exhibited the debrominating activity toward (α-bromoisovaleryl)urea. Both NADPH-cytochrome P450 reductase and cytochrome P450 were needed for the reconstitution of the debrominating activity; the omission of either of these components resulted in a significant decrement or abolition of the activity (Table 4).

These results led us to a conclusion that a cytochrome P450 system plays an essential role in the microsomal debromination of (α-bromoisovaleryl)urea to (3-methylbutyryl)urea.

Table 4. Debromination of (α-bromisovaleryl)urea by a reconstituted cytochrome P450 system.

System	(3-Methylbutyryl)urea formed (nmol min^{-1} (g liver)$^{-1}$)	
	P450 1A1	P450 2B1
Complete	1·85 ± 0·07	3·71 ± 0·31
minus P450	ND	ND
minus fp_2	ND	0·12 ± 0·03
minus NADPH	ND	ND
minus DLPC	1·40 ± 0·42	1·47 ± 0·33

Each value represents the mean ± s.d. of three experiments. ND = not detected. fp_2 = NADPH-cytochrome P450 reductase; DLPC = dilauroyl phosphatidylcholine.

Discussion

The present study demonstrates for the first time the involvement of a cytochrome P450 system in the debromination of (α-bromoisovaleryl)urea to (3-methylbutyryl)urea by liver microsomes.

Halothane is metabolized to dehalogenated products under hypoxic conditions. Ahr et al (1982) have reported a stepwise two-electron mechanism of halothane reductive metabolism, which involves the formation of a cytochrome P450-halothane complex, transfer of an electron to the substrate, and concomitant leaving of bromide ion to produce the 2-chloro-1,1,1-trifluoroethyl radical. Abstraction of a hydrogen by the radical then proceeds. Alternatively, further reduction of the radical by a second electron releases a fluoride ion and subsequent β-elimination. A similar mechanism appears to operate in the case of debromination of (α-bromoisovaleryl)urea. However, since the hypnotic is a monohalogenated compound, only one-electron reduction may occur. The reductive pathway leads to the formation of the (3-methylbutyryl)urea radical and bromide ion, and abstraction of a hydrogen from the milieu leads to the debrominated product, (3-methylbutyryl)urea (Fig. 1).

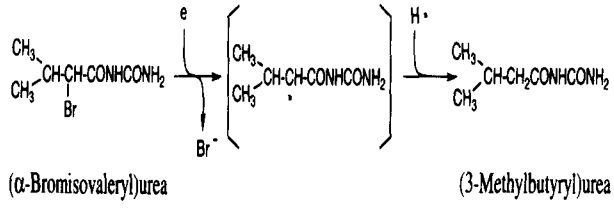

FIG. 1. A possible mechanism for reductive debromination of (α-bromisovaleryl)urea.

Recent studies have established the multiplicity of hepatic microsomal cytochrome P450 enzymes which differ in substrate specificity, molecular weight, and spectral and immunological properties. Therefore, it was of interest to compare the debrominating activities toward (α-bromoisovaleryl)urea of different types of cytochrome P450. Cytochromes P450 1A1 and P450 2B1 used in the present reconstitution study are cytochrome P450 species inducible by 3-methylcholanthrene and phenobarbitone, respectively. The activity of the former was lower than that of the latter. However, the differences were not remarkable in view of their profoundly different substrate specificities in oxidative reactions. The results suggested that (α-bromoisovaleryl)urea as well as oxygen interacts directly with the heme group of cytochrome P450 and receives an electron from NADPH via NADPH-cytochrome P450 reductase to form the debrominated product.

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