Further Studies on Reductive Metabolism of Acetohexamide in Heart

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Species and sex differences of acetohexamide reductase activity were investigated using the cytosolic fraction of heart homogenate. The activity in the rabbit was considerably higher than that in the other species (guinea pig, hamster, rat and mouse). No sex difference of the activity was observed in any of the species tested. Ketone-containing drugs (daunorubicin, befunolol and levobunolol) other than acetohexamide were little reduced in the cytosol of rabbit heart. Some aldehyde reductase inhibitors (phenobarbital, valproate and chlorothiazide) were found to decrease the acetohexamide reductase activity in the cytosol of rabbit heart.

Keywords acetohexamide; reductive metabolism; heart; species difference; sex difference; ketone-containing drug; aldehyde reductase inhibitor

Although many tissues in the body are capable of metabolizing drugs, most drugs are mainly metabolized in the liver. Recently, we have demonstrated that in the reductive metabolism of acetohexamide, the cytosol of rabbit heart exhibits an approximately 2-fold higher activity than that of rabbit liver, and the enzyme which catalyzes acetohexamide reduction in the heart may differ from that in the liver.[1,2] This finding that the heart contributes markedly to drug metabolism was of great interest. The purpose of the present study was to elucidate species and sex differences of acetohexamide reductase activity using the cytosolic fraction of heart homogenate, and to compare the reductive metabolism of some ketone-containing drugs including acetohexamide in the cytosol of rabbit heart. The effects of aldehyde reductase inhibitors on acetohexamide reductase activity in the cytosol of rabbit heart were also investigated.

Experimental

Materials Acetohexamide was supplied by Shionogi & Co., Ltd. Hydroxyhexamide was synthesized from acetohexamide according to the method of Girgis-Takla and Chroneos.[3] Daunorubicin (Meiji Seika Co., Ltd.), befunolol hydrochloride (Kaken Pharm. Co., Ltd.), levobunolol hydrochloride (Warner–Lambert, Inc.), fenbufen (Lederle (Japan), Ltd.), valproate sodium (Kyowa Hakko Co., Ltd.) and chlorothiazide (Merck (Japan), Ltd.) were gifts from the manufacturers. Phenobarbital was obtained from Wako Pure Chemical Industries, Ltd. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. All other chemicals were of reagent grade.

Preparation of Cytosolic Fraction Male and female rabbits (Japanese White, 2.0—3.0 kg), guinea pigs (Hartley, 280—380 g), hamsters (Syrian, 90—125 g), rats (Wistar, 160—290 g) and mice (ddY, 28—31 g) were fasted for about 24 h before experiments, but drinking water was freely available. The rabbits were exsanguinated from the carotid artery, and the other animals were killed by decapitation. The heart and liver were excised carefully, and then homogenized with a Potter–Elvehjem homogenizer in 3 volumes of 0.01 M phosphate buffer containing 1.15% KCl (pH 7.4). The homogenates were centrifuged at $10000 \times g$ for 20 min and the resulting supernatants were centrifuged at $105000 \times g$ for 60 min to obtain the cytosolic fraction. All these procedures were done at 0—4 °C. The cytosolic fraction was assayed for enzyme activity.

Assay of Enzyme Activity 1) In assaying the acetohexamide reductase activity, the incubation mixture consisted of acetohexamide (1.0 mM), $NADP^+$ (0.25 mM), glucose-6-phosphate (6.25 mM), glucose-6-phosphate dehydrogenase (0.25 unit), $MgCl_2$ (6.25 mM), cytosolic fraction of heart or liver homogenate and 0.1 M phosphate buffer (pH 7.4) in a final volume of 2.0 ml. The reaction was started by the addition of the cofactor, and the mixture was incubated at 37 °C for 10 min under aerobic conditions. The reaction was stopped by the addition of 0.5 ml of 1 N HCl to the mixture. The reduction product (hydroxyhexamide) was determined by high-performance liquid chromatography (HPLC).[4] Each reaction mixture was extracted with 5.0 ml of benzene–ethyl acetate (1 : 1, v/v) containing fenbufen as the internal standard. After centrifugation at 3000 rpm for 10 min, the organic phase (4.0 ml) was evaporated *in vacuo* and the residue was dissolved in acetonitrile (0.3 ml) and subjected to HPLC. HPLC was carried out using a Hitachi 655A-11 HPLC apparatus equipped with a LiChrosorb RP-18 column (250 × 4 mm i.d., Cica-Merck) and a Hitachi 638-41 UV monitor (230 nm). Acetonitrile–0.2% acetic acid (47 : 53, v/v) was used as a mobile phase at a flow rate of 1.0 ml/min.

2) The activity of the enzyme which reduces ketone-containing drugs was spectrophotometrically determined by recording the rate of NADPH oxidation at 340 nm in the incubation mixture. The incubation mixture consisted of ketone-containing drug (1.0 mM), NADPH (0.25 mM), cytosolic fraction of heart or liver homogenate, and 0.1 M phosphate buffer (pH 7.4) in a final volume of 3.0 ml. One unit of enzyme activity was defined as the amount that catalyzes oxidation of 1 μmol of NADPH/min at 25 °C. In the reduction of ketone-containing drugs, one munit/mg protein corresponds to 1 nmol/min/mg protein.

3) Protein concentration was determined by the method of Lowry *et al.*[5] with bovine serum albumin as the standard.

Statistics The results were analyzed statistically with Student's unpaired *t*-test.

Results

Species and Sex Difference of Acetohexamide Reductase Activity in Cytosol of Heart Figure 1 shows species and sex differences of acetohexamide reductase activity in the cytosolic fraction of heart homogenate from some experimental animals. The activity in the cytosolic fraction of heart homogenate from the rabbit was considerably higher

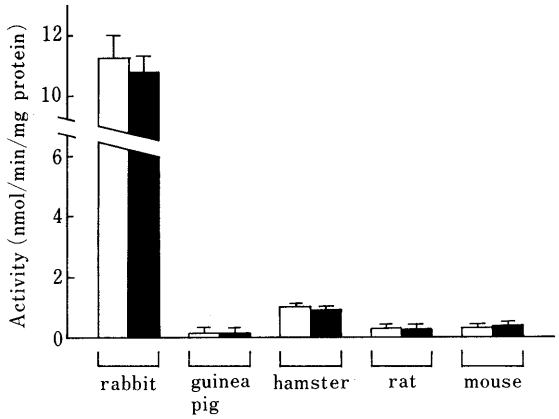

Fig. 1. Species and Sex Differences of Acetohexamide Reductase Activity in the Cytosol of Heart

□, male; ■, female. Each bar represents the mean ± S.D. of 6 experiments. No significant difference was observed in any of the species between the male and the female.

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TABLE I. Reductive Metabolism of Acetohexamide and Some Ketone-Containing Drugs in the Cytosols of Rabbit Heart and Liver

Drug (1.0 mM)	Activity (munit/mg protein)	
	Heart	Liver
Acetohexamide	5.90±0.36	2.67±0.40
Daunorubicin	0.32±0.20	2.90±0.31
Befunolol	0.42±0.42	3.01±0.55
Levobunolol	0.47±0.37	2.59±0.31

Each value represents the mean±S.D. of 3 experiments.

TABLE II. Effect of Phenobarbital, Valproate and Chlorothiazide on Acetohexamide Reductase Activity in the Cytosols of Rabbit Heart and Liver

Inhibitor	Concn. (mM)	Relative activity (%)	
		Heart	Liver
None		100.0	100.0
Phenobarbital	0.1	81.2± 7.7	101.1±4.5
	1.0	45.7± 5.0	102.6±2.3
Valproate	0.1	83.2±12.8	96.2±6.7
	1.0	64.2± 7.5	90.6±9.9
Chlorothiazide	0.1	64.3± 5.2	66.4±6.8

Each value represents the mean±S.D. of 2—3 experiments.

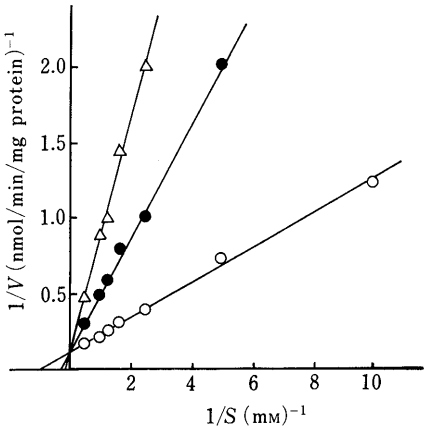

Fig. 2. Lineweaver–Burk Plot for Acetohexamide Reductase Activity in the Cytosol of Rabbit Heart

○, in the absence of phenobarbital; ●, in the presence of phenobarbital (0.9 mM); △, in the presence of phenobarbital (2.0 mM). Each point represents the mean of 2—3 experiments.

than that in the guinea pig, hamster, rat or mouse. Furthermore, no sex difference of the activity was observed in any of the species tested.

Reductive Metabolism of Ketone-Containing Drugs in Cytosols of Rabbit Heart and Liver The reductive metabolism of ketone-containing drugs such as daunorubicin, befunolol and levobunolol was examined in the cytosols of rabbit heart and liver. As shown in Table I, these ketone-containing drugs, unlike acetohexamide, were little reduced in the cytosol of rabbit heart. In contrast, the cytosol of rabbit liver had the ability to reduce all the ketone-containing drugs including acetohexamide. Ahmed *et al.*[6] have also reported a similar result for daunorubicin reductase activity in the cytosols of rabbit heart and liver.

Effect of Inhibitor on Acetohexamide Reductase Activity in Cytosols of Rabbit Heart and Liver In our previous paper, we pointed out that an aldehyde reductase may be involved in the reductive metabolism of acetohexamide in the cytosol of rabbit heart.[1] Phenobarbital, valproate and chlorothiazide are known to inhibit potently aldehyde reductases.[7-10] Table II shows the effects of these inhibitors on acetohexamide reductase activity in the cytosols of rabbit heart and liver. As expected, these inhibitors evidently decreased the activity in the cytosol of rabbit heart; chlorothiazide also decreased the activity in the cytosol of rabbit liver. In addition, phenobarbital was found to inhibit competitively the acetohexamide reduction in the cytosol of rabbit heart, as shown in Fig. 2. The values of apparent kinetic constants (K_m and V_{max}) of acetohexamide reduction were 1.15±0.08 mM and 9.65±0.92 nmol/min/mg protein, respectively, and the value of inhibition constant (K_i) for phenobarbital was 0.39±0.04 mM.

Discussion

In general, enzymes which catalyze the reduction of ketone-containing drugs have been reported to be localized in the cytosolic fraction of tissue homogenates including the liver.[6,11-13] Thus, species difference in the reductive metabolism of acetohexamide in the heart was examined using the cytosolic fraction. Among the species tested, only the rabbit displayed a significant acetohexamide reductase activity in the cytosol of the heart (Fig. 1).

Information concerning the reduction of ketone-containing drugs in the cytosol of the heart has been very limited. In the present study, we have provided evidence that although acetohexamide can be reduced in the cytosol of rabbit heart, daunorubicin, befunolol and levobunolol are little reduced (Table I). These results suggest that ketone-containing drugs other than acetohexamide may be poor substrates for the enzyme which catalyzes acetohexamide reduction in the cytosol of rabbit heart.

In an attempt to elucidate the properties of the acetohexamide reducing enzyme present in the cytosol of rabbit heart, the effects of aldehyde reductase inhibitors such as phenobarbital, valproate and chlorothiazide on the activity were examined. These inhibitors evidently decreased the activity (Table II), supporting our suggestion that the acetohexamide reducing enzyme can be classified as an aldehyde reductase.[1] Erwin and Deitrich have demonstrated that phenobarbital produces a noncompetitive inhibition for bovine brain aldehyde reductase.[7] A similar phenomenon was observed for the inhibition of sheep liver aldehyde reductase by phenobarbital.[14] However, in the present study, phenobarbital inhibited competitively acetohexamide reduction in the cytosol of rabbit heart (Fig. 2). Therefore, the enzyme which catalyzes acetohexamide reduction in the cytosol of rabbit heart appeared to be different from bovine brain aldehyde reductase[7] or sheep liver aldehyde reductase.[14] Further investigations including purification of the acetohexamide reductase and examination of the enzyme kinetics are in progress at this laboratory.

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