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Studies on Metabolic Reduction of Befunolol in Rabbit Liver and Kidney

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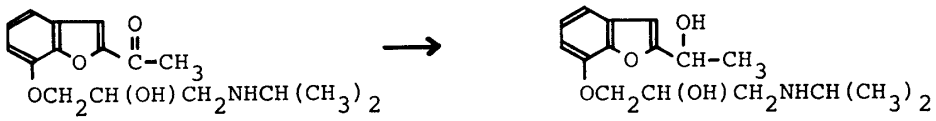
The metabolic reduction of befunolol was studied using rabbit liver and kidney preparations. Befunolol was mainly reduced in the cytosols of liver and kidney and its reduction was mediated by an NADPH (nicotinamide adenine dinucleotide phosphate, reduced form)—dependent enzyme with a pH optimum in the range of 6.0 to 7.0. Rather similar K_m (Michaelis constant) and V_{max} (maximal velocity) values were observed for befunolol reduction in the cytosols of liver and kidney, and plant flavonoids (quercitrin, quercetin) and sulfhydryl reagents markedly inhibited the befunolol reduction. These findings indicate that the metabolic reduction of befunolol in the liver and kidney is catalyzed by the same carbonyl reductase. Furthermore, it was suggested that the befunolol-reducing enzyme might be a 3α-hydroxysteroid dehydrogenase on the basis of the inhibitory effects of various nonsteroidal anti-inflammatory drugs.

Keywords——befunolol; metabolic reduction; rabbit tissue; cytosolic fraction; nonsteroidal anti-inflammatory drug; inhibitory effect; carbonyl reductase

Befunolol possesses a potent β-adrenoceptor-blocking activity and is used in the treatment of arrhythmia.[1] In humans and some animals, befunolol is known to be reduced to dihydrobefunolol which is a pharmacologically active metabolite (Fig. 1).[2] Some properties of the befunolol-reducing enzyme have been examined using rabbit liver.[3] However, the physiological role of the enzyme is still uncertain, and no attempt to characterize the enzyme present in rabbit kidney has been made. Since the kidney often has the ability to reduce readily xenobiotic ketones, it is important to elucidate the contribution of the kidney to befunolol reduction. The purpose of this study was to examine and compare the properties of the befunolol-reducing enzyme, using rabbit liver and kidney. The inhibitory effects of nonsteroidal anti-inflammatory drugs on the metabolic reduction of befunolol were also investigated.



befunolol dihydrobefunolol

Fig. 1. Metabolic Reduction of Befunolol to Dihydrobefunolol

Experimental

Chemicals——Befunolol hydrochloride [2-acetyl-7-(2-hydroxy-3-isopropylaminopropoxy) benzofuran hydrochloride] and dihydrobefunolol hydrochloride were supplied by Kaken Pharm. Co., Ltd., (Osaka, Japan). Acetaminophen (Yamanouchi Pharm. Co., Ltd., Tokyo, Japan), diclofenac sodium (Fujisawa Pharm. Co., Ltd., Osaka, Japan), flufenamic acid, indomethacin (Taisho Pharm. Co., Ltd., Tokyo, Japan), ibuprofen (Nisshin Pharm.

Co., Ltd., Osaka, Japan), mefenamic acid, protizinic acid (Yoshitomi Pharm. Industries Ltd., Osaka, Japan), fenbufen (Lederle (Japan) Ltd., Tokyo, Japan), flurbiprofen (Mitsubishi Chem. Industries Ltd., Tokyo, Japan) and ketoprofen (Hisamitsu Pharm. Co., Ltd., Saga, Japan) were provided by the manufacturers. Nicotinamide adenine coenzymes (reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD)), flavin adenine dinucleotide (FAD), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SKF 525-A was kindly supplied by Professor H. Yoshimura (Kyushu University, Japan). Other enzyme inhibitors were obtained from Wako Pure Chemical (Osaka, Japan). All other chemicals used in this study were guaranteed reagents.

Preparation of Subcellular Fractions——Male albino rabbits weighing 2.0—3.0 kg were fasted for 38—42 h prior to the experiments, but drinking water was allowed *ad libitum*. The animals were exsanguinated from the carotid artery and the tissues were carefully removed. The tissues were homogenized with 0.01 M phosphate buffer containing 1.15% KCl (pH 7.4) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at $10000 \times g$ for 20 min and the resulting supernatant was centrifuged at $113000 \times g$ for 60 min to obtain the cytosol. The cytosolic fraction was used in the assay for enzyme activity. When individual subcellular fractions were desired, each rabbit tissue was homogenized and prepared according to the method of Hogeboom.[4] That is, the homogenates were centrifuged at $700 \times g$ for 10 min. The resulting supernatants were centrifuged at $5000 \times g$ for 10 min to sediment a mitochondrial fraction and finally at $113000 \times g$ for 60 min to obtain microsomal pellets and cytosolic supernatant. The sedimented pellets at each step were washed once with the same homogenizing buffer. All steps involving homogenization and centrifugation were carried out at 0—4 °C.

Enzyme Assay——In assaying the befunolol-reducing activity, the typical incubation mixture consisted of 2.0 mM befunolol, 0.25 mM NADP, 6.25 mM glucose-6-phosphate, 6.25 mM $MgCl_2$, 0.25 unit of glucose-6-phosphate dehydrogenase, 0.1 M phosphate buffer (pH 7.4), and enzyme preparation in a total volume of 2.0 ml, unless otherwise noted. The reaction was started by the addition of each enzyme preparation, and continued at 37 °C for 10 min under aerobic conditions. The reaction was stopped by the addition of 1.0 ml of 2 N NaOH to the reaction mixture. For determining the effect of pH on the activity, we used the following 0.1 M buffers with the indicated pH ranges; phosphate (pH 5.5 to 8.0), Tris–HCl (pH 7.5 to 9.0). The reduction product (dihydrobefunolol) was determined by high-performance liquid chromatography (HPLC). Protein concentration was determined by the method of Lowry *et al.*[5] with bovine serum albumin as the standard.

Determination of Dihydrobefunolol——Each reaction mixture was extracted with 7.0 ml of diethyl ether. After centrifugation at 3000 rpm for 10 min, the supernatant (organic phase) was evaporated with the internal standard (sulfadimethoxine) *in vacuo*, and the residue was dissolved in the mobile phase of HPLC 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 655A-21 UV monitor (245 nm). Acetonitrile–0.02 M phosphate buffer (pH 2.5) (1 : 3) containing 5 mM sodium 1-hexanesulfonate was employed as a mobile phase at a flow rate of 1.0 ml/min.

Results

Tissue Distribution

The distribution of befunolol-reducing activity in the cytosols of various rabbit tissues is shown in Fig. 2. Although the activity was widely distributed among the cytosols of all the tissues tested, the highest activity was observed in the liver or kidney. Similar results have been reported for the tissue distribution of enzymes that catalyze the metabolic reduction of ketone-containing drugs such as daunorubicin and oxisuran.[6]

Subcellular Localization

The befunolol-reducing activity was examined in the subcellular fractions of rabbit liver and kidney. As shown in Table I, the activity was mainly localized in the cytosolic fractions of the liver and kidney. In the microsomal fractions of the liver and kidney, however, the activity was not detected.

Cofactor Requirement

The cofactor requirement for befunolol reduction in the cytosols of rabbit liver and kidney is shown in Table II. The befunolol-reducing activity in the liver and kidney essentially required NADPH as a cofactor. Other cofactors were much less effective or were ineffective at supporting the enzymatic activity. Thus, the befunolol-reducing enzyme in the liver or kidney

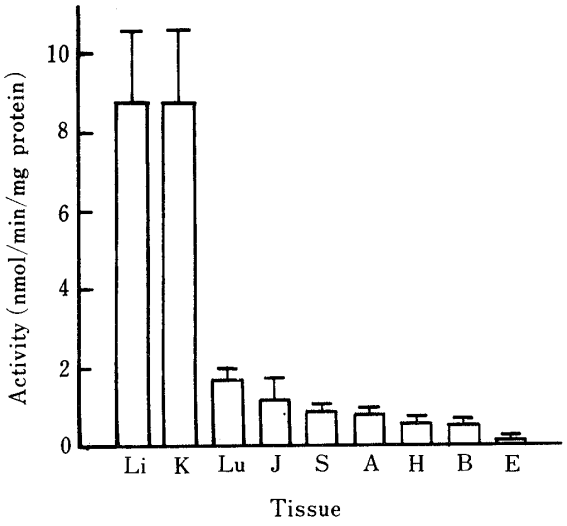


Fig. 2. Tissue Distribution of Befunolol-Reducing Activity

Each value represents the mean ± S.D. ($n=10$). Li, liver; K, kidney; Lu, lung; J, jejunum; S, spleen; A, adrenal gland; H, heart; B, brain; E, eye.

TABLE I. Subcellular Localization of Befunolol-Reducing Activity

Fraction	Activity (nmol/min/mg protein)	
	Liver	Kidney
Nuclei	0.28 ± 0.26	n.d.
Mitochondria	0.60 ± 0.52	n.d.
Microsomes	n.d.	n.d.
Cytosol	8.73 ± 1.63	8.74 ± 1.67

Each value represents the mean ± S.D. ($n=3$). n.d.; not detected.

TABLE II. Cofactor Requirement of Befunolol-Reducing Activity

Cofactor	Activity (nmol/min/mg protein)	
	Liver	Kidney
None	n.d.	n.d.
NADPH	7.99 ± 1.78	6.54 ± 0.83
NADH	0.50 ± 0.11	0.28 ± 0.07
NADP	0.36 ± 0.09	n.d.
NAD	0.20 ± 0.17	0.15 ± 0.12
FAD	n.d.	n.d.

Each value represents the mean ± S.D. ($n=3$). Concentration of cofactors was 0.25 mM. n.d.; not detected.

TABLE III. Apparent Michaelis Constant (K_m) and Maximal Velocity (V_{max}) of Befunolol Reduction

Tissue	K_m (mM)	V_{max} (nmol/min/mg protein)
Liver	1.14 ± 0.51	11.7 ± 4.4
Kidney	1.35 ± 0.23	18.9 ± 2.8

Each value represents the mean ± S.D. ($n=3$).

appears not to be an alcohol dehydrogenase, which would require NADH as a cofactor.

Optimal pH

The pH dependence of befunolol reduction by rabbit liver and kidney cytosols was examined. The pH optima of befunolol reduction in these two tissues were between 6.0 and 7.0 (data not shown).

Kinetics

Table III shows the apparent Michaelis constants K_m and V_{max} values for befunolol-reducing activity in the cytosols of rabbit liver and kidney. These values were calculated from Lineweaver-Burk plots. The liver and kidney gave rather similar K_m and V_{max} values, suggesting similarities between the befunolol-reducing enzymes in the two organs.

Effect of Inhibitors

The effects of various inhibitors on the metabolic reduction of befunolol in the cytosols of rabbit liver and kidney are summarized in Table IV. Pyrazole and barbiturates were used as potent inhibitors of alcohol dehydrogenase and aldehyde reductase, respectively. The plant

TABLE IV. Effect of Various Inhibitors on Befunolol-Reducing Activity

Inhibitor	Concn. (mM)	Relative activity (%)	
		Liver	Kidney
Pyrazole	10.0	92.1±8.5	93.6± 3.0
Barbital	1.0	86.3±2.1	97.6± 3.3
Phenobarbital	1.0	84.9±6.7	97.0± 4.9
Quercitrin	0.1	35.0±6.2	15.3± 0.3
Quercetin	0.1	56.0±2.3	49.5± 7.9
*p*CMB	0.1	44.3±6.1	65.6±13.6
DTNB	1.0	19.3±5.9	7.4± 2.0
EDTA	1.0	102.4±4.7	100.9± 8.6
SKF 525-A	1.0	97.3±4.6	95.6± 5.7

Each value represents the mean±S.D. ($n=3$).

TABLE V. Effect of Nonsteroidal Anti-inflammatory Drugs on Befunolol-Reducing Activity

Drug	Relative activity (%)	
	Liver	Kidney
Acetaminophen	61.9±10.0	60.2±3.2
Diclofenac sodium	16.0± 2.2	18.5±7.8
Flufenamic acid	21.8± 5.2	22.4±2.9
Ibuprofen	63.8± 6.6	63.9±2.7
Indomethacin	31.9± 1.0	27.6±0.4
Mefenamic acid	27.6± 1.7	27.9±0.6
Fenbufen	40.9± 4.6	44.6±3.9
Flurbiprofen	45.8± 4.9	42.0±2.3
Ketoprofen	39.5± 4.9	27.4±1.1
Protizinic acid	25.6± 0.8	21.0±0.6

Each value represents the mean±S.D. ($n=3$). Concentration of nonsteroidal anti-inflammatory drugs was 1.0 mM.

flavonoids quercitrin and quercetin were effective inhibitors of lens aldose reductase[7] and some carbonyl reductases.[8] Pyrazole and barbiturates had no effect on befunolol-reducing activity, whereas quercitrin and quercetin markedly inhibited befunolol reduction in the liver and kidney. A microsomal enzyme inhibitor, SKF 525-A, did not affect befunolol-reducing activity. Furthermore, although sulfhydryl reagents *p*-chloromercuribenzoic acid (*p*CMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) inhibited befunolol reduction in the liver and kidney, a metal-chelating reagent ethylenediaminetetraacetic acid (EDTA) did not.

Effect of Nonsteroidal Anti-inflammatory Drugs

The effects of various nonsteroidal anti-inflammatory drugs on the metabolic reduction of befunolol were examined by using cytosols of rabbit liver and kidney. As shown in Table V, indomethacin, an inhibitor of prostaglandin E_2 9-keto-reductase[9] or human brain carbonyl reductase,[8a] markedly inhibited befunolol reduction in the liver and kidney. Other nonsteroidal anti-inflammatory drugs also inhibited the reduction.

Discussion

On the basis of the data on subcellular localization, cofactor requirement, optimal pH

and kinetic characteristics, it was suggested that the metabolic reduction of befunolol is catalyzed by the same enzyme in the cytosols of rabbit liver and kidney. Since the befunolol-reducing enzyme preferentially utilized NADPH as a cofactor, it appeared not to be an alcohol dehydrogenase. To further elucidate the properties of the enzyme, the effects of specific inhibitors on the befunolol-reducing activities of rabbit liver and kidney cytosols were examined. Pyrazole, a well known inhibitor of alcohol dehydrogenase, had no effect on the activities. This supports the above assumption that alcohol dehydrogenase is not involved. Barbiturates, potent inhibitors of aldehyde reductase, also did not inhibit befunolol reduction, indicating that aldehyde reductase is not involved. On the other hand, quercitrin and quercetin, which provide an additional way to distinguish carbonyl reductase from alcohol dehydrogenase or aldehyde reductase, markedly inhibited befunolol reduction. In addition, the befunolol reduction was evidently inhibited by sulfhydryl reagents but not a metal-chelating reagent. These findings indicate that the befunolol-reducing enzyme can be classified as a carbonyl reductase, as described by Wermuth[8a] and Sawada *et al.*[8b]

Our previous papers have demonstrated that the heart contributes greatly to the metabolic reduction of an oral antidiabetic drug acetohexamide, and the acetohexamide-reducing enzyme in the heart is different from that in the liver or kidney.[10] However, in the case of befunolol reduction, the contribution of the heart was negligibly small.

Sawada *et al.* purified four reductases (F_1, F_2, F_3 and F_4) from rabbit liver cytosol by various chromatographic techniques.[8b] The reductases F_1 and F_3 were the enzymes responsible for the reduction of xenobiotic aldehydes and ketones. Furthermore, based on substrate specificity and apparent K_m values for steroids, the reductases F_1 and F_3 were identified as 3 (17) β-hydroxysteroid dehydrogenase and 3α-hydroxysteroid dehydrogenase, respectively. Tanaka *et al.*[11] have pointed out that the loxoprofen-reducing enzyme purified from rabbit liver cytosol is identical with 3α-hydroxysteroid dehydrogenase reported by Sawada *et al.*[8b]

Recently, it has been reported that 3α-hydroxysteroid dehydrogenase from rat liver cytosol is potently inhibited by many nonsteroidal anti-inflammatory drugs.[12] In this study, we found that a variety of nonsteroidal anti-inflammatory drugs inhibit the befunolol-reducing enzyme in rabbit liver and kidney cytosols. Thus, it is suggested that the befunolol-reducing enzyme probably corresponds to a 3α-hydroxysteroid dehydrogenase, although further investigations including purification of the enzyme should be conducted.

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