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AGE-DEPENDENCE IN CAPACITY-LIMITED UPTAKE KINETICS OF PROPRANOLOL BY ISOLATED RAT HEPATOCYTES*

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Abstract—Overall initial uptake rates of propranolol by isolated rat hepatocytes were analyzed to give both apparently linear and saturable processes. The rate constant for the linear transport process in 7- and 24-week old rats was approximately 0.01 ml·sec^{-1}·(10^6 cells)$^{-1}$. For the capacity-limited uptake process of propranolol, the kinetic parameters in 7-week-old rats were estimated as V_{max} = 0.609 μg·(10^6 cells)$^{-1}$·sec^{-1} and K_m = 8.17 μg/ml, whereas in 24-week-old rats, V_{max} = 0.348 μg·(10^6 cells)$^{-1}$·sec^{-1} and K_m = 9.40 μg/ml. The overall uptake rate, when the hepatocytes were incubated with 20 μg/ml of propranolol, was reduced with age of the rats between 5 or 7 and 24 weeks and declined gradually thereafter, A higher cell to medium concentration ratio for the initially, net transported component of propranolol in relatively young (5- to 11-week-old) rats suggested an age-dependent saturable uptake process. This was supported by the direct comparison of the saturable uptake rates between 7 and 24 weeks. Therefore, the saturable sinusoidal transport (i.e. saturable uptake by the hepatocytes) may be considered one of the most important mechanisms determining the age-dependence in the intrinsic hepatic clearance of this drug noted in our previous report.

Systemic clearance of propranolol has been reported to be significantly lower in aged people, both normal subjects and patients of 70–80 years than in young adults of about 30 years [1–3]. In rats, it has also been reported that both systemic and intrinsic hepatic clearances are reduced in relatively aged animals [4]. This age-dependence of the hepatic clearance of propranolol has been suggested to be directly related to age-dependent changes in liver blood flow [4]. Furthermore, both our previous studies *in vivo* [5] and *in vitro* [6] of propranolol in rats have demonstrated non-linear hepatic elimination kinetics. Rane *et al.* [7] have reported that under first-order conditions the activity of the rat hepatic drug-metabolizing enzymes (i.e. intrinsic hepatic clearance) is closely approximated by the ratio of the kinetic parameters, V_{max} and K_m, for several drugs *in vitro*, including propranolol. There have been no reports, however, of a factor that determines age-dependence of the intrinsic hepatic clearance of propranolol, except our previous report demonstrating a significant age dependence of the metabolic rate of this drug in perfused liver *in vitro* [6].

Drug metabolism in isolated hepatocytes has been thought to correlate better with *in vivo* drug metabolism than with metabolism in 9000 *g* supernatant fractions or microsomes [8]. Inasmuch as the first, and prerequisite, step for hepatic metabolism of propranolol, which is its transport into liver cells and thus translocation across the hepatocyte sinusoidal membrane, may limit the rate of its hepatic removal from the circulation (intrinsic hepatic clearance), it seems very important to study effects of age on uptake or transport kinetics and mechanisms of this drug by rat hepatocytes.

The present investigation, therefore, was designed to characterize and compare the initial uptake kinetics for propanolol by isolated hepatocytes at various initial substrate concentrations in different age groups (5- to 52-week-old) of rats.

MATERIALS AND METHODS

Materials. Propranolol hydrochloride (*dl*-racemate, Sumitomo Chemical Co., Osaka, Japan) was donated by ICI-Pharma Ltd. (Osaka, Japan). Other chemicals and reagents were purchased from the folllowing sources: ^3H_2O (sp. act. 1 mCi/ml) from the New England Nuclear Corp. (Boston, MA, U.S.A.); collagenase type I (from *Clostridium historiticum*, EC 3.4.24.3) from the Boehringer Co. (Mannheim, GmbH, F.R.G.); bovine serum albumin (Fraction V) from the Sigma Chemical Co. (St. Louis, MO, U.S.A.); silicone oil (d = 1.02) from the Shin-etsu Chemical Co. (Tokyo, Japan); and trypan blue from the Wako Pure Chemical Co. (Nagoya, Japan). All other chemicals used were of analytical grade.

Animals. All animals used in this study were male Wistar rats purchased from the Shizuoka Laboratory Animal Farm (Hamamatsu, Japan) and fasted overnight before the experiment as reported previously [4, 6]. The animal ages expressed in weeks after birth (body weight), were as follows: 5 (105–135 g); 7 (200–226 g); 11 (350–377 g); 15 (382–400 g); 24 (455–495 g); 36 (568–610 g); and 52 (596–695 g).

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Preparation and incubation of isolated hepatocytes. Isolated hepatocytes were prepared by a slight modification by Baur *et al.* [9] of the original procedure of Berry and Friend [10]. The detailed procedure for this collagenase (0.05%) perfusion method, including the compositions of the main buffer solutions, i.e. perfusion buffer (pH 7.30), wash buffer (pH 7.40) and incubation buffer (pH 7.40) which were oxygenated with 95% O_2–5% CO_2, has been reported previously [11–13].

A stock solution of propranolol was prepared in the incubation buffer to give final concentrations of 1–200 μg/ml. The effect of initial substrate concentrations on the uptake rate was examined in both 7- and 24-week-old rats, whereas the effect of liver aging on the uptake rate was tested using 20 μg/ml as the initial substrate concentration. All the incubation experiments were carried out at 37° under oxygenation with 95% O_2–5% CO_2; each mean data point was obtained from four rats. After 3 min of preincubation of the cells (6.0 to 8.5 mg as cellular protein corresponding to approximately 5.7×10^5 to 7.1×10^5 cells) in 2.8 ml, 0.2 ml of the stock drug solution was added at time zero. No cofactor was added to the incubation mixture.

To determine the overall velocity of initial uptake (V_o), periodical sampling and centrifugation of the incubated mixture were carried out in the same way as reported previously [13]. The amount of propranolol taken into the hepatocytes was quantified by placing the pellet layer in a tube containing 0.1 ml of 4 N NaOH; 0.05 ml of the supernatant layer of the incubated sample was also transferred into another tube containing the same constituent as above. The amount and concentration of propranolol in the pellet and supernatant layers, respectively, were determined by slightly modifying the method of Vervloet *et al.* [14] as described previously [15]. Based on the preliminary material-balance examinations, the constituent taken into the hepatocytes and remaining in the supernatant fraction even at the end of the incubation period (60 sec) was identified as essentially unchanged drug and not the metabolites. The net content of propranolol transported into the hepatocytes was evaluated after correcting for adherent fluid (incubation medium) that passed together with the cell pellets through the silicone oil phase upon centrifugation, in the same manner as described previously [11–13].

Cell viability tests. Cell viability before and after the incubation experiments was determined only by the trypan blue exclusion method [9]. Cell preparations in which more than 97% of the cells excluded the dye were used in the present experiments. Even after 1 min of incubation, the exclusion percentages were still almost at the same levels (ranging from 95 to 97%) as before the incubation.

Determination of cellular protein, cell numbers and aqueous cellular volumes. Cellular protein was determined by the method of Lowry *et al.* [16], with bovine serum albumin as a standard. Cellular protein concentration (X mg/ml) was then converted to the cell numbers in the same unit volume of the medium (Y cells/ml) according to the relationships, $Y = 0.950 \times 10^5 X$ to $Y = 0.835 \times 10^5 X$ for the hepatocytes from 5- to 52-week-old rats, respectively, obtained by the preliminary dilution of the cell suspensions.

Aqueous cellular volume of intracellular water in the pellets was determined by incubating cells with ^3H_2O in the same manner as reported previously [13]. This value ranged from 22.2 (±1.1) to 31.4 (±1.8) μl/10^6 cells for the hepatocytes from 5- to 52-week-old rats.

RESULTS

Time course of propranolol uptake by isolated hepatocytes. Figure 1 represents the uptake of propranolol per 10^6 cells with time at nine different

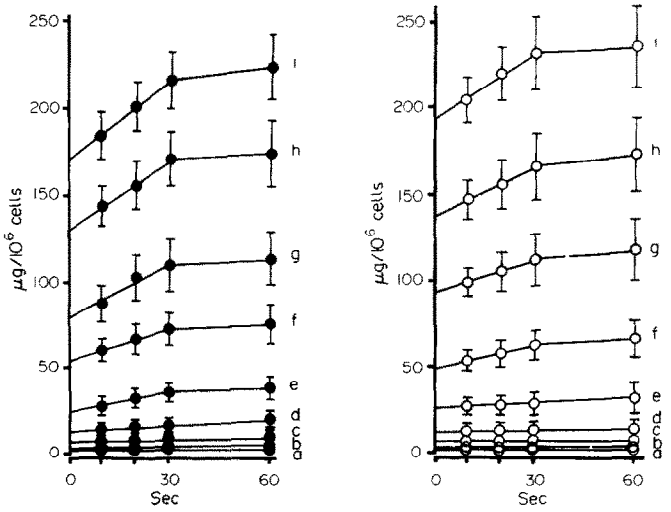

Fig. 1. Time courses of propranolol uptake by isolated rat hepatocytes prepared from 7 (●)- and 24 (○)-week old rats. Uptake is expressed as μg of total drug per 10^6 cells. Incubation was performed at pH 7.4 and 37° using 5.7×10^5 to 7.1×10^5 cells (approximately 6.0 to 8.5 mg as cellular protein) in 3.0 ml buffer. Initial drug concentrations were 1 (a), 2 (b), 5 (c), 10 (d), 20 (e), 50 (f), 100 (g), 150 (h) or 200 (i) μg/ml. Each point is the mean ± S.D. of four experiments (rats).

substrate concentrations ranging from 1 to 200 μg/ml in 7- and 24-week-old rats. Uptake appeared to be linear at all initial concentrations tested for the first 30 sec of the incubation. However, the uptake rates of this substrate at relatively high concentrations tended to be reduced thereafter. The overall initial uptake rate (V_0) was then estimated from the slope of each initial linear portion.

Effect of the initial propranolol concentration on V_0. When the overall initial uptake rate was plotted against the initial propranolol concentration, as shown in Fig. 2, curvilinear profiles were obtained for hepatocyte preparations from both 7- and 24-week-old rats. The overall uptake rate was always larger at 7 weeks than at 24 weeks. These uptake profiles against the substrate concentration were then analyzed in the same manner as described previously for morphine [12], nalorphine [12] or salicylamide [13], since the overall uptake process of propranolol was considered to be a combination of a saturable process and an apparently linear process. In both age groups, the uptake rate was found to increase almost linearly when the initial substrate concentrations were above 50 μg/ml. An almost identical rate constant [approximately 0.01 ml·(10^6 cells)$^{-1}$·sec^{-1}] was estimated for these linear transport processes in 7- and 24-week-old rat hepatocytes. The linear portion which intersected the origin was then subtracted from the overall uptake rate at each substrate concentration to yield the initial uptake rate for the saturable component, which is plotted as the curve shown in Fig. 3a. Single reciprocal linear transformations of these mean data yielded straight lines (Fig. 3b). Kinetic parameters were determined as V_{max} = 0.609 and 0.348 μg·(10^6 cells)$^{-1}$sec^{-1} and K_m = 8.17 and 9.40 μg/ml for 7- and 24-week-old rats respectively.

Effect of the initial propranolol concentration on its intracellular accumulation. The net uptake amount of propranolol at 30 sec in 7- and 24-week-old rats was converted to the intracellular concentration using the estimates for the corresponding cellular aqueous volume in the same manner as before [12, 13]. The extent of initial intracellular accumulation of propranolol, expressed as a cell to medium concentration ratio (C/M ratio), is presented in Fig. 4. This C/M ratio was inversely dependent on the substrate concentration, ranging from approximately 40 to 9 with increasing initial concentration in 7-week-old rats and from 30 to 7 in 24-week-old rats.

Effect of age on V_0. This experiment was carried out at an initial propranolol concentration of 20 μg/ml, where both linear and saturable transport processes were involved to almost the same extent in 7-week-old rats (Fig. 2). The overall initial uptake rate against the age of the rats is represented in Fig. 5. The uptake rate was relatively large in the younger rats (5–7 weeks) and then decreased remarkably with age until 24 weeks, followed by a gradual decline.

Effect of age on intracellular accumulation. The C/M ratio at 30 sec after incubation with 20 μg/ml

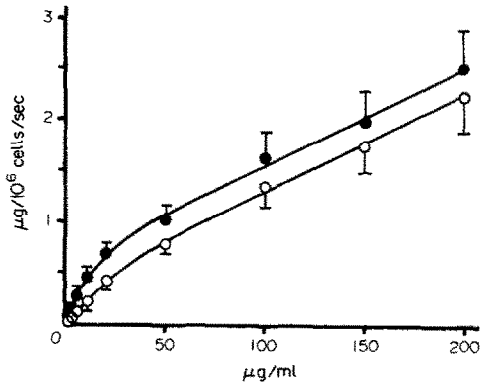

Fig. 2. Effect of initial substrate concentration on the overall initial uptake rate of propranolol by isolated rat hepatocytes prepared from 7 (●)- and 24 (○)-week-old rats. The overall uptake rate was estimated from the slope of the linear portion in Fig. 1 and expressed as μg·(10^6 cells)$^{-1}$·sec^{-1}. Each point is the mean ± S.D. of four experiments. The linear regression lines for 50–200 μg/ml propranolol are Y = 0.0098X + 0.610 and Y = 0.0102X + 0.351 for 7 (r = 0.990)- and 24 (r = 0.992)-week-old rats, respectively, where Y is the overall uptake rate and X the initial concentration.

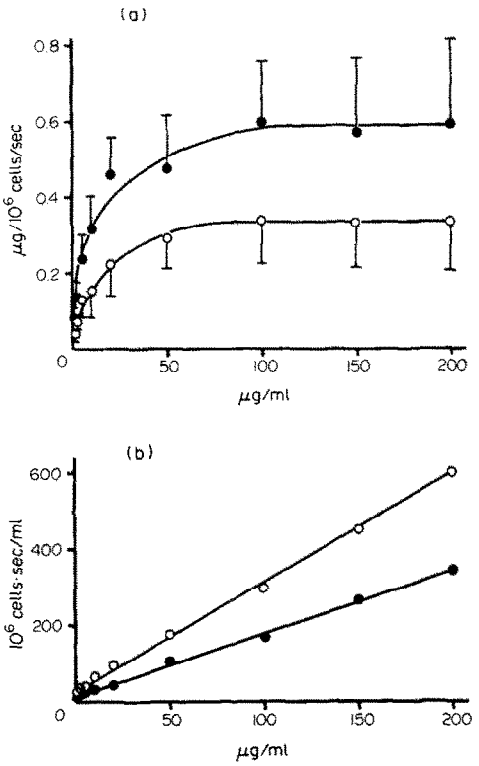

Fig. 3. (a) Saturable uptake rate by isolated hepatocytes from 7 (●)- and 24 (○)-week-old rats at various propranolol concentrations. This rate was estimated by subtracting each simple diffusion rate from each overall uptake rate. Each point is the mean ± S.D. of four experiments. (b) Single-reciprocal plots for the mean saturable uptake rate (ordinate) against initial propranolol concentration. Symbols are the same as those in (a). Linear regression analysis yielded the relationships expressed as Y = 1.64X + 13.4 and Y = 2.87X + 27.0 in 7 (r = 0.998)- and 24 (r = 0.996)-week-old rats, respectively, where Y is the initial substrate concentration divided by the saturable uptake rate and X is the initial concentration. Hence, the uptake kinetic parameters are estimated as follows: V_{max} = 0.609 and 0.348 μg·(10^6 cells)$^{-1}$·sec^{-1} and K_m = 8.17 and 9.40 μg/ml in 7- and 24-week-old rats respectively.

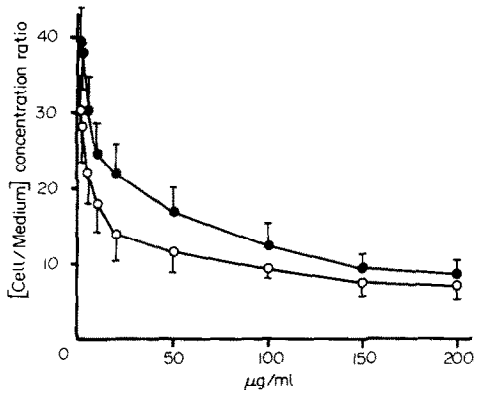

Fig. 4. Cell/medium concentration ratio of propranolol at various initial concentrations in 7 (●)- and 24 (○)-week-old rats. Cellular drug concentration for the net uptake was obtained by subtracting the non-specifically bound amount at t = 0, which was the intercept in Fig. 1, from the overall uptake amount at 30 sec and then dividing this net amount by the respective aqueous cellular volume per unit cells. Each point is the mean ± S.D. of four experiments.

of propranolol, estimated in the same manner as described above, was plotted against the age of the rats (Fig. 6). As expected from the above results with age-dependent change in the overall uptake rates, the C/M ratio was also reduced extensively with age, ranging from about 22 to 9 for the hepatocytes from 5- or 7- to 52-week-old-rats.

DISCUSSION

Although both significant age-dependence [4] and dose-dependence [5] of intrinsic hepatic clearances as well as of total body clearance of propranolol have been demonstrated in rats, the only published data supporting these observations are our previous *in vitro* findings that perfused liver isolated from relatively young rats eliminates this drug much more rapidly than liver from aged rats and that the clearance by the perfused liver is also highly dependent

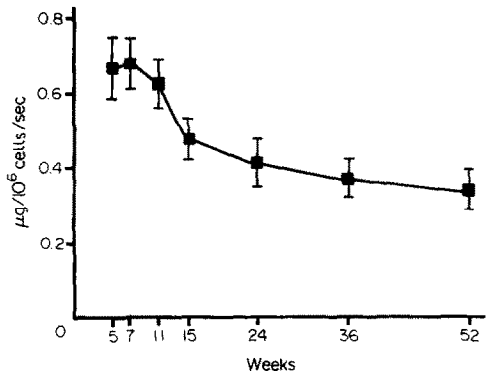

Fig. 5. Effect of age on the overall initial uptake rate of propranolol by isolated rat hepatocytes. Initial substrate concentration was 20 μg/ml. Incubation conditions were the same as described in Fig. 1. The overall initial uptake rate was calculated in the same manner as mentioned in Fig. 2. Each point is the mean ± S.D. of four experiments.

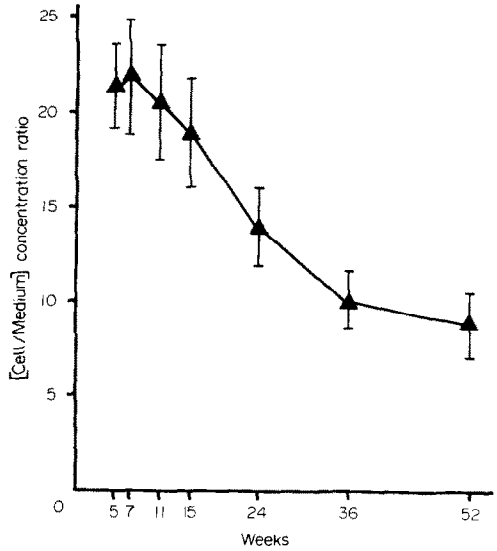

Fig. 6. Effect of age on the cell/medium concentration ratio of propranolol when rat hepatocytes were incubated with 20 μg/ml of the substrate. Cellular drug concentration for the net uptake was calculated in the same manner as described in Fig. 4. Each point is the mean ± S.D. of four experiments.

on the initial substrate level in the perfusate [6]. The total body clearance of propranolol has also been reported to be reduced in aged subjects or patients [1–3]. However, there have been no reports implicating a determining factor in the age-dependent intrinsic hepatic clearance of this drug.

Comparative studies using intact animal, perfused liver and liver slice models to describe hepatic uptake and biliary excretion of many compounds show that kinetic characterization of the sinusoidal uptake system apart from the metabolic and biliary excretory systems is not possible with these experimental models [17]. However, isolated hepatocytes have an advantage over other models for the study of hepatic sinusoidal uptake processes, because the amount of a given compound taken into the hepatocytes can be determined at relatively early and short, time intervals (usually within 1 min) before the biliary excretory, or metabolic process significantly interferes with the uptake process. It was evident from the preliminary experiments that metabolism of propranolol in hepatocytes from rats in any age group was not significant within the first 60 sec of the incubation.

In the present study, the initial uptake kinetics of propranolol by the hepatocytes was characterized in 7- and 24-week-old rats. An apparent plateau-like profile of the overall uptake amount by the hepatocytes during the last half of the incubation time (30–60 sec) when they were incubated with relatively high initial concentrations of this drug (Fig. 1) may be due to a time-dependent decrease in the capacity-limited (saturable) uptake. This time-dependence presumably was not caused by a periodical deterioration of cellular function, since cell viability was not lost after incubation. A similar time-dependence was observed in the initial uptake of salicylamide by rat hepatocytes [13].

Uptake of propranolol by isolated rat hepatocytes was driven by both capacity-limited (saturable) and apparently linear transport processes. The present evidence for the substrate concentration dependence in both initial uptake rate and C/M ratio of this drug by rat hepatocytes is considered to be qualitatively relevant to our previous *in vivo* [5] and *in vitro* [6] results for hepatic clearance. An uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (0.1 mM), and reduced incubation temperature (25°) were found to inhibit the initial uptake of propranolol (20 μg/ml) by the hepatocytes, each decreasing the V_0 of the substrate by 50% or more. Of the kinetic parameters estimated for the capacity-limited uptake process in 7-week-old rats, V_{max} [0.609 $\mu g \cdot (10^6 \text{ cells})^{-1} \cdot sec^{-1}$] seemed to be appreciably larger in comparison to those obtained for other drugs such as ouabain [11], morphine [12], nalorphine [12] and salicylamide [13] in 7- to 9-week-old rats, suggesting a relatively high uptake capacity for propranolol by hepatocytes (Fig. 3, a and b).

Both the V_{max} and the C/M ratio were larger in 7-week-old rats than in 24-week-old rats. Furthermore, the overall uptake rate of propranolol by the hepatocytes was relatively high in the young (5–7 weeks) rats. This age-dependence is suggested to be due principally to the possible age-dependent change in the capacity-limited uptake rate, since the linear transport rate was not affected by aging between 7 and 24 weeks. Similar but more distinct age-dependence was observed in the C/M ratio.

In conclusion, the results of the present *in vitro* propranolol uptake experiments with rat hepatocytes suggest that the capacity-limited uptake process may play an important role in the age-dependence of the intrinsic hepatic clearance of this drug.

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