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AGE-DEPENDENT DECREASE IN THE HEPATIC UPTAKE OF TAUROCHOLIC ACID RESEMBLES THAT FOR OUABAIN

A POSSIBLE ROLE OF SURFACE MEMBRANE PROTEIN MOBILITY

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Abstract—Isolated hepatocytes were prepared from Wistar-derived male rats of different ages (4, 12 and 27–29 months) by the collagenase perfusion method. The hepatic uptake rate of taurocholate (TC) for the saturable fraction was calculated by subtracting the non-saturable fraction from the total hepatic uptake. The V_{max} and the apparent affinity constant K_m were computed for the saturable fraction by means of non-linear regression. The V_{max} (nmol/mg protein/min, mean ± SE) for young rats (N = 6) was 2.15 ± 0.11, whereas in old rats (N = 4) the value was 50% lower (1.16 ± 0.11, $P < 0.005$). In contrast, K_m (μM) values were not significantly different between young (25.88 ± 1.90) and old (30.34 ± 4.96) rats. There was a significant inverse linear relationship ($r = 0.79$; $P < 0.01$) between the age of rats and the uptake velocity (nmol/mg/mg protein/min) at 1 μM of TC, suggesting a steady and almost linear decrease of TC uptake velocity with age. The rate of decrease per month (2.1%) was quite close to the value for ouabain uptake (2.8%) previously found by the authors. Furthermore, a marked linearity was observed between the average values for TC uptake rates for three age groups and corresponding lateral diffusion constants of hepatocyte plasma membrane proteins previously obtained by the authors using the fluorescence recovery after photobleaching method. The results support our previous proposal that protein mobility within the hepatocyte surface membrane may play at least a partial role in regulation of carrier-mediated hepatocyte uptake functions for various materials.

The biliary excretion of i.v. injected ouabain (a non-metabolizable neutral glycoside) decreases with age in an almost linear fashion in rats of both sexes [1–3], largely owing to a steady decrease in the hepatic uptake of ouabain [3]. The lateral mobility of proteins (lateral diffusion constant) in the hepatocyte surface membrane, as measured by the fluorescence recovery after photobleaching (FRAP) technique, also decreases with age in a linear fashion, in both rats [2, 4] and mice [5]. Furthermore, pretreatment with spironolactone (SP) was shown to increase both the biliary excretion of ouabain and the lateral diffusion constant of hepatocyte surface membrane proteins, in rats of three different ages [2].

Interestingly, SP pretreatment was reported to decrease (rather than increase) the lipid fluidity of hepatocyte surface membranes, as assessed by a fluorescence anisotropy method using diphenylhexatriene (DPH) [6, 7]. On the basis of these studies, the working hypothesis has been proposed that protein mobility in the hepatocyte surface membrane as suggested from the results of our FRAP study, is a significant regulatory mechanism for carrier-mediated membrane functions, such as hepatic uptake of materials [2]. If our hypothesis is correct, the linear decrease with age in the hepatic uptake observed for ouabain may also exist for other materials having transport mechanisms different from that for ouabain.

To test this hypothesis, in the present study we examined the hepatic uptake of taurocholic acid (TC) by isolated hepatocytes obtained from rats of different ages. TC was chosen because it is generally believed that the uptake mechanisms for the two substances are different. The receptors for TC and ouabain differ [8, 9], and TC uptake is mainly Na^+ dependent [10, 11], whereas ouabain uptake is Na^+ independent [12, 13].

MATERIALS AND METHODS

Tritiated water (0.25 mCi/g), [carbonyl-^{14}C]dextran (1.24 mCi/g, molecular weight 50,000–70,000), and [carbonyl-^{14}C]taurocholic acid (46.7 mCi/mmol) were purchased from New England Nuclear (Boston, MA). The possible contamination of the [^{14}C]dextran preparation by lower molecular weight materials which might have penetrated the hepatocytes was examined by means of ultrafiltration using Centricon membrane filters (Amicon, Danvers, MA) as previously reported [3]. It was judged that no correction for this factor was necessary. Sodium taurocholate (TC) was from the Sigma Chemical Co. (St Louis, MO). The chemical purity of this compound was more than 98%, as assessed by HPLC. As an incubation medium, the modified Waymouth MB752/1 medium (Gibco, Grand Island, NY) was added to 25 mM HEPES (Dojindo Laboratories, Kumamoto, Japan) as described previously [3]. The pH (7.4) and osmolality (300 mOsm/kg) of the medium were adjusted to physiological levels by adding 1 N NaOH solution and NaCl, respectively. All chemical agents were of analytical grade.

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Male Wistar rats of three different ages (young, 4 months; middle-aged, 12 months; old, 27–29 months) were used. These animals were bred and raised under specific pathogen-free (SPF) conditions at the aging farm of the institute. The husbandry conditions [14], the pathological condition [15] observed in the latter period of their lives, and their survival rates [15] have been reported elsewhere. Isolated hepatocytes were obtained from these rats by the collagenase perfusion method as described previously [3]. After washing, the hepatocytes were stored in suspension (about 2.0×10^6 cells/mL) at 4° in the incubation medium for about 2 hr before uptake study. TC uptake velocity determination was performed by the modified method as described by Iga and Klaassen [16].

The hepatocyte suspension (about 10 mL) was preincubated for 3 min at 37° under an atmosphere of 95% O_2 and 5% CO_2 with constant shaking (10 oscillations/min). One hundred microliters of incubation medium containing about 6–13 nCi of [^{14}C]TC with unlabeled TC of nine different concentrations (1–750 μM) was placed in a 0.5-mL centrifuge tube containing 50 μL of 3 M NaOH solution in the bottom of a tube, covered by a 100 μL silicone layer with a density of 1.015 which was prepared from silicone oil (d = 1.05, Aldrich Chemical Company, Milwaukee, WI) and decahydronaphthalane (d = 0.876; Wako Pure Chemical Industry, Osaka, Japan) as reported previously [3]. This silicone layer separated the TC fraction and NaOH layers before and during the incubation period. The centrifuge tube was preincubated for 3 min and 100 μL of hepatocyte suspension was added quickly to the TC solution. After incubation for 5, 10, 15, 20 or 25 sec, hepatocytes were separated from the incubation medium and sedimented to the bottom of the tube by centrifugal filtration and lysed in the NaOH solution. The cell fraction lysed in NaOH solution was added with Aquazol II (New England Nuclear), and the specific radioactivity was counted by a scintillation counter [3]. Uptake of TC by the hepatocytes was calculated from the specific radioactivity of cell fractions after appropriate correction for water adhering to the hepatocyte surface by using ^3H_2O and [^{14}C]dextran [3]. Using a cell preparation obtained from the liver of one animal, uptake rates for nine different concentrations were determined for each measurement.

Hepatocytes viability was estimated by the Trypan blue exclusion test. Preparations with below 90% viability were not used for the experiments. The concentration of cellular protein was determined using a portion of the cell suspension used for each incubation experiment by the method of Lowry *et al.* [17]. One milliliter of hepatocyte suspension was washed three times with a 0.9% NaCl solution. After centrifugation, the cell pellets were lysed by addition of 8 mL of H_2O and 2 mL of a 5 M NaOH solution and were used for protein concentration determination. Bovine serum albumin was used as a standard.

As is shown in Fig. 2, a non-linear relationship was obtained between the initial uptake velocity and bile salt concentrations. As the rate of uptake became linear with substrate concentrations above 100 μM for all studies, the overall uptake process was considered to be a combination of a saturable process and a linear (non-saturable) one mediated by a simple diffusion, as reported in previous studies [9–11, 13]. The rate of hepatic uptake of TC from the saturable fraction was obtained by dissociating a saturable fraction from a non-saturable fraction. To separate the relative contribution of each of these processes, an apparent diffusion constant (k) for the non-saturable fraction, was calculated from the slopes of the linear portion of each curve above 100 μM by applying the least-squares linear regression analysis. The hepatic uptake rate of the non-saturable fraction at each concentration of TC (i.e., k times concentration) was subtracted from the overall uptake rate at each concentration to yield the initial velocity of the saturable component. V_{max} and K_m were calculated from the relations between the initial uptake rate of the saturable fraction and each TC concentration in the medium by means of a non-linear regression as described by Wilkinson [18] with minor modifications for a Vax-11 computer. We then selected the best fit to the Michaelis–Menten equation.

Statistical analysis for kinetic parameters was performed by the method of Cleland [19]. The least-squares linear regression analysis was also applied to evaluate the relation between the uptake rate at a given concentration and rat age. For all studies, P values lower than 0.05 were judged to be significant.

RESULTS

Figure 1 shows examples of the sequential changes in the hepatic uptake of TC at several different concentrations as examined in hepatocyte preparations from a young rat. The uptake was linear against time up to 25 sec over a wide range of TC concentrations in both young and old rats. From the slope of the linear portion of the hepatic uptake, the rate of initial hepatic uptake was calculated.

Figure 2 shows an example of the relationship between the uptake rate of TC and its concentration in the medium. The relationship can be analysed as a sum of saturable and non-saturable components for TC uptake. After subtraction of the non-saturable fraction from the total uptake, the relation between the uptake rate and the concentration in the medium for the saturable fraction followed the Michaelis–Menten kinetics.

Figure 3 shows the saturation kinetics for TC uptake by isolated hepatocyte prepared from young and old rats, using TC uptake rates of the saturable component. The V_{max} values (nmol/mg protein/min, mean ± SE) calculated from these relations were 2.15 ± 0.11 (N = 6), 1.45 ± 0.32 (N = 4), and 1.16 ± 0.11 (N = 4) for young, middle-aged and old rats, respectively. The V_{max} value in old rats was significantly lower than that in young rats ($P < 0.005$). In contrast, K_m (μM) values were not significantly different among the different age groups ($P > 0.5$) (25.88 ± 1.90, 30.34 ± 1.45, 30.34 ± 4.96 for young, middle aged and old rats, respectively).

Figure 4 shows the relationship between the initial uptake velocity of TC by hepatocytes (Y) and the age of animals (X) at a TC concentration of 1 μM. A significant linear relationship ($Y = 77.11 - 1.597X$; $r = -0.788$; $P < 0.005$) was obtained. The rate of

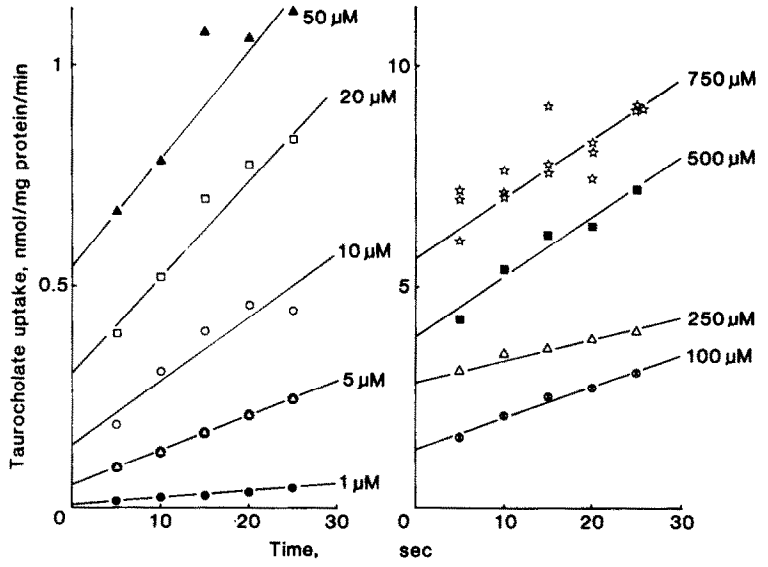

Fig. 1. Time course of TC uptake at various taurocholate concentrations by isolated hepatocytes prepared from a young Wistar rat.

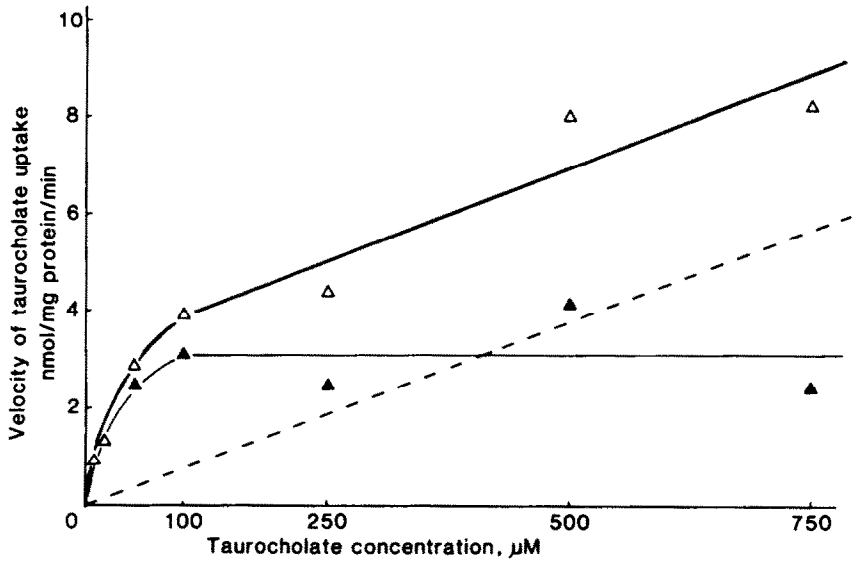

Fig. 2. An example of the relation between the initial TC uptake rates and TC concentrations by isolated hepatocytes prepared from a young male Wistar rat. (△) Total initial uptake rates measured directly from the cell suspension radioactivity. (▲) Calculated uptake rates for the saturable fraction. Values were obtained by subtracting the uptake rate of the non-saturable fraction from the total uptake rate. Uptake rates of the non-saturable fraction were obtained as the products of the diffusion constant (k), obtained from the linear regression of the original values above 100 μM in TC concentration and corresponding TC concentrations shown as broken line.

decrease in uptake velocity of 2.1% per month was obtained by the calculation.

Figure 5A shows the relationship between TC uptake velocity at 1 μM (Y) and the lateral diffusion constant of hepatocyte surface membrane proteins (X) as previously determined by the FRAP technique [2], in three corresponding age groups. A marked linearity between the two independently determined parameters was found ($Y = -107.66 + 67.52X$, $r = 0.984$).

Figure 5B shows the same relationship for ouabain uptake velocity previously found by the authors [2, 3] ($Y = -115.15 + 63.70X$, $r = 0.978$). Again, a marked linearity can be observed.

DISCUSSION

The results of the present study have shown that the initial uptake rate of TC for the saturable fraction by isolated rat hepatocytes decreases in an almost linear fashion with age, as previously shown for ouabain uptake [3]. The rate of decrease per month for the hepatic uptake rate of TC (2.1%) was surprisingly close to the value for ouabain (2.8%) previously found in our laboratory [3]. Furthermore,

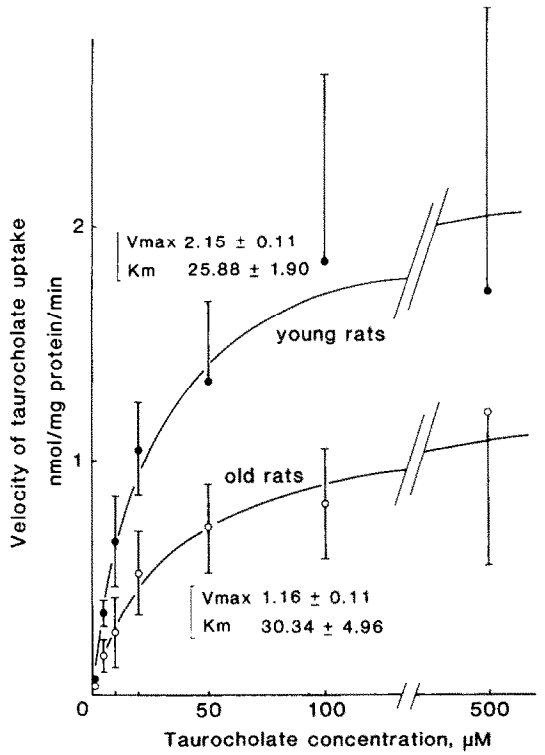

Fig. 3. Kinetic studies of initial hepatic uptake rates of TC in young (4 months, ●, N = 6) and old (27–29 months, ○, N = 4) rat groups for the saturable fraction of TC uptake. Vertical bar indicates SD. Kinetic parameters were calculated by a non-linear regression analysis. Values are presented as mean ± SE. N indicates the number of rats in each age group.

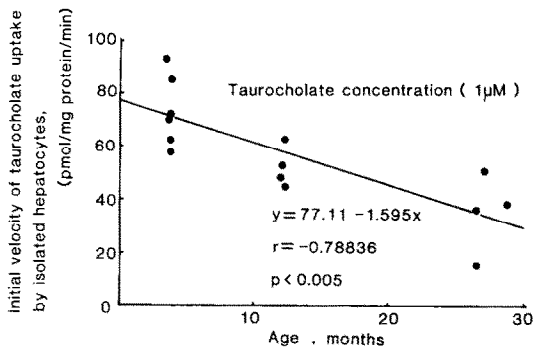

Fig. 4. The relationship between the initial uptake velocity of TC by hepatocytes (Y) and the age of animals (X) at a TC concentration in the medium of 1 μM.

the 50% decrease in V_{max} with unaltered K_m value in old rats compared with values in young rats found in the present experiment for TC also agrees with our previous study on ouabain uptake kinetics [3].

Generally speaking, the V_{max} of a carrier-mediated transport system is considered to be the function of the number of carrier unit and the diffusion constant of the carrier-ligand complex [20]. The decrease in the V_{max} value with unaltered K_m is therefore interpreted as owing to a decrease in either the carrier unit number or the diffusion constant (or to a combination of both). If the transport system requires an expenditure of energy, the decrease in energy may also affect the V_{max}.

If the decline in the hepatic uptake rate of TC is related to alteration of Na^+,K^+-ATPase activity during aging, the decline in the uptake rate for ouabain must therefore be explained in terms of a mechanism totally different from that of TC, because ouabain uptake is Na^+ independent [12, 13]. If this is the case, the agreement of the degree of V_{max} decrease with age for the two substances may be only a coincidence. Alternatively, the V_{max} decrease in TC uptake may be due to an age-dependent decrease in the carrier unit number or the mobility of the carrier–ligand complex [20]. However, the carrier systems postulated for these materials in previous reports remain a matter of controversy. Until recently, it had been relatively well established that the carrier for bile salts (anions) uptake is different from that for ouabain, a neutral compound [8, 9, 21]. However, a recent study by Petzinger *et al.* [22] has suggested that these two different compounds may share a common transport protein for their hepatic uptake processes. If indeed, the carrier proteins for TC and ouabain are the same, as maintained by Petzinger *et al.* [22], the comparable decrease in V_{max} values for these two substances during aging might be most easily explained on the basis of the decrease in their common carrier unit numbers. At present, however, it is not clear whether the carrier proteins for TC and ouabain are really the same. It appears rather unlikely that these two materials completely share the same carrier, because the developmental changes in the hepatic uptakes of these materials are totally different [23, 24]. If they are different, therefore, the V_{max} decrease should be explained as the result of the independent decrease in numbers of the respective (and different) carrier units for the two materials. Again, the agreement of the magnitude of their decreases should be a coincidental phenomenon.

A third possibility, that the decrease in the diffusion constant of the carrier–ligand complex during aging is the cause, requires special consideration, because we have previously shown that the lateral mobility of hepatocyte surface membrane proteins as assessed by FRAP technique, decreases in a linear fashion with rat age [2, 4]. Although membrane proteins previously studied by our FRAP technique are a complex of proteins bound to oxidized riboflavin [25, 26], and are not specific proteins directly mediating the transports of TC and ouabain, there is some theoretical and experimental basis that the mobilities of cell surface membrane proteins are generally more restricted as the age advances [4, 27–29]. If, indeed, the membrane protein movement is progressively restricted during the aging process by the decrease in passive permeability of the membrane for potassium [4, 27, 28], cross-linking of membrane proteins [28] and or changes in lipid composition, it is not unreasonable to assume that the mobility of the membrane proteins involved with the transports of materials are also affected during aging as suggested by our previous FRAP studies [2, 4, 5]. Assuming that the mobilities of both carrier proteins are similarly altered during aging, as suggested by our previous studies [4], and that such an alteration regulates

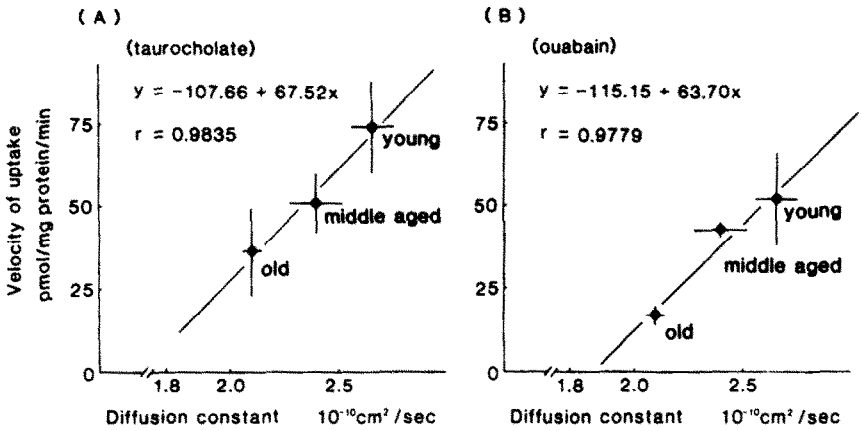

Fig. 5. The relationship between TC uptake velocity at 1 μM and the diffusion constant of hepatocyte surface membrane proteins, as previously determined by our FRAP method [2] in three different age groups (A) and a similar relationship for ouabain uptake rate (B). The latter figure comprises the results of previous studies of ouabain uptake [3] and FRAP study [2].

the hepatic uptake of materials, our results on both TC and ouabain uptake in aging rat livers can be more plausibly explained as owing to a common mechanism.

Surprisingly linear and similar relations found between the uptakes for these materials and the lateral diffusion constant of hepatocyte surface membrane proteins, as assessed by our FRAP system (Fig. 5A, B), are consistent with our working hypothesis that the changes in membrane protein mobility that occur during aging may be a significant factor for regulating the hepatic uptake rates for many different materials that are mediated by membrane-located protein carriers.

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