Korean J. Toxicol.
Vol. 7, No. 2, pp. 173-181, 1991

A STUDY ON MEMBRANE TRANSPORT OF BRAZILIN USING PRIMARY CULTURED RAT HEPATOCYTES

Chang-Kiu Moon, Kwang Sik Park, Jin Ho Chung, Seong Gon Kim
Myung-Kyu Chung and Chang-Hyun Moon*

College of Pharmacy, Seoul National University, Seoul 151-742
**College of Medicine, Ajou University, Soowon 440-649, Korea*
(Received November 20, 1991)
(Accepted 1991)

ABSTRACT: *Membrane transport of brazilin was investigated in primary cultured rat hepatocytes. Brazilin was transported into hepatocytes very slowly and reached plateau at about 60 minutes. Saturation of transport process was not observed and the transport was not affected by ouabain, metabolic inhibitors, and its structural analog. The amount of brazilin transported into hepatocytes was decreased when the environmental temperature was decreased. These results suggest that brazilin might be transported into hepatocytes by simple diffusional process.*
Key words: *brazilin, membrane transport, hepatocytes culture*

INTRODUCTION

For the pharmacological and toxicological effects, drugs or chemicals have to bind to the surface of the target cell membrane and/or have to be transported into the intracellular compartment (Bloom, *et al.*, 1982).

Membrane transport is usually mediated by one of the three different mechanisms such as simple diffusion, active transport and facilitated diffusion (Alberts, *et al.*, 1989): In simple diffusion, movement occurs with an electrochemical gradient without expenditure of energy. On the other hand, active transport consists of movement against an electrochemical gradient at the expense of cellular energy. This type of "uphill" transport is coupled directly to the hydrolysis of ATP and is represented by the sodium pump (Na^+-K^+ ATPase) mechanisms. Facilitated diffusion of "downhill" active transport is also a primary process. It is not energy dependent. Solute movement is facilitated by a carrier, possibly through permeability modification.

Investigation of the hepatic membrane transport of xenobiotics is very important, because the liver is of major importance in mammalian metabolism of xenobiotics and the first organ presented with absorbed xenobiotics in gastrointestinal tract. Hepatic membrane transport might be influenced by plasma protein binding, the

rate of metabolism of the xenobiotics, and in some cases by the extent of bile flow. In this study, hepatic membrane transport of brazilin was investigated in primary cultured rat hepatocytes. *In vitro* study using hepatocyte culture technique has some advantages over the *in vivo* set up; no extrahepatic distribution is occurring, no influence of plasma and bile flow is present, and calculation of kinetic constant is facilitated.

MATERIALS AND METHODS

Materials

Adenosine triphosphate (ATP), carbonylcyanide-*m*-chlorophenyl hydrazone (CCP), fetal bovine serum (FBS), Hank's balanced salts solution (HBSS) and other culture-tested reagents were purchased from Sigma Chemical Co., and tritium labelled brazilin was supplied from New England Nuclear.

Isolation and Culture of Primary Rat Hepatocytes

Male SD rats weighing about 200g were supplied from the Experimental Animal Breeding Center of Seoul National University and were given free access to a standard pellet diet and water. Isolated hepatocytes were prepared by a collagenase perfusion technique as described by Berry and Friend and Seglen with slight modification. The rat was anesthetized with sodium pentobarbital, the abdomen was opened, and the portal vein was cannulated. The liver was perfused with Ca^{2+}, Mg^{2+} free-HBSS for 5 minutes. During the perfusion, the chest was opened and the thoracic portion of the inferior vena cava was cannulated via the right atrium. The inferior vena cava was ligated just above the level of the renal veins. This diverted the outflow of liver perfusate to the inferior vena cava cannula above liver, then the perfusion solution was returned to the perfusion bottle for recirculation through the liver of the animal. The perfusion buffer was maintained at 37℃ and gassed continuously with 95% O_2 and 5% CO_2 throughout the experiments. After circulation of the perfusion buffer was established, collagenase (Type IV, 0.05% w/w) was added to the recirculating perfusion buffer and perfusion was continued until the liver had been digested. Following digestion, the liver was removed, transferred to perfusion buffer and separated into pieces by shaking slowly. The resulting cell suspension was filtered through two layers of nylon mesh and centrifuged at 50 g for 4 minutes. The pellet was washed two or three times by centrifugation and the final sediments was resuspended in final culture medium. Viability of each hepatocytes preparation was determined by trypan blue exclusion test and only preparations where more than 90% of cells excluded the dye were used. The cell suspension was diluted to 1.0×10^6 cells/m*l* in the Waymouth medium containing FBS (10%) and 3 m*l* of that suspension was pipetted into a collagen coated dish. For cell attachment, hepatocytes were incubated for 4 hours at 37℃ in a CO_2 incubator (5% CO_2, 95% air), and then the medium was replaced with serum free medium.

Determination of Transported Brazilin

Hepatocytes which had been in culture for 48 hours in Waymouth (serum free) were washed rapidly with phosphate-buffered saline at 37℃. Excess fluid was blotted and 3.0 m*l* of phenol red free HBSS prewarmed to 37℃ was added. Tritium labelled brazilin (1 μCi/m*l*) with appropriate amount of cold brazilin and other chemicals were added according to the aim of experiments. At the end of incubation, hepatocytes were washed rapidly three times with PBS at 4℃, excess fluid was blotted and cells were lysed with 1 m*l* of 1% aqueous solution of sodium lauryl sulfate. Lysed hepatocytes were dispersed by using micropipette. 100 μ*l* of the dispersed solution was taken for the determination of protein and 500 μ*l* of the solution was placed into Bray's solution for the measurement of radioactivity of transported brazilin. Values were expressed as brazilin uptake (nmol of pmol/mg protein of hepatocytes) (Hardison and Weiner 1980; Kozik and McCormick, 1987; Eaton and Klassen, 1978).

RESULTS AND DISCUSSION

Time Course of Brazilin Transport in Cultured Rat Hepatocytes

Time course of brazilin uptake is shown in Fig. 1. Brazilin was transported into hepatocytes very slowly and reached plateau at about 60 minutes. In order to determine the initial velocity of brazilin transport, linearity of brazilin uptake was investegated. As seen in Fig. 2 and 3 the process of uptake was found to be linear within the 5 minutes. The rates of brazilin uptake at various concentrations ranging from 10 to 90 μM were measured. Slope of each line represents the initial velocity uptake (Vo) for each concentration of brazilin. The positive y-intercepts at higher

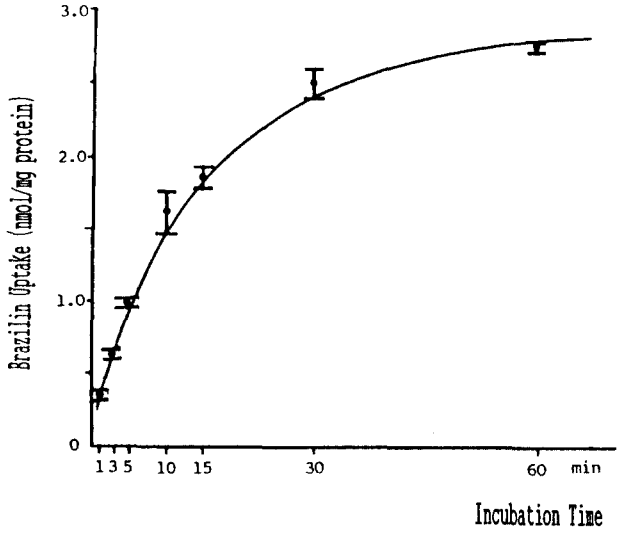

Fig. 1. Time course of brazilin uptake into hepatocytes. Cells were incubated in HBSS (phenol red-free) at 37℃ in the presence of brazilin (3 μCi/10 μM/3 m*l*).
Experimental data are mean values from at least three cell culture plates.

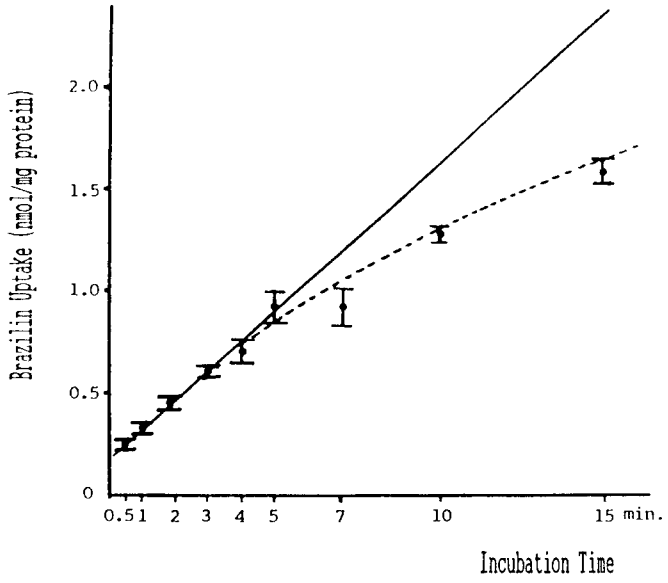

Fig. 2. Linearity of brazilin uptake. The process of uptake was linear within the 5 min range. Experimental data are mean values from at least three cell culture plates.

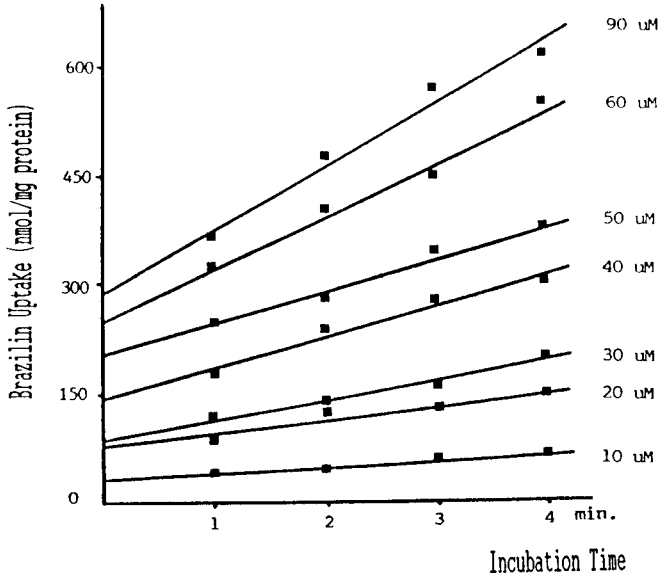

Fig. 3. Initial uptake experiment at different brazilin concentrations. Slopes represent initial velocity of uptake for each concentration.

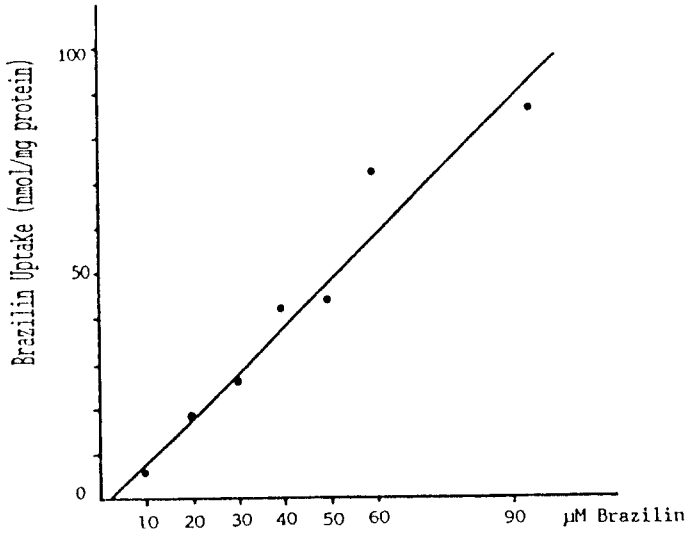

Fig. 4. Plot of initial velocity of uptake (Vo) vs. brazilin concentration(So).

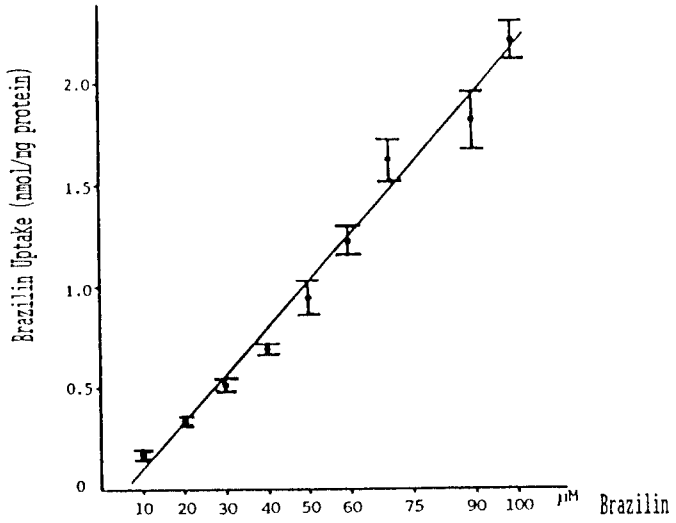

Fig. 5. Accumultion of brazilin at different concentrations. Cells were incubated with brazilin at its various for 60 minutes. Experimental data are mean values from at least three cell culture plates.

concentrations probably represent either undercorrection for the adherent fluid or nonspecific binding to the cell surface (Eaton and Klassen, 1978). In the plot of initial velocity of brazilin uptake (Vo) vs. its concentrations in the medium (So), any hyperbolic curve was not observed and all of the curves showed linearity. Therefore it was considered that there might be no saturation in the transport process of brazilin and total accumulation of brazilin in hepatocytes was increased

linearly proportional to the increase of its concentration as shown in Fig. 4 and 5. Based on these kinetic data, it is considered that brazilin might be transported by simple diffusional process.

Mechanism Studies

The uptake process was affected by temperature as seen in Fig. 6. It is generally known to us that the influence of temperature on the uptake process is caused not by the decrease of energy generation but by the increase of membrane rigidity and the decrease of cell volume. Generally, simple diffusion has a low temperature coefficient and active transport system is extremely sensitive to alterations in environmental temperature.

Na^+-K^- ATPase, which plays a major role in active transport, was inhibited by ouabain, but any significant effect of this enzyme on the uptake process of brazilin was not found as seen in Fig. 7. To examine whether brazilin transport is dependent on metabolic energy, brazilin was added to the cells preincubated with three different metabolic inhibitors such as antimycin A which blocks electron transport in the span from cytochrome b to c, rotenone which blocks the electron transport near the initial step, and CCP which is an uncoupler influencing the brazilin transport pattern. But Fig. 8 shows that any serious effect on brazilin transport pattern was not observed as generally shown in active transport pattern. This result indicates that some energy was required for the membrane transport of brazilin. If same protein carrier might play a major role in the brazilin transport, there would be a possibility that is analogue might use the same carrier, too. Hematoxylin was used as a brazilin analogue, and it was found that hematoxylin had no effect on the transport of brazilin (100 μM) at the concentration range from 25 μM to 500 μM. (Fig. 9).

Once brazilin was transported into cells, metabolism would be occurred by

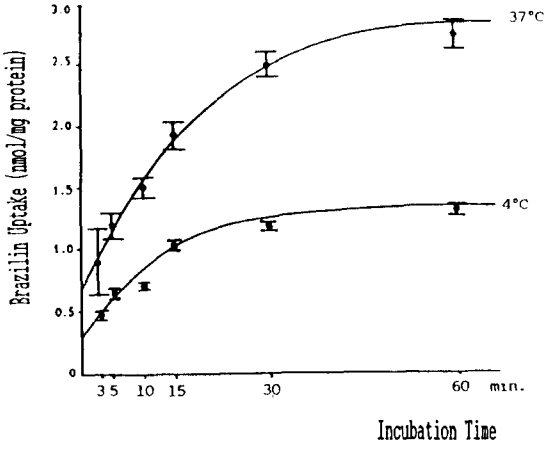

Fig. 6. Effect of temperature on brazilin uptake. Cells were incubated with brazilin (100 μM) at 37℃ and 4℃. Experimental data are mean values from at least three cell culture plates.

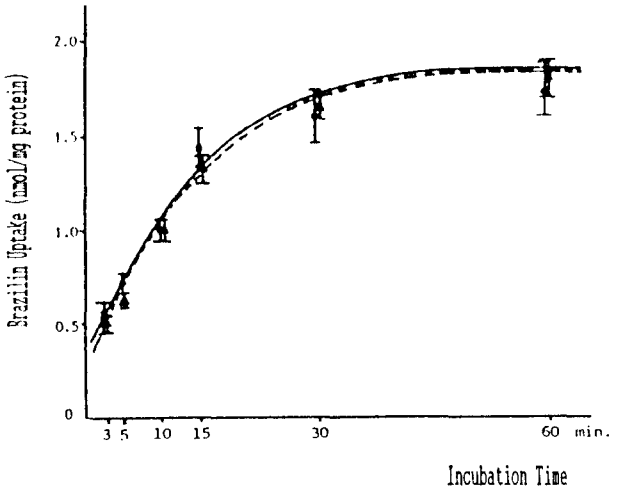

Fig. 7. Effect of ouabain on brazilin uptake. Ouabain (1 mM) was preincubated for 15 min before the addition of brazilin. —— normal ---- ouabain. Experimental data are mean values from at least three cell culture plates.

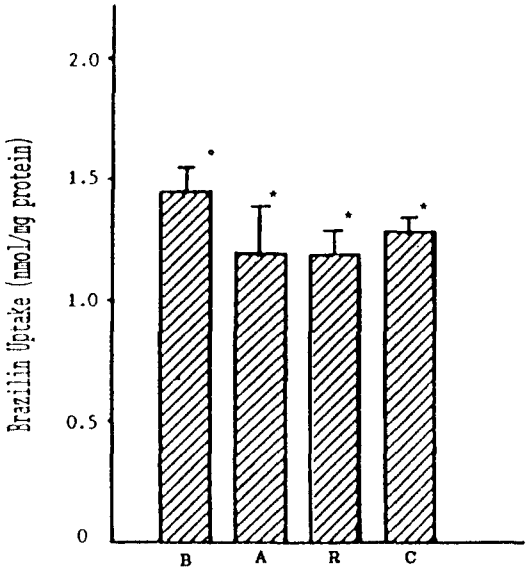

Fig. 8. Effect of metabolic inhibitors on brazilin uptake. Cells were preincubated with metabolic inhibitors for 5 min before the addition of brazilin and were further incubated with brazilin for 10 minutes. Brazilin concentration was 100 μM, and inhibitor concentrations were; antimycin A, 5 μg/l; rotenone, 10 μM; CCP, 2 μM.

B; Brazilin, A; Brazilin + Antimycin A, R; Brazilin + Rotenone, C; Brazilin + CCP. Comparisons of the groups were made by a one-way analysis of variance followed by Duncan's test ($p<0.05$). Groups with different symbol are significantly different by one-way ANOVA/Duncan.

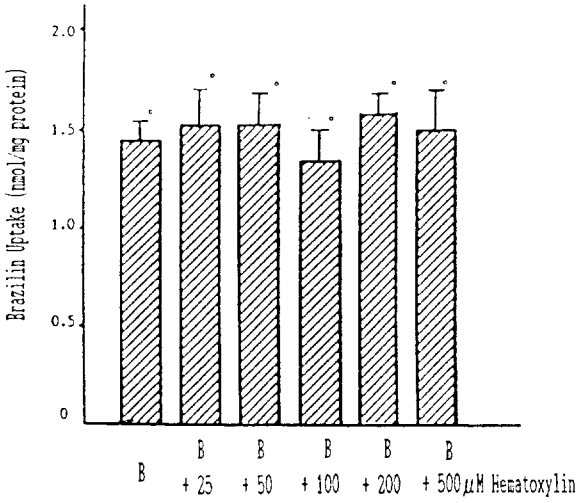

Fig. 9. Effect of an analogue of brazilin, hematoxylin, on brazilin uptake. Brazilin (100 μM) was cotransported with hematoxylin (25, 50, 100, 200, 500 μM) for 10 minutes. B; Brazilin. Comparisons of the groups were made by a one-way analysis of variance followed by Duncan's test ($p<0.05$). Groups with different symbol are significantly different by one-way ANOVA/Duncan.

certain metabolizing enzyme systems. Then transport of brazilin may be influenced by its intracellular metabolites. Therefore it is very important in transport study to confirm the presence of metabolites (Kozik and McCormick, 1987; Tipping and Kelterer, 1981). The intracellular metabolites of brazilin were extracted with ethanol and were developed onto TLC plate. The presence of three metabolites and parent compound was confirmed by measuring the radioactivity (data not shown). Structure identification of metabolites is now under the experiment.

Although the possibility, that biotransformation of brazilin in the hepatocytes might affect the transport, was not excluded, it is considered that brazilin might be transported by simple diffusional pathway.

ACKNOWLEDGEMENT

This work was partially supported by the Grant from RCNDD, KOSEF.

REFERENCES

Berry, M.N. and Friend, D.S. (1969): High yield preparation of isolated rat liver parenchymal cells; A biochemical and fine structural study, *J. Cell. Biol.*, **43**, 505.

Bloom, A., Scaf, A.H.J. and Meijer, D.K.F. (1982): Hepatic drug transport in the rat, *Biochem. Pharmacol.*, **30**, 1553.

Bruce Alberts, Dennin Bray, Julian Lewis, Martin Raff, Keith Roberts, and James

D. Watson (1989): Membrane transport of small molecules in *Molecular Biology of the Cell* sec. ed., Garland Publishing, Inc., NY and London.

Eaton, D.L. and Klassen, C.D. (1978): Carrier-mediated transport of ouabain in isolated hepatocytes, *J. Pharm. Exp. Therapeutics*, **295**(2), 480.

Hardison, W.G.M. and Weiner, R. (1980): Taurine transport by rat hepatocytes in primary culture, *Biochemica et Biophysica Acta*, **598**, 145.

Kozik, A. and McCormick, D.B. (1987): Mechanism of pyridine uptake by isolated rat liver cells, *Arch. Biochem. Biophys.*, **229**, 187.

Seglen, P.O. (1972): Preparation of rat liver cells, *Exp. Cell Res.*, **74**, 450.

Tipping, E. and Kelterer, B. (1981): The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes, *Biochem. J.*, **195**, 441.