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Binding cells of ^{125}I-iodoamphetamine in rat liver

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We recently reported that transrectal or intestinal portal scintigraphy with ^{123}I-iodoamphetamine (IMP) could be a useful method for the non-invasive and quantitative evaluation of the portosystemic shunt in portal hypertension, but what cells in the liver trap IMP has not been clarified. This study was aimed at elucidating whether IMP was extracted by parenchymal cells, sinusoidal endothelial cells, Kupffer cells or fat storing cells. Each type of liver cell was isolated from rats and cultured. The cells were incubated with ^{125}I-IMP and the radioactivity of the lysate was determined. Nonspecific binding was assessed in the presence of an excess of unlabeled IMP, and specific binding was determined by subtracting the nonspecific from total binding. Specific binding observed in parenchymal cells, endothelial cells and Kupffer cells was 70.2 ± 0.4, 4.2 ± 1.4 and 2.3 ± 0.8 pmol/well, respectively, but no specific binding was observed in fat storing cells. The binding in parenchymal cells was much higher than that in endothelial cells or Kupffer cells ($p < 0.005$). In addition, the binding to parenchymal cells reached equilibrium within 20 min and was not saturable over the concentration range tested (0.5–10 μM). These findings indicate that IMP is mostly extracted by parenchymal cells in the liver.

Key words: ^{125}I-iodoamphetamine, ^{123}I-iodoamphetamine, binding assay, liver, hepatocyte

INTRODUCTION

^{123}I-IODOAMPHETAMINE (IMP) is a clinically useful radioactive tracer in assessing brain[1,2] or lung perfusion,[3–5] because intravenously administered IMP is taken up by these tissues and retained. IMP introduced into the intestinal tract is absorbed and carried to the liver via portal circulation. It is nearly then completely extracted by the liver on the first pass,[6] but if portosystemic shunting is developed, for example in patients with portal hypertension, part of the IMP escapes extraction by the liver and is trapped by the lungs. Portosystemic shunt could therefore be evaluated non-invasively and quantitatively by transintestinal portal scintigraphy with IMP. We recently reported that the portosystemic shunt index, calculated by dividing the counts obtained in the lungs by the counts obtained in the liver and lungs, is clinically useful and reliable in evaluating the degree of portosystemic shunting.[7] As the mechanism of hepatic extraction of IMP is not clear, it is important and interesting to know what cells in the liver trap IMP. In the lungs, IMP binds to endothelial cells.[8] On the other hand, what cells in the liver trap IMP has not been clarified. This study was therefore designed to elucidate whether IMP was extracted by parenchymal cells or nonparenchymal cells (sinusoidal endothelial cells, Kupffer cells and fat storing cells).

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MATERIALS AND METHODS

Cell culture

Male Sprague-Dawley rats (Charles River Japan, Inc., Osaka, Japan; 450–550 g) were kept under specific pathogen free conditions and allowed free access to laboratory chow and tap water. The liver was isolated under

pentobarbital anesthesia (60 mg/kg of body weight, i.p.) and perfused in a non-recirculating system with Krebs-Ringer-HEPES buffer (115 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 25 mM HEPES, 1 mM $CaCl_2$, pH 7.4, 37°C) containing 0.02% collagenase (Wako, Osaka, Japan) for 14 min, following 10 min of perfusion with calcium-free Krebs-Ringer-HEPES buffer containing 0.5 mM EGTA (pH 7.4, 37°C, saturated with 95% O_2: 5% CO_2).[9] Liver cells were dispersed by gently shaking in Hanks balanced salt solution (HBSS; pH 7.4, 37°C) containing 0.1% bovine serum albumin (BSA). The cell suspension was filtered through a nylon mesh and centrifuged at 50 × *g* for 2 min. The supernatant was centrifuged at 200 × *g* for 3 min and nonparenchymal cell rich supernatant was collected for further isolation of non-parenchymal cells. Parenchymal cells were purified from the pellet by low speed centrifugation, and resuspended in culture medium DME/F-12 (GIBCO Laboratories Life Technologies Inc., Grand Island, NY) containing 15% fetal calf serum (Boehringer Mannheim Biochemicals, Indianapolis, IN), 15 mM HEPES, 100 U/m*l* penicillin G, 100 mg/m*l* streptomycin sulfate, 20 nM dexamethasone and 4 g/m*l* insulin and seeded on 12-well culture plates coated with collagen type I (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at an initial density of 5×10^5 m*l*/well. Parenchymal cells were cultured at 37°C in 5% CO_2/air. Non-adherent cells were removed 4 hr later by replacement with fresh culture medium. Sinusoidal endothelial cells, Kupffer cells and fat-storing cells (Ito cells) were separated by centrifugal elutriation.[10] The nonparenchymal cell rich fraction was introduced into a separation chamber of a J-21-B elutriator rotor in a J-21 Beckman centrifuge (Beckman Instruments, Inc., Palo Alto, CA) at a flow rate of 15 m*l*/min (4°C). The rotor speed was 2,500 rpm (750 × *g*). Sinusoidal endothelial cells and Kupffer cells were elutriated at 22 m*l*/min and 40 m*l*/min, respectively. The fraction elutriated at 15 m*l*/min was reintroduced into the elutriator (4°C, 3,250 rpm, 10 m*l*/min) and fat-storing cells were separated at 18 m*l*/min. The non-parenchymal cells were suspended in DME/F-12 supplemented with 15% fetal calf serum, 15 mM HEPES, 100 U/m*l* penicillin G and 100 mg/m*l* streptomycin sulfate. Endothelial cells were seeded on 12-well culture plates (Becton Dickinson & Co., Lincoln Park, NJ) coated with fibronectin (Sigma Chemical Co., St. Louis, MO) at an initial density of 2×10^6 m*l*/well and cultured at 37°C in 5% CO_2/air. Non-adherent cells were removed 3 hr later by replacement with fresh culture medium. Kupffer cells were seeded on 12-well culture plates (Nunc, Inc., Naperville, IL) at an initial density of 1.5×10^6 m*l*/well and cultured at 37°C in 5% CO_2/air. Non-adherent cells were removed 1 hr later by replacement with fresh culture medium. Fat-storing cells were seeded on 12-well culture plates coated with collagen type I at an initial density of 2×10^6 m*l*/well and cultured at 37°C in 5% CO_2/air. Non-adherent cells were removed 20 hr later by replacement with fresh culture medium. To maintain each cell culture, the culture medium was changed daily. To assess the purity of each cell culture, each type of liver cell was distinguished by light microscopic morphology.[11] Sinusoidal endothelial cells and Kupffer cells were identified by uptake of acetyl-LDL[12] and phagocytosis of latex beads with a diameter of 1 μm,[13] respectively. Fat-storing cells were judged by the autofluorescence of vitamin A stored in the cells.[11]

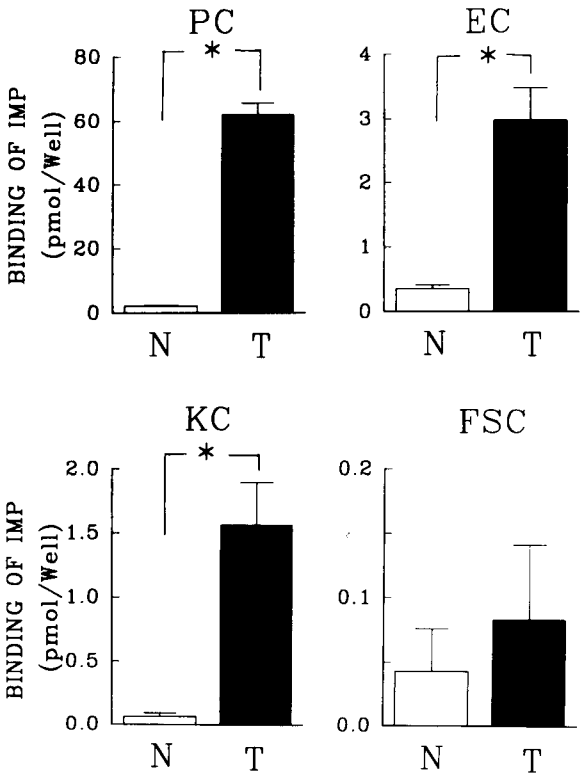

Fig. 1 Binding of ^{125}I-IMP to each cultured liver cell. Parenchymal cells (PC), sinusoidal endothelial cells (EC), Kupffer cells (KC) and fat-storing cells (FSC) were isolated and cultured on 12-well plates for 2 days as described in Methods. Each cell was incubated for 20 min at 37°C with 1 M ^{125}I-IMP in the presence (N, nonspecific binding) or the absence (T, total binding) of excess unlabeled IMP (1 mM). The data are from a typical experiment repeated three times and are expressed as the mean ± S.E.M. of triplicate determinations.
*, $p < 0.05$ for comparison with values in the presence of 1 mM unlabeled IMP (Student's t-test).

Binding assay

^{125}I labeled (37 MBq/m*l*) and unlabeled IMPs were kindly donated by Nihon Mediphysics Co., Ltd. (Takarazuka, Japan). Each type of liver cell was used for the binding assay at 2 days of culture when the cells became confluent. Cultured cells were preincubated in HBSS with 0.3% BSA (pH 7.4) at 37°C for 10 min. Then the cells were incubated in HBSS with 0.3% BSA and 1 μM ^{125}I-IMP at 37°C for 20 min unless otherwise stated. After the incubation with ^{125}I-IMP, the cells were rapidly rinsed with

Table 1 Specific binding of ^{125}I-IMP to liver cells

	Specific binding (pmol/well)
Parenchymal cell	70.2 ± 8.4
Sinusoidal endothelial cell	4.2 ± 1.4
Kupffer cell	2.3 ± 0.8
Fat-storing cell	not detectable

Experimental procedures were as described in Figure 1. Specific binding was determined by subtracting nonspecific from total binding. Each value is the mean ± S.E.M. of three individual experiments.

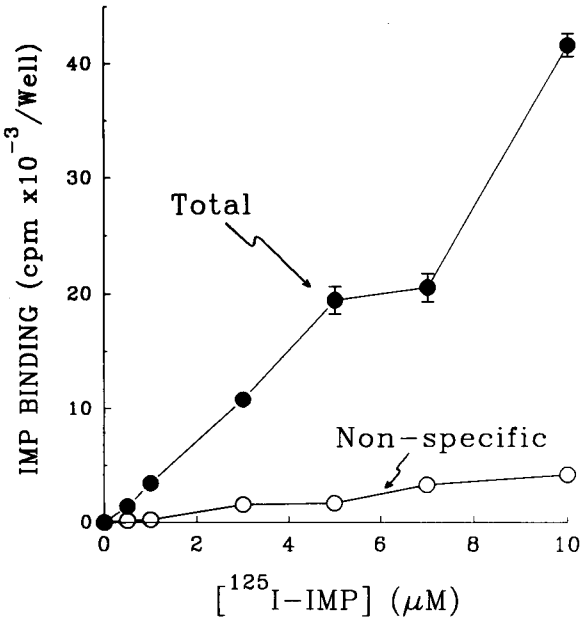

Fig. 2 Binding of ^{125}I-IMP to cultured rat parenchymal cells as a function of ^{125}I-IMP concentrations.
Parenchymal cells were isolated and cultured on 12-wells plates as described for 2 days in Methods. The cells were incubated for 20 min at 37°C with various concentrations of ^{125}I-IMP and in the absence (●) or the presence (○) of excess unlabeled IMP (3 mM). The data represent a typical experiment repeated four times and are expressed as the mean ± S.E.M. of triplicate determinations.
*, $p < 0.05$ for comparison with values in the presence of 3 mM unlabeled IMP (Student's t-test).

three 1.5 m*l* volumes of ice-cold HBSS and lysed with 2N NaOH. The radioactivity in the lysate was determined with an Aloka ARC 300 gamma counter (Aloka Co., Tokyo, Japan). Nonspecific binding was estimated from measurements of binding in the presence of excess unlabeled IMP (1 or 3 mM). Specific binding was determined by subtracting the nonspecific binding value from the total binding to the cells and culture plate. Each binding assay was conducted in triplicate.

RESULTS

The purity of the parenchymal cell culture and fat storing

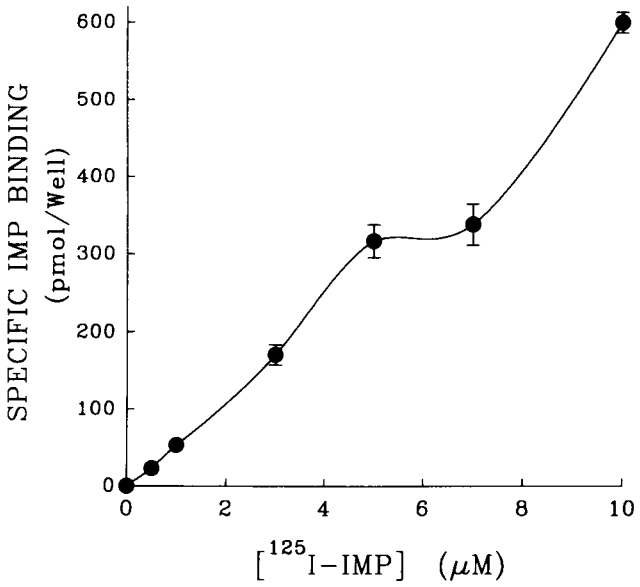

Fig. 3 Concentration-response curve for specific binding of ^{125}I-IMP to cultured rat parenchymal cells.
Experimental procedures were as described in the legend to Figure 2. Parenchymal cells were incubated with various concentrations of ^{125}I-IMP indicated on the abscissa. Nonspecific binding was determined in the presence of excess (3 mM) unlabeled IMP. Specific binding was determined by subtracting nonspecific from total binding. Data are the mean ± S.E.M. of four individual experiments.

cell culture was more than 90 and 95%, respectively. The purity of the endothelial cell culture and Kupffer cell culture was about 85%. Most of the contaminant cells in the parenchymal cell, endothelial cell and Kupffer cell cultures were fat storing cells. Each liver cell became confluent at 2 days of culture. Figure 1 shows the binding of ^{125}I-IMP to each liver cell after 20 min of incubation with 1 μM ^{125}I-IMP. The total binding was significantly higher than the nonspecific binding in the parenchymal cells ($p < 0.0001$), endothelial cells ($p < 0.01$) and Kupffer cells ($p < 0.05$). On the other hand, in fat storing cells there was no statistically significant difference between the total and nonspecific binding, indicating that fat storing cells did not have specific binding sites. Table 1 shows the specific binding of ^{125}I-IMP to parenchymal cells, endothelial cells and Kupffer cells. Since fat storing cells had no specific binding and comprised most of the contaminant cells in the culture of the parenchymal cell, endothelial cell or Kupffer cell, the values seemed to reflect the specific binding to each liver cell itself. The specific binding to the parenchymal cells was much higher than that to the endothelial cells or Kupffer cells ($p < 0.005$). Parenchymal cells constitute more than 90% of the total volume of liver cells and account for approximately 65% of the total number of cells in the liver.[11] These findings therefore suggest that IMP mostly binds to parenchymal cells in the liver. The pharmacological properties of the

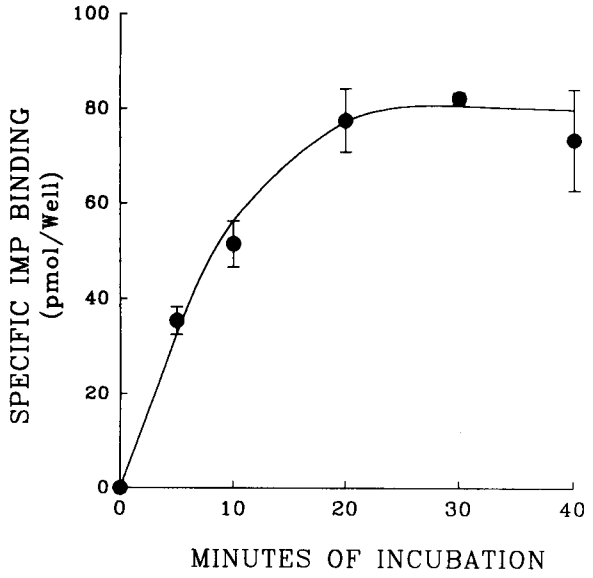

Fig. 4 Time-dependency of specific binding of ^{125}I-IMP to cultured rat parenchymal cells.
Parenchymal cells were incubated with 1 M ^{125}I-IMP at 37°C for various times indicated on the abscissa. Nonspecific binding was determined in the presence of excess (1 mM) unlabeled IMP. Specific binding was determined by subtracting nonspecific from total binding. Data are the mean ± S.E.M. of three individual experiments.

binding to PC were further characterized. Figure 2 shows the concentration-response curve for the binding of ^{125}I-IMP to parenchymal cells. As IMP was not dissolved at a concentration of more than 3 mM in HBSS at pH 7.4, the binding could be tested in the concentration range between 0 and 10 μM of ^{125}I-IMP. The cells were incubated for 20 min with ^{125}I-IMP at 37°C. The total and specific binding was increased with increasing concentrations of the ligand in the incubation medium. The total binding was significantly higher than the nonspecific binding at all concentrations tested, indicating that there was specific binding in the parenchymal cells in this concentration range. Figure 3 shows the specific binding of ^{125}I-IMP to parenchymal cells. The specific binding was increased steadily by increasing the ^{125}I-IMP concentration in the concentration range less than 5 μM. The curve leveled off in the concentration between 5 and 7 μM, but the specific binding was increased at a higher concentration (10 μM) and was unsaturable in the concentration range tested. The concentration-response curve indicated that there were binding sites with low affinity (Kd > hundreds μM) and high capacity in the parenchymal cells, and suggests that the parenchymal cells have higher affinity binding sites with a Kd of about 4 μM. In addition, the specific binding to the parenchymal cells, incubated with 1 μM ^{125}I-IMP at 37°C, equilibrated within 20 min and the equilibrium was maintained at least until 40 min (Fig. 4).

DISCUSSION

The images of the lungs were obtained after the introduction of ^{123}I-IMP into the intestinal tract of patients with a portosystemic shunt.[7] In the lungs, IMP is proposed to bind to nonspecific amine receptors in the endothelial cells.[8,14] Kosuda et al. performed an experimental study on IMP lung uptake by microautoradiography in rats given ^{125}I-IMP.[15] They reported the presence of increased grain density in the interstitial space including the capillary lumen and interalveolar septum, but not in the epithelial cells nor in the muscular layer of the respiratory bronchiole. By contrast, what cells in the liver trap IMP has not been clarified. When we performed liver microautoradiography by ^{125}I-IMP after the portal injection, the grain density was observed along the sinusoidal walls and around the parenchymal cells, suggesting that IMP binds to several types of liver cells,[16] but the experiment could not clarify what cells in the liver trap IMP due to the poor resolution or make possible quantitative analysis. We therefore examined the binding of IMP to each liver cell culture. The current study demonstrates that IMP binds to parenchymal cells, sinusoidal endothelial cells and Kupffer cells but not to fat-storing cells, and suggests that IMP is mostly extracted by parenchymal cells.

Tanaka et al. characterized IMP binding sites in human lung tissue by using a radioligand binding assay with ^{125}I-IMP as the ligand.[17] They reported that there were two binding sites with Kd values of 53 ± 2 and 4687 ± 124 nM in the lung membrane, and that the binding was inhibited by several amines such as ketamine, dopamine and propranorol. Moreover, the Kd value of the IMP binding site in the synaptosome was reported to be 56 μM.[18] The binding was also blocked by several amines. In this study, the specific binding to hepatic parenchymal cells was unsaturable in the concentration range tested. The concentration-response curve indicated that there were binding sites with low affinity (Kd > several hundreds μM) and high capacity in the parenchymal cells, and suggests that parenchymal cells had higher affinity binding sites with a Kd value of approximately 4 μM. We did not perform a displacement-type experiment, but the addition of ketamine has been reported to decrease the ^{123}I-IMP liver uptake following the portal injection of IMP in rats.[19] The IMP binding to parenchymal cells was time-dependent and reached equilibrium within 20 min. In the lung membrane, the equilibrium of the binding was reached after 15 min.[17] The Kd values of the IMP binding sites therefore had a wide range among these different tissues. The binding sites to IMP with a Kd value of around 4 μM in the liver seemed to be similar to those in the lungs and synaptosome. The Kd value of more than 100 μM observed in the liver might be higher than those reported in the lungs and synaptosome, but the characteristics of the binding were very similar to those in the other tissues. Howlett et al.[20] reported that the rat striatum had spiperone

binding sites with Kd values of 31 and 325 pM. The Kd values of the tryptamine binding site in brain tissue were reported to be around 5 nM.[21] The Kd values observed in the parenchymal cells were much higher than those of other amine binding sites, suggesting nonspecific amine binding sites. The mixed function oxidase system in microsomes, accompanied with the interaction of IMP with cytochrome P-450, was involved in the process of IMP metabolism in the liver.[22] The content of cytochrome P-450 is rapidly decreased in collagenase-prepared parenchymal cells following culture.[23] IMP metabolism should therefore be decreased in our parenchymal cell culture. The binding in the parenchymal cells might be affected by the culture conditions.

We have emphasized the clinical relevance of the portosystemic shunt index, evaluated by transintestinal portal scintigraphy with IMP.[7] Portal blood flow carries nutrients and hormones to the liver and plays a most important role in maintaining hepatic functions. Portosystemic shunting therefore causes or augments hepatic insufficiency due to the reduced hepatic blood flow.[24] Quantitative measurement of the shunting helps to understanding the pathophysiology of the various liver diseases and allows rational selection of treatment for patients with portal hypertension. We recently showed that portosystemic shunting from the superior mesenteric vein or the inferior mesenteric vein could be estimated with duodenal[25] or rectal[7] administration of IMP in patients with liver cirrhosis, respectively. In the present study, we characterized the specific binding sites to IMP in parenchymal cells cultured from the normal liver, but the characteristics of the binding sites might be changed in the liver of patients with liver disease. Indeed, in the brain tissue from cirrhotic patients with hepatic encephalopathy, tryptamine binding site densities were significantly decreased, though the binding site affinities were within normal limits[21]. If the density or affinity of the binding sites in the liver of patients with liver disease is reduced, some IMP carried to the liver through the portal circulation might escape being trapped by the liver. Further experiments are needed to clarify whether such parameters regulating IMP that are trapped in the liver are affected in the cirrhotic liver.

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