In Vitro Investigation of Amyloid-β Hepatobiliary Disposition in Sandwich-Cultured Primary Rat Hepatocytes

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ABSTRACT

Failure in amyloid-β (Aβ) systemic clearance across the liver has been suggested to play a role in Aβ brain accumulation and thus to contribute largely to the pathology of Alzheimer’s disease (AD). The purpose of this study was to characterize in vitro the transport mechanisms of Aβ40 across the liver using sandwich-cultured primary rat hepatocytes (SCHs) and to determine its biliary clearance (CLbile) and biliary excretion index (BEI%). 125I-Aβ40 BEI% was time dependent and reached steady state at 30 minutes, with an average value of 29.8% and a CLbile of 1.47 ml/min per kilogram of body weight. The role of low-density lipoprotein receptor-related protein-1 (LRP1) in mediating the basolateral uptake of 125I-Aβ40 in SCHs was assessed using receptor-associated protein (RAP, 2 μM). A significant reduction in 125I-Aβ40 BEI% and CLbile with RAP was observed, demonstrating a major contribution of LRP1 in mediating hepatic uptake of intact 125I-Aβ40 via transcytosis. Furthermore, activity studies suggested a lower role of receptor for advanced glycation end products (RAGE) in 125I-Aβ40 hepatic uptake. Verapamil (50 μM) and valsarpodar (20 μM) significantly reduced 125I-Aβ40 BEI%, indicating a role for P-glycoprotein (P-gp) in the biliary excretion of 125I-Aβ40 in SCHs. LRP1- and P-gp-mediated 125I-Aβ40 biliary excretion was inducible and increased BEI% by 26% after rifampicin pretreatment. In conclusion, our findings demonstrated that besides LRP1, P-gp and, to a lesser extent, RAGE are involved in 125I-Aβ40 hepatobiliary disposition and support the use of enhancement of Aβ hepatic clearance via LRP1 and P-gp induction as a novel therapeutic approach for the prevention and treatment of AD.

Introduction

Alzheimer’s disease (AD) is a complex neurodegenerative disease and the most common cause of dementia (Sagare et al., 2012). AD is characterized by specific neuropathological lesions, including progressive deposition of the intracellular neurotoxic form of the amyloid-β (Aβ) peptides Aβ40 and Aβ42 and extracellular Aβ as senile plaques throughout the brain (Makarova et al., 2004). It has been suggested that elevated levels of brain Aβ monomers result in the formation of neurotoxic Aβ oligomers, which contribute largely to the pathogenesis of this disease (Zhao et al., 2012). Aβ is produced by enzymatic cleavage, mediated by β- and γ-secretases of the transmembrane protein amyloid precursor protein (Cam and Bu, 2006). The high levels of Aβ observed in the brain of AD patients are due mainly to overproduction of Aβ or failure of its clearance from the brain (Sommer, 2002). As a peptide, Aβ40 has poor passive membrane permeability and depends on a transport system to pass through endothelial cells of the blood-brain barrier (BBB) (Banks et al., 2003). Low-density lipoprotein receptor-related protein-1 (LRP1) is the major receptor that mediates removal of Aβ across the BBB in its free form (Deane et al., 2004) or bound to chaperone molecules such as apolipoprotein E. P-glycoprotein (P-gp), expressed on the luminal side of the BBB, mediates the efflux of Aβ into the peripheral circulation (Cirrito et al., 2005; Abuznait and Kaddoumi, 2012). It has been reported that the expression of LRP1 and P-gp at the BBB is decreased and expression of the receptor of advanced glycation end product (RAGE), which influxes circulating Aβ into the brain across the BBB, is increased in AD patients, which favors Aβ accumulation inside the brain (Deane et al., 2003, 2012; Donahue et al., 2006; Sagare et al., 2007; Castellano et al., 2012). In addition to the role of Aβ transport proteins, several Aβ-degrading enzymes contribute significantly to Aβ clearance. Numerous in vitro and in vivo studies support the physiologic role of the insulin-degrading enzyme, neprilysin, endothelin-converting enzyme-1 and -2, among others, in Aβ degradation (Eckman and Eckman, 2005; Saito and Leisring, 2012). Plasma Aβ, including Aβ that is effluxed from the brain, undergoes rapid clearance mainly through the liver in its free or plasma lipoprotein-bound form (Tamaki et al., 2006; Marques et al., 2009). Recently, several studies have reported that endogenous soluble circulating low-density lipoprotein receptor-related protein (sLRP) plays an important role in the clearance of systemic Aβ “sink activity” and maintenance of Aβ brain homeostasis (Quinn et al., 1997; Sagare et al., 2007). Second, LRP normally binds 70–90% of circulating plasma Aβ and forms complexes that are unable to enter the brain across the BBB and favor its elimination mainly through the liver (Deane et al., 2008; Sagare et al., 2011); however, in AD, sLRP1 is mostly oxidized, which prevents its uptake by the liver. This work was supported by a fellowship from the Ministry of Higher Education of the Libyan government; and the Libyan North American Scholarship Program (to L.A.M.).

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ABBREVIATIONS: Aβ, amyloid-β; AD, Alzheimer’s disease; BBB, blood-brain barrier; BEI%, biliary excretion index; BSA, bovine serum albumin; CDF, 5-(and-6)-carboxy-2’,7’-dichlorofluorescin; CDFDA, 5-(and-6)-carboxy-2’,7’-dichlorofluorescin diacetate; CLbile, biliary clearance; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HBSS, Hanks’ balanced salt solution; LRP, low density lipoprotein-receptor related protein; PBS, phosphate-buffered saline; P-gp, P-glycoprotein; RAGE, receptor for advanced glycation end products; RAP, receptor-associated proteins; Rh123, rhodamine 123; RIPA, radioimmunoprecipitation assay (buffer); SCH, sandwich-cultured primary rat hepatocyte; sLRP, soluble low-density lipoprotein receptor-related protein; TCA, trichloroacetic acid.

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binding to circulating αβ40 and causes a sharp increase in free αβ40 plasma levels (Sagare et al., 2011).

LRP1 is expressed abundantly in the liver, mainly in hepatocytes, and is considered the major receptor that clears αβ from the general circulation (Tamaki et al., 2006). Receptor-associated protein (RAP) has shown to inhibit αβ40 liver uptake in rats by 48%, indicating the role of LRP1 in mediating the hepatic uptake of 125I-αβ40 from the circulation (Tamaki et al., 2006). In another study, portal infusion of insulin increased the expression of hepatic LRP1 at the cell membrane in a time-dependent manner and induced the apparent hepatic uptake of αβ40 in a concentration-dependent manner (Tamaki et al., 2007). Several studies have demonstrated the involvement of liver as a main pathway in the clearance of αβ from the peripheral circulation and have shown that faulty clearance of plasma-free αβ contributes largely to AD (Ghiso et al., 2004; Sagare, 2007; Sutcliffe et al., 2011). These studies, however, focused on LRP1’s contribution to the hepatic clearance of αβ. Although LRP1 was determined to be the major pathway for αβ systemic clearance, other mechanisms of transport in the liver that play roles in αβ hepatic clearance need further investigation. Hence, in the current study, we aimed to characterize the mechanism of αβ40 hepatic uptake, as well as biliary excretion, and to address the role of P-gp, LRP1, and RAGE in this process using sandwich-cultured primary rat hepatocytes (SCHs), which might contribute to the vectorial transport of substrates. Since P-gp and RAGE are expressed in the brain and contribute to αβ brain homeostasis, we hypothesized that both of these transport systems, in addition to LRP1, mediate the hepatic uptake and biliary elimination of αβ40.

Materials and Methods

Collagenase (type I, class I), rat-tail collagen (type I), Dulbecco’s modified Eagle’s medium (DMEM), and insulin were purchased from Invitrogen (Carlsbad, CA). Rifampicin, caffeine, 1,1,1,3,3,3-hexafluoro-2-propanol, rhodamine123 (Rh123), dexamethasone, bovine serum albumin (BSA), 10× DMEM, soybean trypsin inhibitor, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic monoiodinated and nonoxidized 125I-αβ40 (human, 2200 Ci/mmol) was purchased from PerkinElmer (Boston, MA). [14C]Salicylic acid and [3H]taurocholate were obtained from American Bio-reagents with the bicinchoninic acid method were reagents with the bicinchoninic acid method were validated from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Total protein measurement’s reagents with the bicinchoninic acid method were from Pierce (Rockford, IL). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Preparation of Sandwich-Cultured Primary Rat Hepatocytes

All culture plates were precoated with a thick collagen layer as a matrix for the attachment of hepatocytes. Twenty-four- and six-well plates were coated with 300 and 1400 μl of 50 μg/ml collagen in 0.2 M acetic acid solution, respectively, and left to dry overnight inside the hood. Plates were then neutralized and washed twice with sterile distilled water, and then 0.5 ml/well of DMEM was added and the plates were incubated at 37°C for 2 to 3 hours to hydrate the collagen layer before seeding the cells. Hepatocytes were seeded at 0.7 × 10^6 cells/ml and allowed to attach for 1 to 2 hours. The medium was then aspirated and washed twice with sterile distilled water, and 0.5 ml/well of DMEM was added and the plates were incubated at 37°C for 6 hours. Medium was then replaced with serum-free medium supplemented with 10% ITS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mg/l insulin, and 1 μm dexamethasone and incubated for 6 hours. Medium was then replaced with serum-free medium supplemented with 1% ITS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 μm dexamethasone. Twenty-four hours later, the medium was removed and hepatocytes were overlaid with 0.5 ml/well Matrigel (BD Discovery Labware, Franklin Lakes, NJ) in ice-cold medium and incubated in the same buffer for 37°C for 24 hours, after which medium was replaced with fresh medium each day up to day 4, when activity studies or protein extractions for Western blot were performed.

Isolation of Primary Rat Hepatocytes

The isolation and purification procedures of hepatocytes from animals’ livers were done under aseptic conditions. Each animal was anesthetized with an intraperitoneal injection of 0.06 g/kg of ketamine/0.012 g/kg of xylazine; then the liver portal vein was cannulated, and perfusion was started after the previously described two-step collagenase perfusion technique (Seglen, 1976). First, the liver was perfused with 450 ml of Ca2+-free Hanks’ balanced salt solution (HBSS) buffer containing 1 mM EGTA and 0.022 mg/ml heparin neutralized to pH 7.4 at a flow rate of 35 ml/min, immediately after which the liver was perfused with 350 ml of standard HBSS supplemented with 5 mM CaCl2, 100 U/ml collagenase type I, and 70 mg of soybean tryptic inhibitor at a flow rate of 30 ml/min. All solutions and tubing were maintained at 37°C and saturated with 95% O2/5% CO2. After the perfusion step, the liver was excised and put in a dish containing warm DMEM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mg/ml insulin, and 1 μm dexamethasone and placed on ice to reach 4°C. The liver capsule was stripped slowly, and cells were released by gently combing the liver with a steel comb. Cells were filtered through 70-μm mesh, and hepatocytes were separated from the other liver cell population by centrifugation at 50g for 5 minutes twice, followed by centrifugation with 35% Percoll solution to remove dead cells. Hepatocytes were resuspended in serum-free medium, and viability was determined using the trypan blue method.

Validation of Sandwich-Cultured Primary Rat Hepatocytes Model

Light Microscopy Imaging. To confirm the integrity of canaliculair networks, phase-contrast microscopy images were taken on day 4 of SCHs using a Nikon Eclipse TS100 inverted microscope from EquipNet, Inc. (Canton, MA).

Fluorescence Microscopy Imaging. We used 5-(and-6)-carboxy-2',7'-dichlorofluorescin diacetate (CDFDA) as a canalicular marker in SCHs on day 4. CDFDA is passively uptaken into hepatocytes and metabolized into the fluorescent metabolite 5-(and-6)-carboxy-2',7'-dichlorofluorescin (CDF), which is actively effluxed into the canalicular space via multidrug resistance–associated protein 2 (Liu et al., 1999b). In these experiments, a culture plate was rinsed with standard HBSS and incubated in the same buffer for 10 minutes. After aspiration of the buffer, 2 μM CDFDA dissolved in HBSS containing 5 mM CaCl2 was added to the cells and incubated for 5 minutes, followed by aspiration and washing four times with ice-cold standard HBSS. Images were then captured for accumulated CDF into canalicular spaces using inverted microscope Olympus 1x71 (Center Valley, PA) at 20× magnification.

Cumulative Uptake of [3H]Taurocholate and [14C]Salicylic Acid in SCHs as Positive and Negative Controls. Respectively. On day 4 of SCHs, cells were washed with warm standard HBSS (i.e., containing 5 mM CaCl2) or Ca2+-free HBSS containing EDTA and then incubated in their respective buffers for 5 minutes. Subsequently, 1 μM of either [3H]taurocholate or [14C]salicylic acid was dissolved in HBSS with or without calcium and added to the cells. After 10 minutes of incubation at 37°C, the plates were placed on...
ice and uptake was ended by aspirating the incubation medium and washing the wells four times with ice-cold standard HBSS. Cells were lysed by adding 200 μl radiomimnonoprecipitation (RIPA) buffer (25 mM Tris HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxy, 0.1% SDS, pH 7.6) containing a 1% protease inhibitors cocktail, sonicated for 30 seconds, and incubated on ice with shaking for 2 hours. Samples were analyzed using a Wallac 1414 WinSpectral Liquid Scintillation Counter (PerkinElmer). Data were normalized to protein content in each well using a bicinchoninic acid protein assay kit with BSA as standard.

Canaliculal Efflux of Rh123. Rh123 is passively uptaken into hepatocytes, and its biliary excretion is mediated by P-gp (Anneta and Brouwer, 2005). On day 4, SCHs in six-well plates were rinsed twice in standard HBSS and incubated in the same buffer for 10 minutes. Subsequently, the buffer was aspirated and cells were preloaded with 1 μM Rh123 dissolved into standard HBSS for 30 minutes, followed by aspiration of the incubation medium and washing four times with ice-cold HBSS. Afterward, an efflux flux was initiated by incubating the cells in warm HBSS with or without calcium for 30 minutes at 37°C. At the end of the incubation time, aliquots from incubation medium were measured for fluorescent intensity of Rh123 using Synergy 2 microplate reader (Biotek, Winooski, VT) with excitation and emission wavelengths of 485 and 529 nm, respectively. Data acquisition was achieved using Gene5 software (Biotek). Data were normalized to protein content in each well, and the biliary excretion index (BEI%) of Rh123 was calculated using eq. 1, as described in the Data Analysis section.

Western Blot Analysis
Hepatocytes were seeded in six-well plates and maintained up to 4 days in sandwich configuration. For induction studies with rifampicin, hepatocytes were cultured in sandwich configuration for 2 days, and then rifampicin treatment (50 μM) was initiated and added fresh each day up to day 4 of sandwich culture. Subsequently, media were removed and cells were washed twice using ice-cold HBSS, scraped, collected in 1.5-ml Eppendorf tubes and then centrifuged at 2000g for 10 minutes at 4°C. The cell pellets were then resuspended and homogenized in RIPA buffer containing 1% protease inhibitors cocktail and incubated on ice for 1 hour. The samples were then centrifuged at 15,000g for 15 minutes at 4°C. The lysate samples were stored at −80°C for subsequent Western blot analyses. Twenty-five micrograms of protein samples were loaded and resolved using 7.5% SDS-PAGE with a 5% stacking gel at 140 V for 1 hour. Proteins were transferred electrophoretically onto nitrocellulose membranes at 300 mA for 1.5 hours. After that, the membranes were blocked by incubation with 2% BSA on a rocking platform for 1 hour at room temperature, followed by incubation with the primary antibodies RAGE IgG (N-16), P-gp (C-219), LRP1 (light chain), or β-actin (C-11) at dilutions 1:125, 1:200, 1:1500, and 1:3000, respectively, in phosphate-buffered saline (PBS) containing 2% BSA and 0.05% Tween-20 overnight at 4°C. For detection of proteins, membranes were incubated with secondary anti-mouse IgG antibody for P-gp, anti-goat IgG antibody for RAGE and β-actin, or anti-rabbit IgG antibody for LRP1, all labeled with horseradish peroxidase, at 1:5000 dilution for P-gp, RAGE, and β-actin or 1:2000 dilution for LRP1 for 1 hour at room temperature. The blots were developed using a chemiluminescence detection kit (SuperSignal West Femto substrate; Thermo Scientific, Waltham, MA). Quantitative analysis of the immunoreactive bands was performed using Syngene luminescent image analyzer (Scientific Resources Southwest, Inc., Stafford, TX). Protein quantification was performed, and data were expressed as ratio of proteins to β-actin.

Immunocytochemistry of RAGE
SCHs cultured for 4 days were fixed in a 2% glutaraldehyde in PBS solution for 30 minutes at room temperature, followed by incubation with 0.1% Triton X-100 solution for membrane permeabilization. The SCHs samples were placed in a blocking solution of 1% BSA in PBS solution for 1 hour at 37°C. The cells were first exposed to RAGE (A-9) primary mouse monoclonal antibody (Santa Cruz Biotechnology) for 2 hours at 37°C and subsequently exposed to a fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Images were acquired by Nikon Eclipse fluorescent microscope.

Time-Dependent Studies of 125I-αβ40 Accumulation in Conventional and Sandwich-Cultured Primary Rat Hepatocytes
Time-dependent accumulation studies of 125I-αβ40 in SCHs were performed on day 4. Cells were incubated with 0.5 ml of 0.14 nM 125I-αβ40 prepared in standard or Ca2+-free HBSS. After incubation for 5, 15, 30, and 60 minutes, the cells were lysed and counted for radioactivity. To study the effect of calcium on the cumulative uptake of 125I-αβ40, conventional cultured hepatocytes were prepared in a 24-well plate. In this case, hepatocytes were not overlaid with a second matrix layer; rather, they were seeded on hard collagen-coated plates to spread as monolayers. At the same time, hepatocytes were seeded on another plate and overlaid with a Matrigel layer to obtain sandwich configuration. The two plates were incubated for 2 days, when 0.5 ml of 0.14 nM 125I-αβ40 was added to the cells, with or without calcium, and incubated for 5 and 30 minutes at 37°C. Uptake was ended by aspirating the medium and washing the cells with ice-cold standard HBSS while keeping plates on ice. Cells were lysed, and quantitative determination of intracellular 125I-αβ40 accumulations was performed as mentioned. Degradation of 125I-αβ40 was determined by trichloracetic acid (TCA) precipitation assay (Shibata et al., 2000). Cells lysate samples were mixed (1:1 volume) with TCA (final concentration 10%) and centrifuged at 14,000g at 4°C for 10 minutes. Radioactivity in the supernatant was determined using Wallac 1470 Wizard Gamma Counter (PerkinElmer).

Determination of the BEI and Biliary Clearance of 125I-αβ40
All experiments have their own control and were conducted on day 4 of sandwich culture. Twenty-four well plates were preincubated in standard or Ca2+-free HBSS for 5 minutes. Subsequently, 0.5 ml of 0.14 nM 125I-αβ40 in standard or Ca2+-free HBSS was added to cell-culture plates and incubated for 30 minutes at 37°C. Uptake was stopped by placing plates on ice, removing the incubation medium, and washing the cells three times with ice-cold standard HBSS containing 0.2% BSA and once with standard HBSS only to remove BSA residue. Cells were then lysed by the addition of RIPA buffer containing 1% protease inhibitors cocktail, sonicated for 30 seconds, followed by gentle shaking on a rocking platform for 2 hours at 4°C. Intracellular radioactivity of 125I-αβ40 was measured using a Wallac 1470 Wizard Gamma Counter. Data obtained were normalized to protein content. Results were also normalized to 125I-αβ40 non specific Matrigel binding by subtracting 125I-αβ40 bound to Matrigel-coated wells without cells. Nonspecific binding was less than 2%. The obtained data from the accumulation experiments of 125I-αβ40, in the presence or absence of calcium, were used to calculate BEI using eq. 2 and the biliary clearance (CLbile) using eq. 3 described in the Data Analysis section.

Effect of Transport Proteins Inhibition on 125I-αβ40 BEI and CLbile in SCHs
For P-gp inhibition studies, verapamil (50 μM) or volsapard (20 μM) was used; for LRPI and RAGE inhibition, RAP (1 μM) and RAGE IgG antibody (10 μg/ml) were used, respectively. In these experiments, cells were washed with warm standard or Ca2+-free HBSS and incubated in their respective buffers for 5 minutes at 37°C. Then buffers were removed and replaced with 0.5 ml of 0.14 nM 125I-αβ40 alone or with inhibitors and incubated for 30 minutes at 37°C. Subsequently, plates were placed on ice, and medium was removed by aspiration. Cells were washed three times with ice-cold 0.2% BSA in standard HBSS and once with standard HBSS to remove BSA residues. Cells were then lysed and used for 125I-αβ40 quantification by gamma counter.

Effect of P-gp Induction by Rifampicin on 125I-αβ40 BEI and CLbile in SCHs
Twenty-four–well plates of SCHs were treated with or without 50 μM rifampicin dissolved in serum-free DMEM (supplemented with 1% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml dexamethasone) for 48 hours, starting from day 2 of sandwich culture. After the end of treatment on day 4 of culture, medium was removed and replaced with freshly prepared medium and incubated for 8 hours as a washout period. Consequently, medium was removed by aspiration, and cells were rinsed gently with warm standard or Ca2+-free

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HBSS and incubated in their respective buffers for 5 minutes at 37°C/5% CO₂. Cumulative uptake was initiated by adding 0.5 ml/well of 0.14 nM ¹²⁵I-Aβ₄₀ with or without calcium for 30 minutes at 37°C. Similarly, for the inhibition study, verapamil (50 μM) was coinubated with 0.14 nM ¹²⁵I-Aβ₄₀ for 30 minutes. At the end of the experiment, ¹²⁵I-Aβ₄₀ radioactivity was determined in the cell lysate, and BEI% and CLₘₜₑ were calculated according to eqs. 2 and 3, respectively.

**Data Analysis**

The efflux of substrates into canalicular spaces in SCHs (i.e., BEI%) was determined by the cumulative efflux or cumulative uptake method. For Rh123, BEI was determined using the cumulative efflux method (Liu et al., 1999b), in which the cumulative efflux of Rh123 into Ca²⁺-free HBSS (cells + canalicular) and standard HBSS (cells only) was determined. Their difference represents the canalicular efflux as shown in eq. 1:

\[
\text{BEI}\% = \frac{\text{Accumulation into cells + canalicular } (+\text{Ca}^{2+}) - \text{Accumulation into cells } (\text{Ca}^{2+})}{\text{Accumulation into cells + Canalicular } (+\text{Ca}^{2+})} \times 100
\]

\[\text{CL} \_\text{bile} = \frac{\text{Accumulation into cells + canalicular } (+\text{Ca}^{2+}) - \text{Accumulation into cells } (\text{Ca}^{2+})}{\text{AUC} \_\text{medium}}
\]

All biliary clearance values are reported with the unit ml/min per kilogram of body weight based on 200 mg of protein/g of liver and 40 g of liver/kg of rat body weight (Seglen, 1976).

**Statistical Analysis**

Unless otherwise indicated, all data were expressed as mean ± S.E.M. The experimental results were statistically analyzed for significant difference using unpaired two-tailed Student’s t test for two groups, and one-way analysis of variance, followed by the Bonferroni test for analysis of more than two groups. A P value less than 0.05 was considered statistically significant.

**SCH Model Validation.** The formation of canalicular networks was confirmed by light microscopy imaging. Figure 1A shows canalicular space formation as bright white spaces indicated by the small white arrow. The content of the canalicular space is sealed by the tight junctions, as shown in Fig. 1B, where the canalicular marker CDFDA was passively uptaken inside the hepatocytes and rapidly metabolized to the fluorescent CDF, which is effluxed into the canalicular space via multidrug resistance–associated protein 2, expressed on the apical side of hepatocytes, and confirms the formation of canalicular spaces (Liu et al., 1999b). Levels of P-gp, LRP1, and RAGE protein expression in SCHs cultured for 4 days were determined by Western blot analysis. P-gp, LRP1, RAGE, and β-actin protein were detected at 150, 85, 55, and 46 kDa, respectively, as shown in Fig. 2.

The activity of the sandwich culture was further tested by using the bile salt [³H]taurocholate for active transport and [¹⁴C]salicylic acid as a passive diffusion marker that is not excreted in the bile canaliculi. Consistent with other studies in the literature (Liu et al., 1999a), the BEI% of [³H]taurocholate was as high as 52.7% ± 8.2%, whereas for [¹⁴C]salicylic acid, it was approximately zero, as its cellular accumulation with or without calcium was approximately the same (Fig. 3A). The CLₘₜₑ of [³H]taurocholate was also determined in SCHs on day 4 of culture and was 81.0 ± 7.7 ml/min per kilogram of body weight.

The functional activity of P-gp at the apical side of the SCHs was determined by using the specific substrate Rh123. Efflux of 1 μM Rh123 into the incubation buffer in presence or absence of calcium was performed, and the BEI% was calculated to be 19.4% ± 5.2% with a significant difference in its efflux between both incubation buffers (P < 0.05) (Fig. 3B).

**Determination of ¹²⁵I-Aβ₄₀ BEI, CLₘₜₑ, and Cumulative Uptake.** To determine the Aβ hepatic transport mechanisms, ¹²⁵I-labeled Aβ₄₀ was used for sensitive quantification. Bell et al. (2007) showed that ¹²⁵I-labeling of Aβ₄₀ did not alter the transport properties of ¹²⁵I-Aβ₄₀ compared with the unlabeled peptide and that the clearance rates of both peptides were identical. In addition, free Aβ₄₀ instead of Aβ₄₀–sLRP1 complex was investigated in the current studies to imitate mild cognitive impairment and AD conditions where, unlike in normal physiologic condition, sLRP1 is mostly present in oxidized form with negligible binding affinity to Aβ₄₀, causing a sharp increase in free Aβ₄₀ plasma levels (Sagare et al., 2011). Although Aβ₄₂ is more prone to aggregation compared with Aβ₄₀, both Aβ peptides have been implicated in the pathogenesis of AD (Hardy, 2006) and participate in the formation of senile plaques with neurotoxicity potentials (Chiang et al., 2008; Seeman and Seeman, 2011). Aβ₄₀ and Aβ₄₂ are substrates for P-gp and LRP1 (Shibata et al., 2000; Cirrito et al., 2005); thus, the clearance of both peptides is expected to be altered by these transport-protein modulations. In this study, Aβ₄₀ was used in the BEI experiments for practical reasons, as it has a much faster clearance rate than does Aβ₄₂ (Zlokovic et al., 2000; Ito et al., 2006).
To determine the optimum time (when steady state is achieved) for 125I-Aβ40 biliary excretion measurement, time-course accumulations with or without calcium were performed, and biliary excretion was determined at different time points. As shown in Fig. 4, the biliary excretion of 125I-Aβ40 was time dependent and reached steady state at 30 minutes with BEI% and CLbile values of 29.8% ± 2.3% and 1.47ml/min per kilogram, respectively. Thus, all subsequent cumulative uptake and BEI% studies were conducted at 30-minute incubation times. To evaluate the effect of calcium on the cumulative uptake of 125I-Aβ40, conventional hepatocyte culture (i.e., not in sandwich configuration) was seeded in parallel with SCHs and maintained for 48 hours at 37°C. Forty-eight hours of culture time was selected as the viability and functionality of conventionally cultured primary hepatocytes start to decline at longer times (Hewitt et al., 2007; Swift et al., 2010). As shown in Fig. 5A, the difference between 125I-Aβ40 cumulative uptake in the presence or absence of calcium in the conventional model was insignificant at 5 and 30 minutes, suggesting no effect of calcium on 125I-Aβ40 uptake; however, the difference in the cumulative uptake in SCHs, although not different at 5 minutes, was obvious and significant at 30 minutes’ incubation time, demonstrating excretion of 125I-Aβ40 into canalicular spaces that were maintained only in the presence of calcium. Using the TCA assay, the percentage of 125I-Aβ40 intracellular degradation was determined and ranged from 27% to 36% (Fig. 5A, P > 0.05). 125I-Aβ40 intracellular degradation, after 30 minutes of cumulative uptake, decreased significantly (~17%) in SCHs at day 4 compared with 27% degradation at day 2 of sandwich culture (P < 0.05). On the other hand, the BEI% of total 125I-Aβ40 (intact + degraded) increased significantly with the length of culture, with 19.4% ± 3.6% and 27.5% ± 2.9% (P < 0.05) for SCHs at days 2 and 4, respectively (Fig. 5B).

Effect of Transport Proteins Inhibition on 125I-Aβ40 BEI and CLbile in SCHs. Inhibition studies of major 125I-Aβ40 transport proteins were conducted to investigate the effect of such inhibition on 125I-Aβ40 cellular accumulation and biliary excretion. Figure 6 illustrates the effect of P-gp inhibition by verapamil or valsodar on 125I-Aβ40 biliary excretion in SCHs. Verapamil (50 μM) caused a significant reduction in 125I-Aβ40 BEI%, from 27.1% ± 4.1% to 12.4% ± 1.4% (P < 0.05). The role of P-gp mediated canalicular efflux of 125I-Aβ40 was further confirmed with the selective inhibitor valsodar. Similarly, valsodar (20 μM) significantly reduced 125I-Aβ40 BEI% from 27.1% ± 4.1% to 9.6% ± 2.6% (P < 0.01).

Furthermore, the role of LRP1 and RAGE in mediating the 125I-Aβ40 disposition in SCHs was assessed using specific inhibitors. As shown in Fig. 7, RAGE antibody (10 μg/ml) reduced the BEI% and CLbile of 125I-Aβ40 from 27.1% ± 4.6% to 20.6% ± 2.6% and from 1.5 ± 0.6 to 0.9 ± 0.09 ml/min per kilogram of body weight, respectively. Coincubation of 125I-Aβ40 with 2 μM RAP, a known chaperone molecule for LRP1, and a competitive inhibitor for Aβ, as determined previously (Shibata et al., 2000; Qosa et al., 2012), caused a significant reduction in the BEI% and CLbile of 125I-Aβ40 by approximately 2.4-fold (from 27.1% ± 4.6% to 11.2% ± 4.8%) and 2.7-fold (from 1.5 ± 0.06 to 0.5 ± 0.15 ml/min per kilogram of body weight) (P < 0.01), respectively. Both inhibitors, RAP and RAGE-IgG, showed comparable inhibition of Aβ40 cumulative uptake in the presence of calcium compared with control (Fig. 7, black bars), whereas its cumulative uptake in the absence of calcium between the three groups (Fig. 7, gray bars) was not significantly different, indicating that the effect of both inhibitors was on Aβ40 uptake from the sinusoidal side of hepatocytes. However, based on the difference in Aβ40 cumulative uptakes in the presence and absence of calcium, the CLbile of Aβ40 with LRP1 inhibitor was significantly lower than its CLbile with RAGE inhibitor (P < 0.01), suggesting the greater role of LRP1 in Aβ40 cellular uptake compared with RAGE.

On the other hand, TCA studies revealed that P-gp, LRP1, and RAGE inhibition did not alter the degradation % of Aβ40 (~17%) demonstrating that Aβ40 degradation products are not substrates for these transport proteins.
RAGE localization in the SCHs was investigated and confirmed by immunocytochemistry. The results demonstrated RAGE distribution at the sinusoidal side of the SCHs (green fluorescence, Fig. 8) and was not detected at the bile canaliculi (white arrows, Fig. 8).

Effect of Rifampicin Treatment on $^{125}$I-$\alpha$-B40 BEI% and CLbile in SCHs. Rifampicin (50 $\mu$M) treatment significantly increased the expression of P-gp and LRPI by 1.6- and 1.4-fold, respectively, but not RAGE expression (Fig. 2). These increases in expression were associated with enhanced BEI% and CLbile of $^{125}$I-$\alpha$-B40 compared with vehicle-treated cells. The BEI% and CLbile values of $^{125}$I-$\alpha$-B40 were significantly increased, from 30.2% ± 2.2% to 38.2% ± 1.3% and from 1.5 ± 0.15 to 1.8 ± 0.2 ml/min per kilogram of body weight, respectively ($P < 0.05$). P-gp inhibition by verapamil caused a significant decrease in the BEI in both control and in rifampicin-treated cells, with a more pronounced inhibitory effect on rifampicin-treated cells. As shown in Fig. 9, the BEI% of $^{125}$I-A decreased with culture time, an observation that has been reported previously in SCHs with other enzymes, including cytochrome P450 and RAGE, transport proteins that have established functions in $\alpha$B transport across BBB to the hepatic clearance of $\alpha$B40, is poorly understood. Thus, to characterize more fully the role of these transport proteins and their interplay in $\alpha$B40 hepatic clearance, we used primary rat hepatocytes cultured in sandwich configuration.

Fig. 3. (A) Cumulative uptake of 1 nM $[^{14}$C]salicylic acid ($[^{14}$C]SA) and 1 125mM $[^{3}$H]taurocholate ($[^{3}$H]TC) in SCHs at day 4 of culture. Hepatocytes were preincubated for 10 minutes in standard HBSS or Ca2+-free HBSS before cumulative uptake studies were conducted. BEI% and CLbile, (ml/min per kilogram of body weight) were determined after 10 minutes of the addition of $[^{14}$C]SA and $[^{3}$H]TC. Each bar represents the mean ± S.E.M. for replicate triangles from three independent experiments. ***P < 0.001, ns, not significant between substrates cumulative uptake from standard HBSS and Ca2+-free HBSS based on unpaired Student’s t test. (B) Cumulative efflux of Rh123 into standard HBSS and Ca2+-free HBSS for 30 minutes in SCHs at day 4 of culture. Hepatocytes were preloaded with 1 125mM Rh123 dissolved in standard HBSS for 30 minutes at 37°C, followed by efflux study. Bars represent mean ± S.E.M. for triplicates from two independent experiments. Unpaired Student’s t test was used to compare between Rh123 efflux into standard and Ca2+-free HBSS. *P < 0.05.

Discussion

Understanding $\alpha$B hepatic transport mechanisms might unravel novel therapeutic targets to enhance $\alpha$B systemic elimination through the liver and consequently reduce its burden in the brain (Sehgal et al., 2012). Whereas available studies focused primarily on the role of LRP1 in hepatocellular uptake of $\alpha$B40, the contribution of P-gp and RAGE, transport proteins that have established functions in $\alpha$B transport across BBB to the hepatic clearance of $\alpha$B40, is poorly understood. Thus, to characterize more fully the role of these transport proteins and their interplay in $\alpha$B40 hepatic clearance, we used primary rat hepatocytes cultured in sandwich configuration.

SCHs were optimized and validated for formation of canalicular networks by observing the formation of canalicular spaces under light microscopy, as well as by accumulation of the fluorescent metabolite CDF (Fig. 1). Also, BEI% and CLbile measurements of $[^{3}$H]taurocholate and $[^{14}$C]salicylic acid, used as positive and negative controls, respectively, were in agreement with previous work (Liu et al., 1999a; Turncliff et al., 2006), and the functional activity of P-gp, determined using Rh123 as a model substrate, was consistent with previously reported results (Annaert and Brouwer, 2005). For the first time, this in vitro model was successfully used to determine the BEI% of $^{125}$I-$\alpha$-B40. Treating SCHs with $^{125}$I-$\alpha$-B40 for 30 minutes demonstrated significantly higher accumulation of $^{125}$I-$\alpha$-B40 in HBSS containing calcium compared with calcium-free HBSS, with a BEI% of 29.8% ± 2.3%, which is considered moderate compared with $[^{3}$H]taurocholate (52.7% ± 8.2%) and Rh123 (19.4% ± 5.2%) (Fig. 3). The effect of calcium on the difference in $^{125}$I-$\alpha$-B40 uptake in SCHs was rolled out by performing cumulative uptake studies of $^{125}$I-$\alpha$-B40 with or without calcium in primary hepatocytes cultured in conventional culture and was compared with SCHs. Degradation studies by TCA assay suggested biliary excreted $^{125}$I-$\alpha$-B40 was mostly intact (~85%), which was supported by the intracellular degradation and modulation studies; however, the degradation of $^{125}$I-$\alpha$-B40 in SCHs decreased with culture time. Further studies are required to explain this observation, but this decrease could be due to reduced expression of $\alpha$B40-degrading enzymes, such as neprilysin and insulin-degrading enzyme, with culture time, an observation that has been reported previously in SCHs with other enzymes, including cytochrome P450 (Hewitt et al., 2007), or reduced expression of another uptake mechanism coupled with $\alpha$B40 endocytotic degradation.

Consistent with available studies in rats (Tamaki et al., 2006, 2007) and transgenic mice (Sehgal et al., 2012), our data demonstrate that
basolateral LRP1 plays an important role in mediating the hepatic clearance of Aβ40 from the general circulation and were confirmed by inhibition studies. RAP significantly reduced 125I-Aβ40 BEI% and CL bile by 2.4- and 2.7-fold, respectively. Interestingly, the same concentration of RAP (2 μM) was reported to decrease the liver uptake of intravenously administered 125I-Aβ40 by 48% (~2-fold) in rats (Tamaki et al., 2006), which is in agreement with our findings. Another interesting finding is that LRP1 inhibition did not affect 125I-Aβ40 degradation in SCHs (~15% in the absence and presence of RAP), suggesting that LRP1 mediates transcytosis of intact 125I-Aβ40 in SCHs. Similar observations were reported previously by Pflanzner et al. (2011), who used primary mouse brain capillary endothelial cells grown on filters and showed that LRP1 mediates intact Aβ40 transport by transcytosis and plays no role in its degradation in this model.

P-gp is expressed abundantly on the canalicular side of hepatocytes, and Aβ40 is a P-gp substrate (Lam et al., 2001). P-gp at the apical side of BBB has been shown to play a substantial role in controlling Aβ levels in the brain by enhancing its removal from brain to blood and by limiting Aβ access to the brain (Cirrito et al., 2005; Kuhnke et al., 2007; Abuznait et al., 2011). To study the role of P-gp in the canalicular excretion of Aβ40, two potent P-gp inhibitors, verapamil and valsaparod, were used. Verapamil inhibition of P-gp has been reported in various in vitro models, including SCHs (Annaert and Brouwer, 2005), Caco2 cells (Bansal et al., 2009), and the porcine kidney cell line (LCC-PK1) expressing human MDR1 (Sugimoto et al., 2011). Also, valsaparod was demonstrated as an effective P-gp inhibitor in Madin-Darby canine kidney cells transected with MDR1 (MDCK-II-MDRI) and rat brain microcapillary endothelial cells (Sziraki et al., 2011). Both compounds inhibited P-gp efflux function and significantly reduced the BEI% of 125I-Aβ40 with more pronounced effect demonstrated by valsaparod compared with verapamil with 64 and 54% reductions, respectively. The reduction in BEI% with both inhibitors can also be noticed from the significant increase in the accumulation of 125I-Aβ40 in calcium-free HBSS, from 2.0 ± 0.04 to 2.5 ± 0.2 (P < 0.01) and 2.2 ± 0.04 (P < 0.05) fmol/mg protein for valsaparod and verapamil treated cells, respectively (Fig. 6).
In the absence of calcium, the tight junctions are disrupted and the canalicular $^{125}$I-$\text{A}_{\beta 40}$ is released into the medium during incubation, which is consequently removed by washing at the end of the experiment; hence, any increase in $^{125}$I-$\text{A}_{\beta 40}$ accumulation inside the cells in the absence of calcium is due to the specific inhibition of canalicular efflux of $^{125}$I-$\text{A}_{\beta 40}$ through apical P-gp. To our knowledge, this function of canalicular P-gp in the hepatic clearance of $\text{A}_{\beta 40}$ is first shown by this study.

We also tested the role of RAGE in mediating $^{125}$I-$\text{A}_{\beta 40}$ clearance in SCHs. To our knowledge, RAGE localization in hepatocytes has not been identified. In this study, we successfully confirmed its sinusoidal localization in SCHs at day 4 by immunocytochemistry (Fig. 8). Inhibition studies with RAGE antibody showed a reduction by 24 and 36% in $^{125}$I-$\text{A}_{\beta 40}$ BEI% and CL$_{\text{bile}}$, respectively ($P < 0.05$), demonstrating a significantly lesser role in mediating the hepatic clearance of $\text{A}_{\beta 40}$ than that of LRP1.

Collectively, these observations confirm hepatic LRP1 and P-gp as significant players in determining $\text{A}_{\beta}$ systemic levels and suggest that a small decline in their expression or activity could decrease $\text{A}_{\beta}$ systemic clearance and possibly contribute to $\text{A}_{\beta}$ accumulation in the brain. Considering the established role of age-associated decline in LRP1- and P-gp-mediated removal of $\text{A}_{\beta}$ peptides in humans (Vogelgesang et al., 2002) and rats (Silverberg et al., 2010), strategies to upregulate expression or activity of both proteins at the BBB have been investigated as a potential preventive approach (Hartz et al., 2010; Qosa et al., 2012). In vivo brain efflux index studies conducted on C57BL/6 mice treated with rifampicin or caffeine significantly enhanced $\text{A}_{\beta 40}$ clearance by more than 20% compared with control vehicle-treated mice (Qosa et al., 2012). Thus, in the current study, we investigated the effect of upregulation of both proteins by rifampicin on the hepatic clearance of $\text{A}_{\beta 40}$. Rifampicin treatment increased the expression of LRP1 at the basolateral side and P-gp at the apical side of hepatocytes. These results are consistent with our previous study in which treatment of mice with rifampicin for 3 weeks increased LRP1 and P-gp expression at the BBB and was associated with enhanced clearance of $^{125}$I-$\text{A}_{\beta 40}$ across the BBB (Qosa et al., 2012).
2012). Interestingly, and consistent with our in vivo study findings (Qosa et al., 2012), P-gp inhibition with verapamil significantly reduced 125I-Abeta BEI% by 80% in rifampicin-treated cells compared with 43% in vehicle-treated cells, indicating that rifampicin induced another mechanism that plays an important role in 125I-Abeta clearance and was inhibited by verapamil. Further studies are in progress to explain this observation. Accordingly, similar to the BBB’s role in maintaining Aβ homeostasis in the brain, the role of hepatic clearance is very important in maintaining Aβ homeostasis in the body. As the level of LRPI and P-gp expressions decrease with aging and in AD (Toornvliet et al., 2006; Silverberg et al., 2010), the level of Aβ is expected to increase in the general circulation as a result of insufficient systemic clearance (Mackie et al., 1998; Tamaki et al., 2006), which may allow for more Aβ influx into the brain. Our results demonstrated that modulation of LRPI or P-gp alters Abeta biliary clearance and excretion, supporting their important function in regulating Abeta systemic levels; our findings also show that their upregulation presents a novel therapeutic approach for AD prevention and treatment. Furthermore, our findings suggest that although the hepatic disposition of Abeta40 is mediated by several transport proteins, competition with endogenous and exogenous substances that are mainly eliminated via a similar transport system in the liver might hinder Aβ hepatic clearance and contribute to the elevated levels of systemic Aβ observed in Alzheimer patients.

In summary, our data showed that, in addition to LRPI, P-gp is involved in the biliary excretion of Abeta40 in SCHs. Furthermore, the present study demonstrated that Abeta binding in SCHs is mediated mainly by LRPI and to a much lesser extent by RAGE. P-gp is involved not only in the clearance of Abeta40 across the BBB but also in its biliary excretion, and its role has been confirmed by inhibition and induction studies. Furthermore, SCHs provided a useful in vitro model to differentiate between Abeta40 sinusoidal and canalicular disposition and successfully determined its biliary excretion.

Authorship Contributions
Participated in research design: Mohamed, Kaddoumi.
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