Biodistribution of ^{125}I-Insulin: Each mouse was anesthetized (1.5% isoflurane with 4 L/min O_2) and its femoral vein/artery and the internal carotid artery were catheterized (n = 4 mice). A 100 μCi dose of ^{125}I-Insulin was administered via a left internal carotid artery. The animals were transcardially perfused with PBS after 15 min; left and right brain hemispheres were collected, and the radioactivity in each hemisphere was measured in the gamma counter. Alternatively, 100 μCi of ^{125}I-Insulin was administered via the femoral vein and the accumulation of radioactivity in each hemisphere was determined and expressed as a percentage of the total brain radioactivity (n = 4 mice).

Plasma membrane fractionation. Minute™ plasma membrane isolation kit (Invent Biotechnologies, Eden Prairie, MN) was used to obtain plasma membrane fractions from about 50 million cells. The cells were washed with ice cold PBS and were then pelleted by a low-speed centrifugation (1000 rpm, 5 min, 4°C). The pellet was resuspended in 200 μL buffer A containing protease inhibitor cocktail, incubated on ice for 10 min and then vortexed vigorously for 30 seconds. The cell suspension was transferred to the supplied filter cartridge and then centrifuged at 14,000 rpm, 4°C for 30 seconds. The pelleted fraction in the collection tube was resuspended again in the buffer A and then repassed for two more times through the filter as recommended by the manufacturer to increase the final yield of the isolated plasma membrane fraction. After the final re-passing step, the pellet was resuspended in the supernatant and then centrifuged at 3000 rpm at 4°C for one minute to pellet the nuclei. The supernatant from this step was transferred to a fresh 1.5 mL tube and centrifuged at 13,000 rpm for 60 minutes at 4°C. The pelleted fraction was resuspended in 200 μL buffer B supplemented with protease inhibitor cocktail, vortexed and then centrifuged at 10,000 rpm for 5 minutes at 4°C to isolate the organelle

membrane proteins. The supernatant was transferred to a fresh 2 mL microcentrifuge tube, 1.6 mL cold PBS was added, mixed by inversion and centrifuged at 13,000 rpm for 75 minutes at 4°C. The pelleted plasma membrane fraction was ultimately solubilized in 50 µL PBS containing 0.5% Triton X-100.

Western blotting. Protein levels in the whole cell lysates and plasma membrane fractions were quantified by using Bicinchoninic acid (BCA) protein assay kit (Pierce). Equal amounts of protein lysates were resolved by using 4-15% mini-PROTEAN® TGX™ precast gel (Bio-rad), electrotransferred onto a nitrocellulose membrane (Bio-rad) and then blocked with 1% bovine serum albumin (BSA) or 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour at room temperature. The blot was then incubated with one of the primary antibodies against: Low density lipoprotein receptor-related protein 1 (LRP1) (2 µg/ mL, clone 5A6, Calbiochem, EMD Millipore, Danvers, MA), receptor for advanced glycation end products (RAGE) (1:200, N-16, Santa Cruz Biotechnologies Inc., Dallas, TX), P-glycoprotein (P-gp) (1:500, E1Y7B, Cell Signaling Technology®, Danvers, MA) or Scavenger receptor class B member 1 (SRB1) (1:500, EP1556Y, Abcam, Cambridge, MA). Then the blot was washed three times, for 10 minutes each time, with TBST followed by incubation with 1:1000 or 1:1500 dilution of a relevant horseradish peroxidase (HRP)-conjugated secondary antibody -anti-mouse IgG-HRP (for probing LRP), anti-goat IgG-HRP (for probing RAGE) and anti-rabbit IgG-HRP (for P-gp and SRB1) obtained from Santa Cruz Biotechnologies Inc., Dallas, TX for 1 hour at 37 °C. After washing three times with TBST, the blot was then analyzed by autoradiography using SuperSignal® West Dura chemiluminescent substrate (Thermo

Scientific, Waltham, MA).Protein loading in each lane was estimated by incubation with antibodies for housekeeping proteins (GAPDH-HRP (FL-335, 1:500) or Na+K+ATPase ɑ1 (1:250 - 1:1000) obtained from Santa Cruz Biotechnologies inc., Dallas, TX. The auto-rads were scanned using CanoScan LiDE 110 (Canon), and the signal intensities of the protein bands were quantified with Image J software [1].

Immunocytochemistry. Control or insulin (100 mIU/mL, 20 min)-treated hCMEC/D3 cell monolayers, cultured on glass bottom dishes (MatTek Corporation, Ashland, MA), were washed with ice-cold Dulbecco's PBS and fixed with 4% v/v para-formaldehyde in PBS for 1 hour at 4 °C. After fixation, the cells were washed three times with PBS and then blocked with a solution of 1% v/v BSA (EMD Biosciences, Danvers, MA) in PBS at room temperature for 1 hr. Subsequently, the cells were incubated overnight at 4 °C with either anti-LRP antibody (8G1 clone, Santa Cruz Biotechnologies inc., Dallas, TX) or anti-RAGE antibody (RAGE N-16, Santa Cruz Biotechnologies inc., Dallas TX) at 1:10 dilution in blocking buffer. The cells were then washed four times with PBS and then incubated with 2 µg/mL fluorescein-anti-mouse IgG (H+L) or Alexa Fluor® 647-anti-goat IgG (H+L) (Life technologies, Grand Island, NY) in dark for 1 hour at room temperature. For P-gp immunostaining, the monolayers were incubated with the MDR1/ABCB1 antibody (EIYZB, Cell Signaling Technologies, Danvers, MA) at 1:800 dilution. Then the monolayers were incubated with anti-rabbit secondary antibody conjugated with AlexFluor® 647 at 1:500 dilution. The cells were then washed thoroughly with PBS, incubated with Hoechst 33342 as described above and then imaged by confocal microscopy.

Reference

1. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 2012; 9(7)**:** 671-5.