ESM Methods

Harvest of iBECs

Male Sprague Dawley rats were purchased from Charles River (Wilmington, MA, USA) and brains were harvested using previously established methods [1]. Brain stem, pial blood vessels, and meninges were removed. The remaining cortical tissue was mechanically digested in BEC isolation buffer (15 mmol/l HEPES, 5.6 mmol/l NaCl, 5.6 mmol/l KCl, 1.74 mmol/l $CaCl_2$, 1.22 mmol/l $MgCl_2$, 1% BSA, pH 7.4) by dicing with a scalpel and pipetting repeatedly with a glass Pasteur pipet. Enzymatic digestion was done by incubating tissue at 37°C with collagenase (Worthington, Lakewood, NJ, USA), dispase (Worthington), and DNase (New England Biolabs, Ipswitch, MA, USA). Digestion was stopped with ice-cold BEC isolation buffer and myelin was removed by centrifuging with 25% BSA. Pelleted cells were washed in BEC isolation buffer and then plated onto collagen IV-coated plates in BEC media (DMEM [1% glutamate, 4.5 g/l glucose], 10% FBS, Anti-Anti [1X, 50 unit/ml penicillin, 50 µg/ml streptomycin, 125 ng/ml fungizone; Thermo Fisher, Waltham, MA, USA]) supplemented with puromycin (4 µg/ml, decreasing to 2 µg/ml after 3 d) for 5 days to remove contaminating cells [2]. After 5 d in culture, iBECs were in puromycin-free media. Resulting cells (iBECs) were 98% CD31+ by flow cytometry (ESM Fig. 1).

For experiments investigating the effect of HFD feeding, iBECs were isolated from 12-week old SD rats after 4 weeks of HFD or normal chow. For transwell experiments, iBECs were isolated from 3-4 week old male SD rats.

Flow cytometry

iBECs were cultured for 7 days, trypsinised, resuspended in MACS buffer (Miltenyi Biotec, San Diego, CA, USA) and 1 x 10^6 cells were counted for each condition: iBECs only, iBECs with $CD31^+$ antibody (anti-rat CD31/PECAM-1 APC-conjugated antibody, R&D Systems, Minneapolis, MN, USA, FAB3628A-025), iBECs with antibody control (Goat APC, R&D Systems, IC108A). 100,000 events were counted per condition on BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analysed with CFlow Software. iBECs were gated based on forward scatter to detect intact cells versus cellular debris.

Immunofluorescence

iBECs were fixed in 4% PFA, permeabilised in 0.1% triton X, blocked in 10% donkey serum before overnight incubation with primary claudin-5 antibody (Cell Signaling, 1:100), and subsequent secondary antibody anti-mouse Alexa Fluor 488-conjucated secondary antibody (Jackson Immunoresearch 715-545-151, West Grove, PA, USA) and mounted in DAPI mounting media (Vectashield, Burlingame, CA, USA). Images were taken on Zeiss 780 confocal microscope. Astrocytes were fixed in 4% PFA, permeabilised and blocked in 0.1% triton X/5% donkey serum, before overnight incubation with primary GFAP antibody (Cell Signaling 3670, 1:800). Astrocytes were washed with PBS then incubated with anti-mouse, Alexa Fluor 488-conjucated secondary antibody and mounted with DAPI mounting media. Images were taken on a Leica SP5 confocal microscope.

Animal studies

Sprague Dawley rats were purchased from Charles River (Wilmington, MA, USA) and housed at 22°C on a 12-hr light-dark cycle and fed standard laboratory chow or HFD where specified, and water ad lib. Rats were group housed in accordance with institutional recommendations on corn cob bedding and received environmental enrichment.

^{125}I-TyrA14-insulin uptake assay

^{125}I-TyrA14-insulin uptake assay was performed as previously described [3, 4]. Cells were serum starved and treated with 200 pmol/l ^{125}I-TyrA14-insulin for 15 min in HEPES binding buffer (HBB; 100 mmol/l HEPES, 120 mmol/l NaCl, 1.2 mmol/l $MgSO_4$, 5 mmol/l KCl, 8 mmol/l glucose, 1% BSA, pH 7.8). In pharmacological experiments, drugs were added in HBB 30 min prior to ^{125}I-TyrA14-insulin addition. Uptake was stopped by placing the cells on ice. Non-specific binding (NSB) was estimated either as ^{125}I-TyrA14-insulin uptake at 4°C for time course experiment (ESM Fig. 3) or uptake after 30 min pretreatment with IR blocker S-961 (10 nmol/l, NovoNordisk, Copenhagen, Denmark). Cells incubated in cold acid wash buffer (0.5 mol/l NaCl, 0.2 mol/l acetic acid, pH 2.5) for 6 min and washed twice more to remove ^{125}I-TyrA14-insulin bound to the cell exterior. Cells were lysed in 1N NaOH. Radioactivity was quantified with a gamma counter, adjusted for efficiency (80%), and NSB subtracted. Protein was quantified with Bradford reagent and final results are expressed as DPM/mg protein.

RNA/cDNA preparation and RT-PCR

RNA extraction was performed with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Genomic DNA was removed by treatment with 2 U RNase Free DNase I/10µg RNA (New England Biolabs, Ipswich, MA, USA) at 37°C for 20 min. DNA-free RNA (1 µg) was reverse

transcribed using SuperScript® III First-Strand Synthesis System (Invitrogen) with random hexamers, according to manufactures protocol. The resulting cDNA was used in real time PCR assay.

IR (*Insr*) β subunit and IDE (*Ide*) mRNA were measured by real time RT-PCR using an iCycler IQ (Bio-Rad, Hercules, CA, USA) and ABsolute Blue QPCR Mix, SYBR Green, fluorescein (Thermo Fisher). Assay conditions were optimised to generate a single PCR product, confirmed by melt curves. All PCR reactions were further optimised for primer annealing temperature to obtain PCR efficiencies of 95-100%. The primers for *Insr* β subunit were forward (FWD) 5' CTGGACAGAACCCACCTATTT 3', reverse (REV) 5' CTGAAGAGGAAGACGAAGATGAG 3'; *Ide* forward (FWD) 5' GGAGGAACGAAGTCCACAATAA 3', reverse (REV) 5' GTTGAAGCAAGGCTCAGAGATA 3' (Integrated DNA Technologies, Coralville, IA). Primer concentration for real time PCR was 100 nmol/l. Unknown samples were measured using 100 ng reverse transcribed RNA against an external standard curve. All samples, including standards, were measured in triplicate, in the same assay.

Immunoblotting

Cells were placed on ice and washed twice with ice-cold PBS before lysis with M-PER and phosphatase and protease inhibitors (Thermo Fisher). Lysate protein was quantified with Bradford reagent and samples prepared for western blot. hBEC lysates were concentrated using a polyethersulfone membrane microcentrifuge spin column with a 10 kD molecular-weight cut off (Thermo Fisher) prior to addition of SDS and then equal volumes were loaded on gels. RBMVEC and hBEC lysates were run on 4-20% gradient gels and iBEC lysate samples on 10% Criterion gels (Bio-Rad). Gels were transferred onto nitrocellulose membranes, blocked in 5% BSA and blotted for p-Akt (Akt^{Ser473} Cell Signaling, Danvers, MA, USA, 4060), Akt (Cell

Signaling 9272), IR-β (Santa Cruz 711), p-ERK ($ERK^{Thr202/Tyr204}$, Cell Signaling 4376), ERK (Cell Signaling 4695), p-eNOS ($eNOS^{Ser1177}$, Cell Signaling 9570), eNOS (Cell Signaling 9572), GAPDH (Cell Signaling 2118), β-actin (Cell Signaling 4967), α-tubulin (Cell Signaling 2144). Blots probed for phosphorylated proteins were stripped (BP-98, Boston Bioproducts, Boston, MA, USA) before reprobing for total protein concentrations. All primary antibodies were used at 1:1000 dilution during an overnight incubation at 4°C. Secondary antibodies (Cell Signaling anti-mouse 7076, anti-rabbit 7074) were used at 1:2000 before imaging. Quantification of densitometry was performed using ImageJ software. For insulin-stimulated responses, phosphorylated protein was normalised to total protein. Insulin receptor was normalised to GAPDH expression.

In vitro blood-brain barrier co-cultures

Two days prior to co-culture, astrocytes were thawed and seeded (80,000 cells/well) on poly-L-lysine-coated 6-well plates [5]. One day before co-culture, iBECs from 3-4 wk old SD rats were passaged onto transwell inserts (90,000 cells/cm^2, 1.0 µm, PET, EMD Millipore, Billerica, MA, USA) and fresh media was provided to astrocytes. On day 0 of co-culture, iBEC transwells inserts were placed over astrocytes and the apical chamber was given fresh DMEM/F12 with hydrocortisone (550 nmol/l), Anti-Anti (1X) and insulin/transferrin/selenium growth supplement (1X, ITS-G, Thermo Fisher). On day 3 of co-culture, insulin was removed from culture media and iBECs were given DMEM/F12 with hydrocortisone (550 nmol/l, Sigma-Aldrich), RO-20-1724 (17.4 µmol/l, EMD Millipore), cpt-cAMP (250 µmol/l, Abcam, Cambridge, MA), IGF-1 (20 nmol/l, PeproTech, Rocky Hill, NJ, USA), holo-transferrin (68.75 nmol/l, R&D Systems), sodium selenite (38.7 nmol/l, Sigma-Aldrich), and Anti-Anti (1X). This medium maintained barrier integrity. On day 3, astrocytes were fed with an equivolume mixture

of DMEM/F12 with IGF-1-media and conditioned media. On day 5 of co-culture, iBEC monolayer and astrocytes were washed 1x in HBB. Basolateral chamber received equivolume mixture of DMEM/F12 with IGF-1 and conditioned media.

References

[1] Burek M, Salvador E, Forster CY (2012) Generation of an immortalized murine brain microvascular endothelial cell line as an in vitro blood brain barrier model. J Vis Exp 66:e4022

[2] Perriere N, Demeuse P, Garcia E, et al. (2005) Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. J Neurochem 93:279-289

[3] Genders AJ, Frison V, Abramson SR, Barrett EJ (2013) Endothelial cells actively concentrate insulin during its transendothelial transport. Microcirculation 20:434-439

[4] Meijer RI, Gray SM, Aylor KW, Barrett EJ (2016) Pathways for insulin access to the brain: the role of the microvascular endothelial cell. Am J Physiol Heart Circ Physiol 311:H1132-H1138

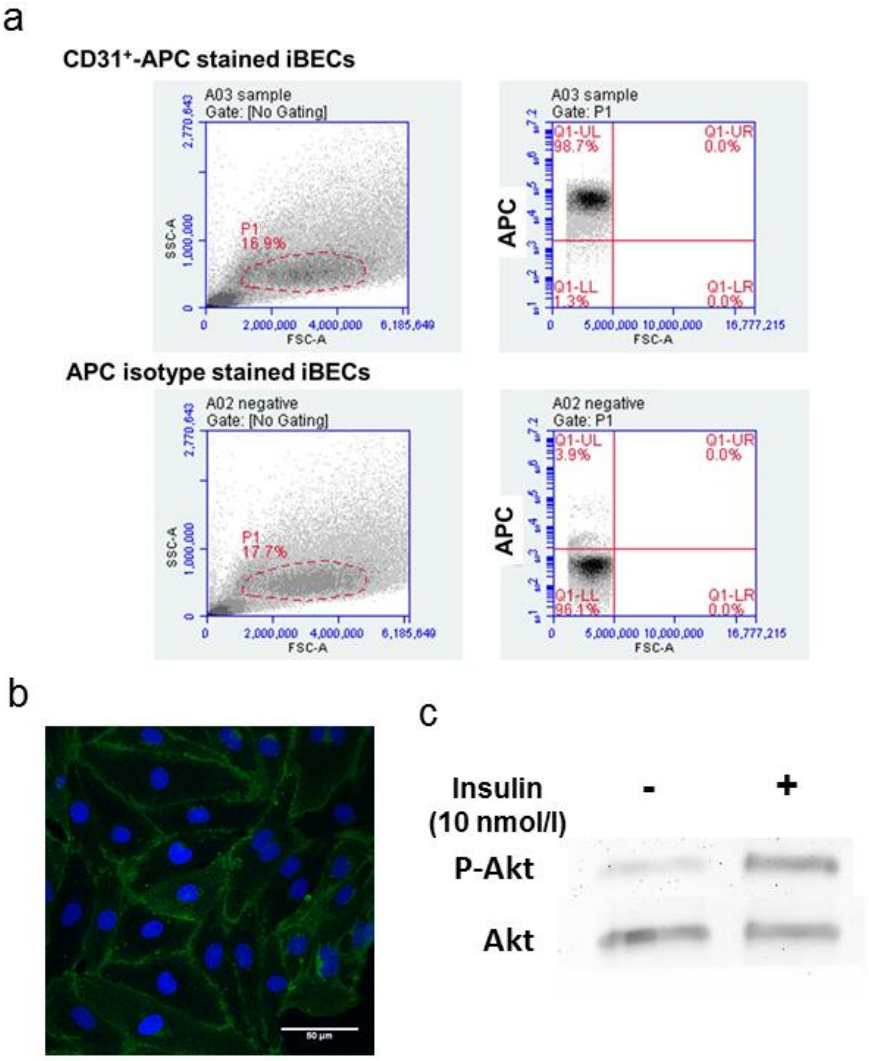
[5] Molino Y, Jabès F, Lacassagne E, Gaudin N, Khrestchatisky M (2014) Setting-up an in vitro model of rat blood-brain barrier (BBB): a focus on BBB impermeability and receptor-mediated transport. J Vis Exp 88:e51278-e51278

ESM Table 1.

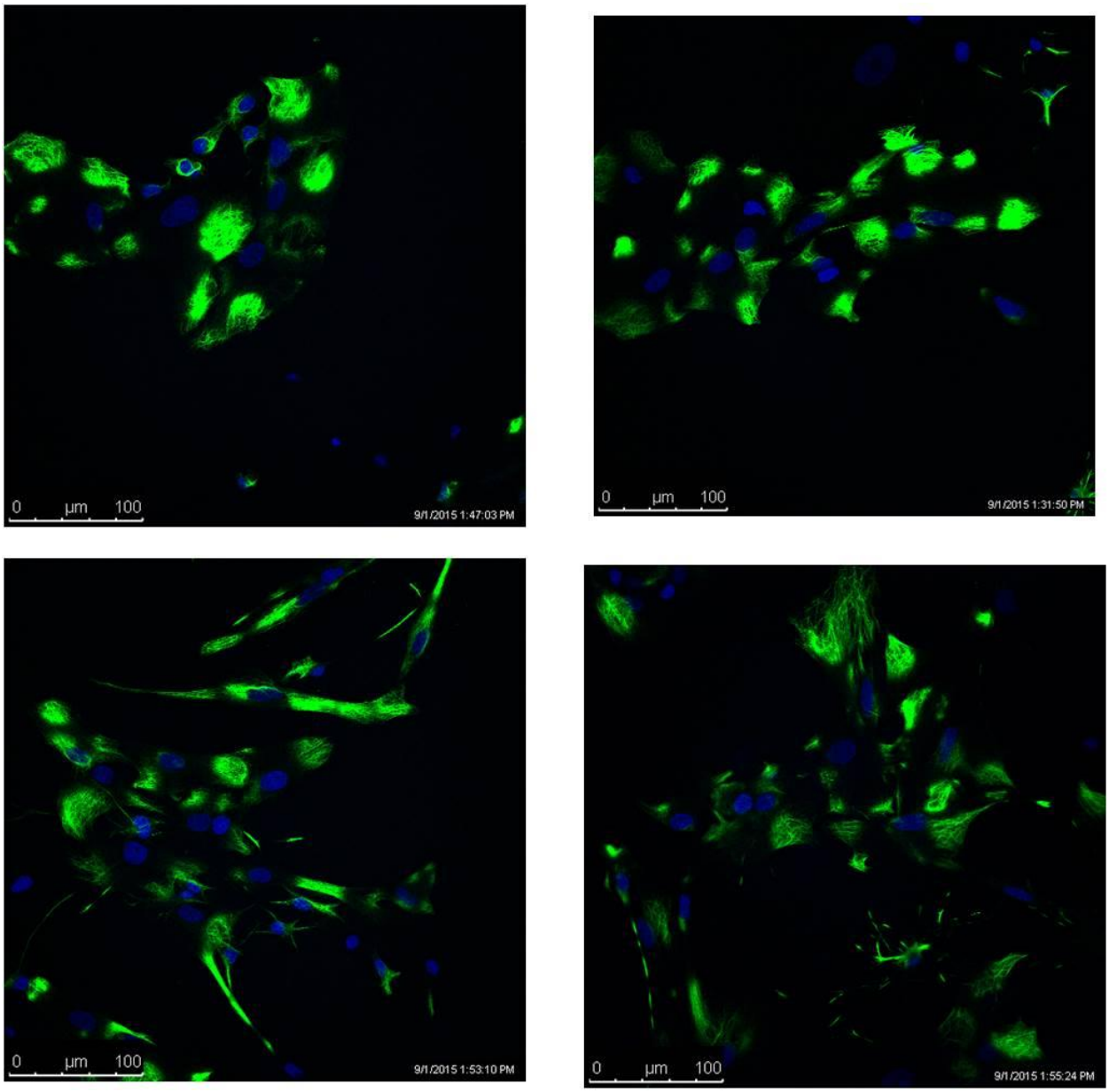
Pharmacological agents and manufacturers used throughout the study are listed below.

Drug	**Product #**	**Manufacturer**	**Location**
Ab-3	GR31L	Calbiochem	San Diego, CA, USA
Dynasore	2897	Tocris	Minneapolis, MN, USA
Filipin	70440	Cayman Chemical	Ann Arbor, MI, USA
Genistein	G6649	Sigma-Aldrich	St. Louis, MO, USA
L-glutamate	0218	Tocris	Minneapolis, MN, USA
N^{ω}-nitro-L-arginine methyl ester (L-NAME)	N5751	Sigma-Aldrich	St. Louis, MO, USA
Methyl-β-cyclodextrin (MβCD)	C4555	Sigma-Aldrich	St. Louis, MO, USA
PD98059	1213	Tocris	Minneapolis, MN, USA
PP1	14244	Cayman Chemical	Ann Arbor, MI, USA
PP2	13198	Cayman Chemical	Ann Arbor, MI, USA
S-961		Novo Nordisk	Copenhagen, Denmark
(*S*)-Nitroso-*N*-acetylpenicillamine (SNAP)	0598	Tocris	Minneapolis, MN, USA
Sodium nitroprusside (SNP)	567538	Calbiochem	San Diego, CA,USA
Thapsigargin	1138	Tocris	Minneapolis, MN, USA
Wortmannin	W1628	Sigma-Aldrich	St. Louis, MO, USA

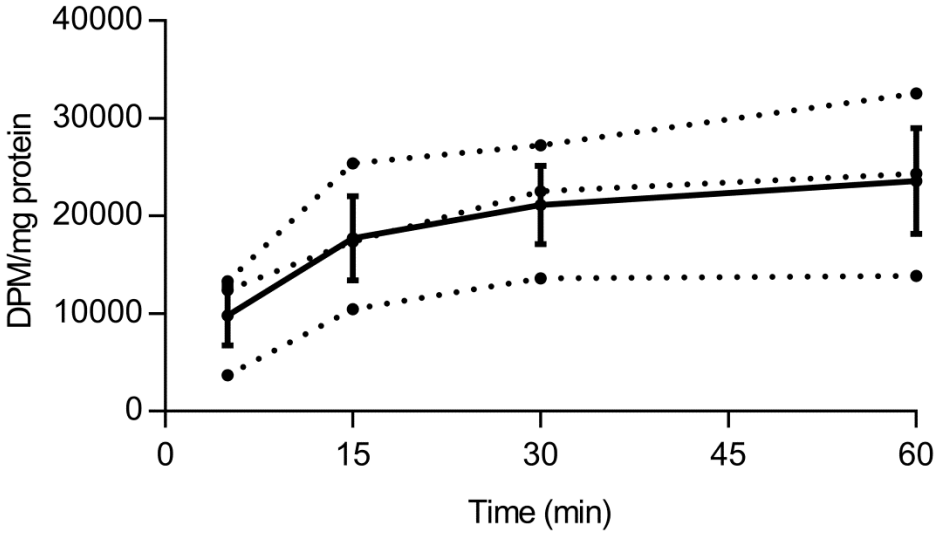
ESM Fig. 1 iBECs are 98% $CD31^+$ based on flow cytometry analysis (**a**). Left panels show forward and side scatter with gating (P1). Right panels show APC signal within P1. CD31-APC stained iBECs showed ~98% $CD31^+$ staining compared to APC isotype stained iBECs (lower panels). iBECs show endothelial morphology and immunofluorescent staining for claudin-5 (tight junction protein, green) and DAPI nuclear staining (blue) (**b**). In response to 15 min of 10 nmol/l insulin stimulation, isolated BECs had increased Akt^{Ser473} phosphorylation compared to vehicle control, indicating the PI3K/Akt pathway was intact (**c**).



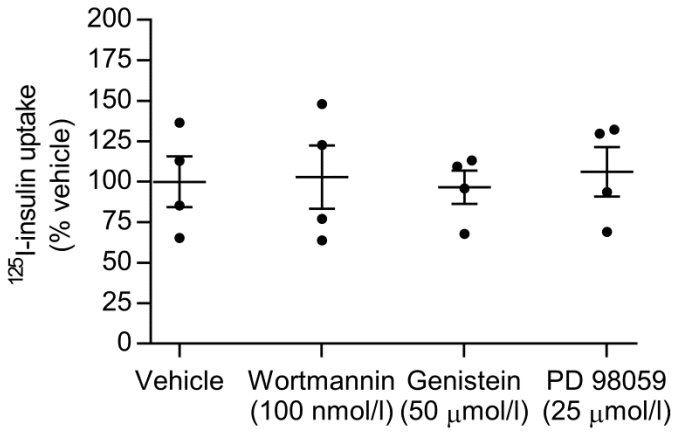
ESM Fig. 2 Isolated astrocytes are GFAP positive. Isolated astrocytes were stained for GFAP (green) and show DAPI nuclear staining (blue). Four separate fields are shown.



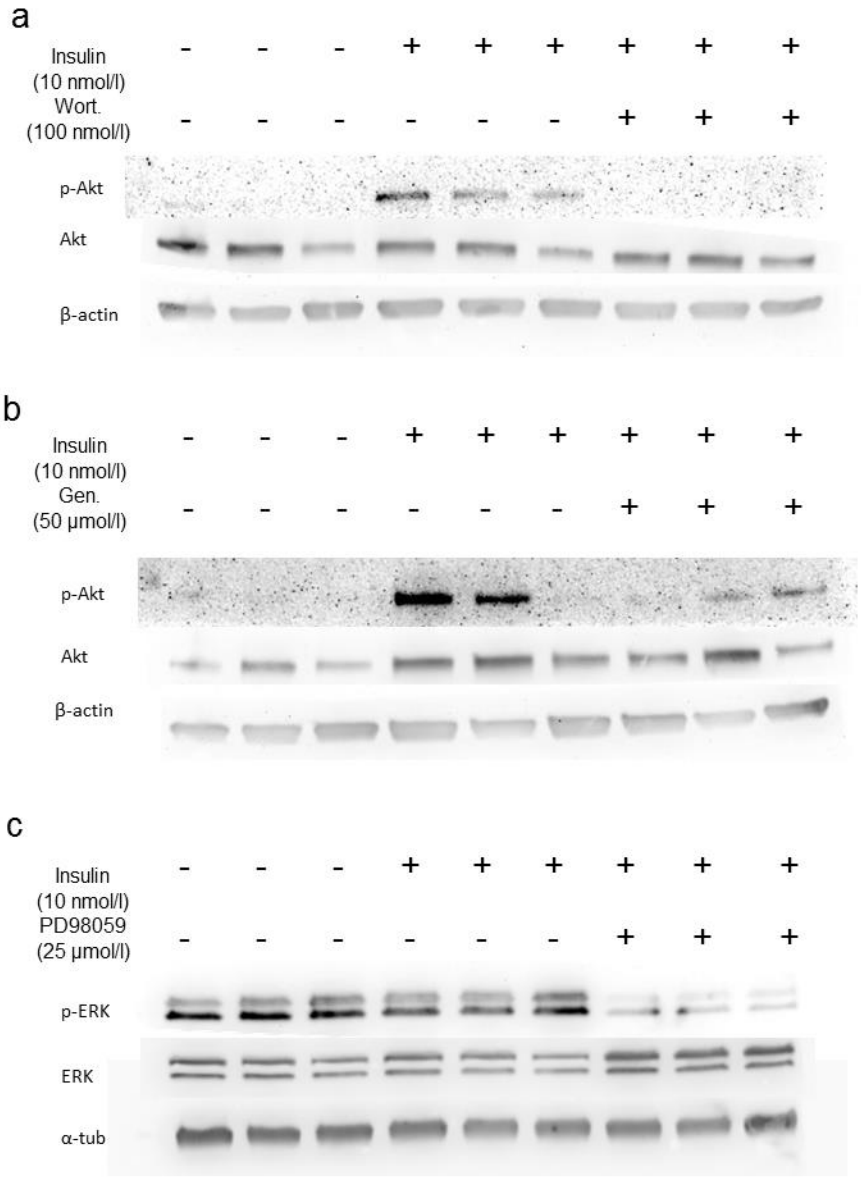
ESM Fig. 3 ^{125}I-TyrA14-insulin uptake is most rapid between 5-15 min and continues over 60 min. RBMVEC were serum starved for 4 h before addition of 200 pmol/l ^{125}I-TyrA14-insulin for 5-60 min at 37°C. Radioactivity was measured as disintegrations per min (DPM) and normalised to protein content. Dotted line indicates individual replicates, solid line indicates mean ± SEM.



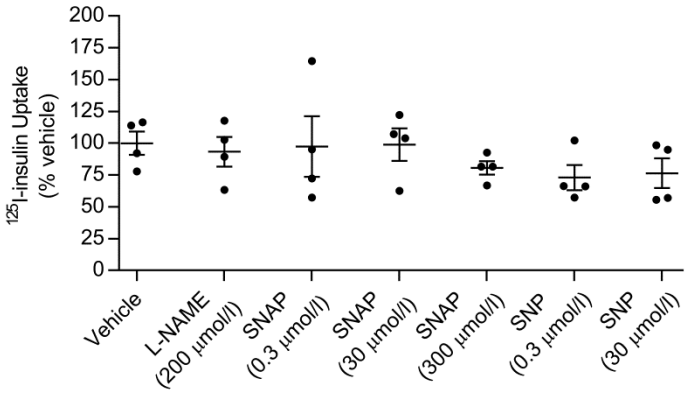
ESM Fig. 4 Wortmannin, genistein, and PD98059 do not inhibit ^{125}I-TyrA14-insulin (^{125}I-insulin) uptake. RBMVECs were serum starved for 2 hours before 30 min pretreatment with drugs and subsequent 15 min incubation with ^{125}I-TyrA14-insulin (200 pmol/l) for 15 min at 37°C. Each circle denotes individual replicate. Data is shown as mean ± SEM, n=4 per condition.



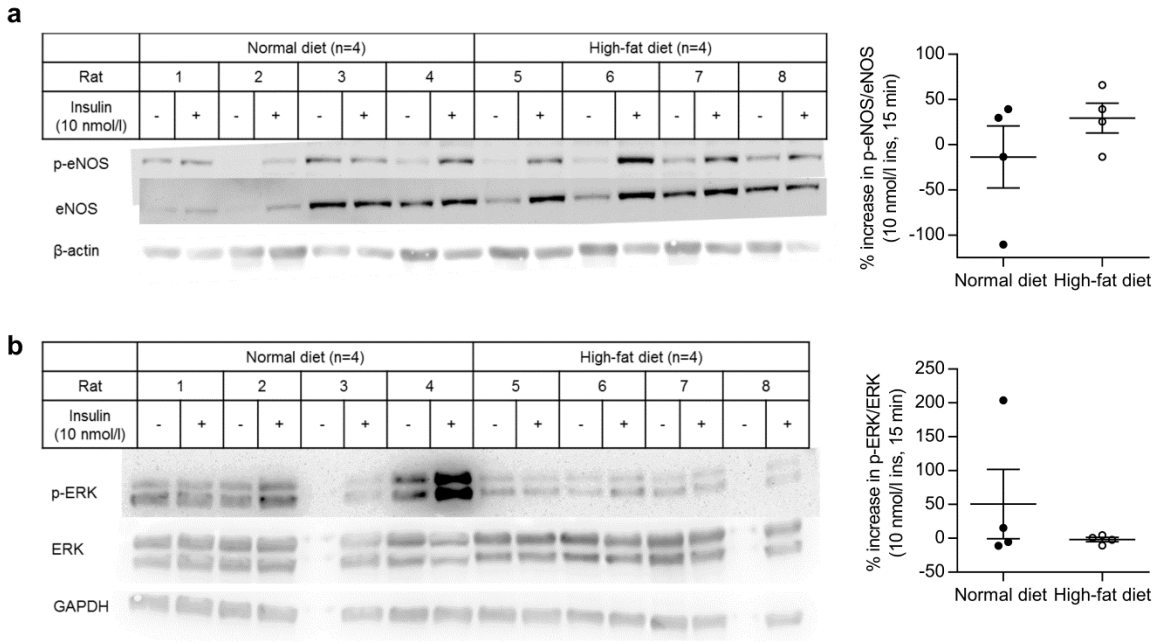
ESM Fig. 5 Wortmannin, genistein, and PD98059 inhibited insulin-stimulated signalling in RBMVEC. RBMVECs were serum starved for 2 h before 30 min pretreatment with wortmannin (wort, a), Genistein (Gen, b), PD98059 (c) and subsequent 15 min incubation with insulin (10 nmol/l) at 37°C. Three individual experiments were run on the same gel.



ESM Fig. 6 ^{125}I-TyrA14-insulin (^{125}I-insulin) uptake was not affected by nitric oxide (NO) inhibitor (L-NAME) or NO donors (SNAP, SNP). RBMVECs were serum starved for 2 hours before 30 min pretreatment with drugs and subsequent 15 min incubation with ^{125}I-TyrA14-insulin (200 pmol/l) at 37°C. Each circle denotes individual replicate. Data is shown as mean ± SEM and were compared using Dunnett's test. n=4 per condition.



ESM Fig. 7 Insulin-stimulated eNOS (**a**) and ERK (**b**) signalling in iBECs is not significantly affected by HFD. iBECs from normal diet and high-fat diet fed rats showed signalling following 15 min insulin (10 nmol/l) treatment for p-eNOS (eNOS^{Ser1177}, **a**) and p-ERK (ERK$^{Thr202/Tyr204}$, **b**). Quantification is phosphorylated protein normalised to total protein.



ESM Fig. 8 Degraded ^{125}I-TyrA14-insulin was not present in the apical transwell chamber. ^{125}I-TyrA14-insulin incubated with or without iBEC for 30 min was collected from the apical chamber (luminal aspect of iBEC), eluted from Sephadex column, and column fractions were quantified in disintegrations per min (DPM). Dotted line indicates no iBECs present, solid line indicates iBECs were present. Thin lines represent individual experiments and thick lines represent means ± SEM; n=3 without iBEC; n=5 with iBECs. ^{125}I-TyrA14-insulin incubated with iBECs eluted in same fraction as ^{125}I-TyrA14-insulin that was not exposed to iBEC. ^{125}I-TyrA14-insulin degraded by HepG2 cells eluted much later, for reference (dotted line, insert).