System-L amino acid transporters play a key role in pancreatic β-cell signalling and function

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Abstract
The branched-chain amino acids (BCAA) leucine, isoleucine and valine, are essential amino acids that play a critical role in cellular signalling and metabolism. They acutely stimulate insulin secretion and activate the regulatory serine/threonine kinase mammalian target of rapamycin complex 1 (mTORC1), a kinase that promotes increased β-cell mass and function. The effects of BCAA on cellular function are dependent on their active transport into the mammalian cells via amino acid transporters and thus the expression and activity of these transporters likely influence β-cell signalling and function. In this report, we show that the System-L transporters are required for BCAA uptake into clonal β-cell lines and pancreatic islets, and that these are essential for signalling to mTORC1. Further investigation revealed that the System-L amino acid transporter 1 (LAT1) is abundantly expressed in the islets, and that knockdown of LAT1 using siRNA inhibits mTORC1 signalling, leucine-stimulated insulin secretion and islet cell proliferation. In summary, we show that the LAT1 is required for regulating β-cell signalling and function in islets and thus may be a novel pharmacological/nutritional target for the treatment and prevention of type 2 diabetes.

Introduction
The branched-chain amino acids (BCAA), leucine, isoleucine and valine, are essential amino acids that play an important role in glucose homeostasis. In vitro, leucine stimulates insulin secretion (Newsholme et al. 2007) and improves insulin secretory function of human islets (Yang et al. 2006). Studies on the effect of diets containing increased BCAA or leucine on glucose homeostasis in vivo have been contradictory, likely due to differences in diet regimens and models. However, there is evidence that high protein diets, a source of BCAA, or leucine supplementation enhances insulin secretion and glucose clearance in healthy adults and type 2 diabetics (Linn et al. 1996, van Loon et al. 2003, Kalogeropoulou et al. 2008, Leenders & van Loon 2011). Moreover, human neonates fed high protein formula milk have significantly raised serum leucine which parallels increased C-peptide (Socha et al. 2011).

Branched-chain amino acids, and particularly leucine, are also critical for the activation of mammalian target of rapamycin complex 1 (mTORC1), as they...
promote the translocation of mTORC1 to GTP-loaded Rheb, its upstream activator located at the lysosome (for review see Bar-Peled & Sabatini 2014). This process is dependent on a complex of proteins including RAGS (RAS-related GTPases), the Regulator, v-ATPase, GATOR (GAP activity towards RAGS) and folliculin. Yet how changes in the amino acid concentration are sensed is still unclear. Although there is growing evidence to suggest that changes in the intra-lysosomal concentration play a significant role. Importantly, mTORC1 is a kinase whose hyperactivation in mice has been shown to stimulate both pancreatic β-cell function and mass (for reviews see Blandino-Rosano et al. 2012, Xie & Herbert 2012). For example, β-cell-specific deletion of tuberous sclerosis complex-2 (Tsc2), a negative regulator of mTORC1, or β-cell-specific expression of constitutively active Rheb, a small G protein which binds to and activates mTORC1, results in increased β-cell mass due to increased proliferation and cell size (Rachdi et al. 2008, Balcazar et al. 2009). Mice expressing constitutively active protein kinase B (PKB) in β-cells also have increased β-cell mass due to increased mTORC1 activity (Elghazi et al. 2007). Therefore, BCAA-dependent activation of mTORC1 may be important in β-cell compensation and hence in protecting against the development of type 2 diabetes.

The effects of amino acids on β-cell signalling, function and mass are likely mediated through changes in their intracellular concentration. Importantly, amino acid transporters play an important part in determining the size and composition of the intracellular amino acids pool. There are several amino acid transporter Systems facilitating transport of large neutral amino acids (i.e., the BCAA and System-L amino acids was made using individual amino acids from Table 1). The dispersed cells were plated on a poly- d-lysine coated plates and incubated in

Materials and methods

Chemicals and media

All chemicals and media were purchased from Sigma-Aldrich unless otherwise stated. EBSS (Earle's Balanced Salt Solution) contained 20 mM glucose, 2 mM l-glutamine and P/S/N (50 µg/mL streptomycin, 50 units/mL penicillin sulphate and 0.1 mg/mL Neomycin) and the pH was adjusted to 7.4 using NaHCO3. MEM essential amino acids solution (1×) was made from a 50× MEM stock (Sigma M5550) and contained 0.1264 g/L l-Arg·HCl, 0.03128 g/L l-Cys·2HCl, 0.042 g/L l-His·HCl·H2O, 0.0525 g/L l-Ile, 0.0524 g/L l-Leu, 0.0725 g/L l-Lys·HCl, 0.0151 g/L l-Met, 0.033 g/L l-Phe, 0.0476 g/L l-Thr, 0.0102 g/L l-Tyr, 0.036 g/L l-Tyr, and 0.0468 g/L l-Val. MEM essential amino acid mixture (1×) without System-L amino acids was made using individual amino acids purchased from Sigma and contained 0.1264 g/L l-Arg·HCl, 0.03128 g/L l-Cys·2HCl, 0.042 g/L l-His·HCl·H2O, 0.0725 g/L l-Lys·HCl, 0.0151 g/L l-Met, and 0.0476 g/L l-Thr. DMEM minus leucine media was made in-house from its constituent components.

Cell culture and treatment

Rat insulinoma 1E (INS1E) cells (Merglen et al. 2004) were maintained in a humidified incubator (95% air, 5% CO2) at 37°C in RPMI-1640 medium containing 11.1 mM glucose, supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS) (Invitrogen, Thermo Scientific Inc, Waltham, MA, USA), 1 mM sodium pyruvate, 10 mM HEPES, 55 µM β-mercaptoethanol and P/S/N (50 µg/mL streptomycin, 50 units/mL penicillin sulphate and 0.1 mg/mL Neomycin). Before treatments, INS1E cells were serum starved overnight (16 h) in CMRL-1066 medium containing 0.5 mM l-glutamine and P/S/N. The cells were treated as described in the figure legends.

Pancreatic islets were isolated from Male Wistar Albino rats weighing 200–250 g and dissociated as described previously (Moore et al. 2011). The dispersed cells were plated on a poly- d-lysine coated plates and incubated in
RPMI-1640 media containing 10% FBS and P/S/N. The cells were treated as described in the figure legends.

**SDS–polyacrylamide gel electrophoresis and Western blot analysis**

SDS–Page and Western blotting were performed as described previously (Moore et al. 2009) using anti-phospho Akt/PKB (Ser473), anti-Akt/PKB, anti-phospho S6K1 (Thr389), anti-phospho ribosomal protein S6 (Ser240/244) and anti-LC3 A/B from Cell signalling Technologies, Beverly, MA, USA, and anti-riboosomal protein S6 obtained from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA.

**Amino acid analysis**

Amino acid analysis was performed essentially as described previously (Evans et al. 2007). After treatments, INS1E or islets of Langerhans were rapidly chilled on ice, rinsed three times with ice-cold 1× PBS to remove extracellular amino acids and deproteinised by scraping with 150 µl of 0.3 M perchloric acid. The resulting lysate was transferred to a microcentrifuge tubes on ice, followed by incubation for 14 hr at 0°C with 0.3 M perchloric acid. The resulting lysate was transferred to a microcentrifuge tubes on ice, followed by incubation for at least 30 min. Precipitated protein was sedimented by centrifugation (10 min, 14,000 g) and retained for total protein assay. The supernatant was filtered through a 0.45 µm microfilter and was immediately used for determination of amino acids on an Agilent 1100 high-performance liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) with Zorbax Eclipse AAA column (4.6×75 mm, 3.5 µm) at 40°C with o-phthalaldehyde/3-mercaptopropionate/9-fluorenylethylchlorofomate precolumn derivatisation and ultraviolet and fluorimetric postcolumn detection.

**RNA isolation for qRT-PCR**

peqGOLD Total RNA Kit (VWR international, Germany) was used in RNA extraction for qRT-PCR according to manufacturer’s protocol. Islets of Langerhans were cultured for 2 days before RNA extraction. 2 µg of total RNA from each extraction were used as a template for reverse-transcription using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). qRT-PCR was then performed on cDNA from 1 ng RNA using gene-specific primers from PrimerDesign (Southampton, UK); Rat LAT1 forward 5'-TTGTTCCGATCTAGCAGATG-3' and reverse 5'-ATTATCCGTCTCCGTTGTCAGT-3'; Rat LAT2 forward 5'-CCAGGTTCTCTCTCCTCCTCTC-3' and reverse 5'-CAAGATTCAATGGCCATCTCGTCTC-3'; Rat LAT3 forward 5'-ACACTGAAGTTTTGTCTCCGTTG-3' and reverse 5'-ACATGCTTATGTGTGTGAC-3'; and Rat LAT4 forward 5'-CAGAAGCGAGACGGAGATG-3' and reverse 5'-TGAGGGCGAGTGGAGGG-3'; and 18s RNA primers forward 5'-GGTGGTTTCCGAGAC-3' and reverse 5'-GCATCTTTTGTTGGAGAAAC-3'.

**Transfection of siRNAs**

Rat islets of Langerhans were dispersed in a 24-well plate and cultured overnight in rat islet growing medium before transfection with Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM medium (Invitrogen) according to the manufacturer’s instructions. For LAT1 knockdown, the cells were transfected for 72 h with 100 nM on-target plus siRNA (Dharmacon) against Slc7a5 (L-092749–01-0005) or 100 nM Silencer Select Pre-Designed siRNA (Invitrogen) against Slc7a5 (s132356).

**Proliferation assay**

Rat islet cell proliferation assays were performed essentially as described previously (Kwon et al. 2006, Xie et al. 2014). A total of 150 rat islets of Langerhans per treatment were used for proliferation assay. Islet cells were starved in RPMI media supplemented with 0.2% FBS and treated with DMEM+16.7 mM glucose in the absence or presence of 0.8 mM L-leucine or 2.5 mM BCH for 48 h; 2 µCi/mL [3H]leucine was added during the last 24 h. The cells were then collected and washed twice with 1× phosphate buffered saline (PBS). DNA was precipitated on ice upon the addition of 5% trichloroacetic acid for 30 min. The samples were centrifuged at 16000×g, 4°C for 10 min and the DNA pellets were then solubilised by adding 0.1 N NaOH and incubated for 30 min at RT. [3H]leucine incorporation was determined by quench-corrected scintillation counting using the LS6500 multi-purpose scintillation counter to detect DPM (disintegrations per minute) in each sample.

**Leucine transport assays**

The cells were preincubated for 10 min in the culture incubator in Leucine-free Krebs Ringer Buffer (KRB), L-[4,5-3H]leucine (Perkin Elmer) plus unlabelled L-leucine were then added to the KRB to give a final radioisotope concentration of 5 µCi/mL and a final total L-leucine concentration of 0.8 mM. BCH was then added to some cultures to give a final concentration of 2.5 mM. The cultures with [3H] were then incubated in the culture incubator for exactly 5 min. The cells were then immediately placed on ice, the medium was aspirated off and the cells were rapidly washed 3× in ice-cold KRB.
0.05 M NaOH was then added to the cells and the lysates transferred to microcentrifuge tubes. The lysates were then incubated at 70°C for 30 min. A fraction of the lysate was then transferred to a scintillation vial containing Ecoscint A scintillant and allowed to stand for at least an hour to allow chemiluminescence to decay before quench-corrected scintillation counting using the LS6500 multi-purpose scintillation counter. \( l^-{[4,5-3H]} \text{leucine} \) transport rate is expressed as DPM/mg protein/5 min.

**Insulin secretion assay**

Following treatments, the test media were collected and centrifuged at 2000 g for 3 min to pellet any detached cells. The supernatants were collected and the insulin concentrations measured using a Rat Insulin Enzyme Immunoassay Kit (Bertin Pharma, A05105–96 wells) with rat insulin as a standard in accordance with manufacturer’s instructions. The absorbance was read at 414 nm on the NOVOSTAR microplate reader.

**Statistical analysis**

The results are expressed as means ± SEM. Data were analysed by ANOVA, followed by Bonferroni correction for all pair-wise comparisons or two-tailed paired T-test using GraphPad Prism 6.0. Significance was assigned at \( P < 0.05 \).

**Results and discussion**

**System-L transporters are critical for mTORC1 activation in the rodent clonal pancreatic \( \beta \)-cell line INS1E**

The System-L amino acid transporters are widely expressed, and members of its family are capable of transporting large neutral amino acids including the BCAA. Therefore, we initially investigated the role of the System-L family of amino acid transporters in signalling to mTORC1 in \( \beta \)-cells.

Clonal rat pancreatic \( \beta \)-cells (INS1E cells) were treated with insulin to activate mTORC1, in the presence or absence of BCH (2-aminobicyclo-(2,2,1) heptanecarboxylic acid), a competitive inhibitor of System-L, or in the presence or absence of the System-L amino acids substrates: \( l^-{\text{Leu}}, l^-{\text{Ile}}, l^-{\text{Val}}, l^-{\text{Phe}}, l^-{\text{Tyr}} \) and \( l^-{\text{Trp}} \) (Fig. 1A). As anticipated, the addition of insulin led to a robust increase in mTORC1 activity as determined by the phosphorylation of its downstream target S6 kinase 1 (S6K1) on Thr389 and a substrate of S6K1, ribosomal protein S6 (rpS6), on Ser240/244. Insulin also stimulated the activation of PKB, as assessed by its phosphorylation on Ser473 (Fig. 1A). The addition of BCH or the withdrawal of System-L substrates (i.e., \( l^-{\text{Leu}}, l^-{\text{Ile}}, l^-{\text{Val}}, l^-{\text{Phe}}, l^-{\text{Tyr}} \) and \( l^-{\text{Trp}} \)) significantly inhibited insulin-stimulated mTORC1 activation. In addition, BCH induced the conversion of cytosolic LC3-I to the lipid-bound LC3-II, which is widely used as the measurement of autophagic flux (Klionsky et al. 2008), indicating that BCH promotes autophagy, possibly through its inhibition of mTORC1 (Jewell et al. 2013). Interestingly, BCH also inhibited insulin-stimulated phosphorylation of PKB. In contrast, the withdrawal of System-L amino acids had no significant effect on the conversion of LC3 or on the phosphorylation of PKB. In summary, these results provide good evidence that the System-L transporters play a critical role in signalling to mTORC1 and may also influence autophagy and PKB activity.

The effects of BCH and the removal of System-L substrates on cellular signalling are possibly mediated by a decrease in the intracellular concentration of BCAA. To investigate this, the effect of BCH or System-L AA withdrawal on the intracellular amino acids pool was determined by HPLC analysis (Fig. 1B). System-L AA withdrawal led to a dramatic decrease in the intracellular concentration of the BCAA, but had no significant effect on the intracellular concentration of the aromatic amino acids, likely reflecting their low rate of catabolism. The addition of BCH caused a significant decrease in the intracellular concentration of leucine and a trend towards a decrease in the other BCAA, indicating the importance of System-L transporters in the uptake of BCAA in \( \beta \)-cells. To directly investigate the role of System-L transporters on BCAA transport into INS1E cells, the effect of BCH on \( {^3H} \)leucine uptake into INS1E cells was also determined (Fig. 1C and E). BCH caused a significant decrease in the uptake of \( {^3H} \)leucine, demonstrating that System-L transporters play an important role in BCAA uptake into INS1E cells.

**The uptake of \( l^-{\text{leucine}} \) through System-L amino transporters is required for mTORC1 activation**

To determine which System-L amino acids are required for signalling to mTORC1, INS1E cells were depleted of System-L amino acids and the effect of the re-addition of each System-L amino acid in turn on the restoration of insulin signalling to mTORC1 was assessed (Fig. 2A). The re-addition of \( l^-{\text{Leucine}} \) significantly restored insulin signalling to mTORC1, whereas the re-addition of \( l^-{\text{Isoleucine}}, l^-{\text{Valine}}, l^-{\text{Phenylalanine}}, l^-{\text{Tyrosine}} \) and \( l^-{\text{Tryptophan}} \) had little-to-no effect on mTORC1. Thus, of
the System-L substrates, only L-Leucine is able to restore insulin signalling to mTORC1. These results confirm and extend previous findings in the rat insulinoma cell line RINm5F and isolated rat islets (Xu et al. 1998).

To investigate whether L-leucine-stimulated mTORC1 activation was indeed dependent upon System-L transporters, INS1E cells depleted of leucine were incubated with 0.8 mM or 0.4 mM L-leucine in the presence or absence of 10 mM BCH (Fig. 2B). As anticipated, the re-addition of leucine stimulated mTORC1 activation as determined by the phosphorylation of S6K1 and rpS6. Surprisingly, leucine re-addition also inhibited the phosphorylation of PKB on S473 (Fig. 2B). This is possibly mediated by a well-characterised mTORC1-dependent feedback mechanism.
Journal of Molecular Endocrinology

System-L transporters and the pancreatic β-cell

A

– + + + + + + + + Insulin System–L AAs (1X)
+ + – – – – – + 1 x AA
L I V F Y W
PKB
P–PKB S473
P–S6 K389
P–S6 S240/244
rpS6

B

– + + – – – – Leucine (0.8 mM)
– + – – + + + Leucine (0.4 mM)
– + – – + + + BCH (10 mM)
P–PKB S473
P–S6 K389
P–S6 S240/244
Total rpS6

C

Leucine (mM)
P–S6 K389
P–S6 S240/244
rpS6

EC50 0.267 x 10⁻³M

Leucine (mM)

D

– + + + + Leucine
+ + + Nifedipine

P–S6 S240/244
Total rpS6

Figure 2

The system–L amino acid substrate leucine is required for signalling to mTORC1 in INS1E cells. (A) Serum starved INS1E cells were incubated for 30 min in EBSS containing 20 mM glucose, 2 mM l-glutamine supplemented with either 1 MEM EAA (+ System–L AAs) or 1 x MEM EAA minus System–L amino acids (− System–L AAs) in the presence or absence of each individual System–L amino acid substrate (single letter amino acid code) as specified. Where indicated, 100 mM insulin (insulin) was also included. (B) BCH inhibits leucine-stimulated mTORC1 activation in INS1E cells. INS1E cells were pre-incubated in DMEM minus l-leucine for 1 h. The cells were then incubated for a further 30 min in DMEM minus leucine supplemented with either 0.8 mM or 0.4 mM leucine in the presence or absence of 2.5 mM BCH. (C) Leucine stimulates mTORC1 in a dose-dependent manner. INS1E cells were pre-incubated in DMEM minus l-leucine for 1 h, followed by 30 min treatment in DMEM minus leucine supplemented with increasing concentration of l-leucine as indicated. Leucine concentration response curves and corresponding EC50 are shown below. The results presented are the mean (± s.e.m) of at least three independent experiments. (D) INS1E cells were pre-incubated in DMEM minus l-leucine for 1 h. The cells were then incubated for a further 30 min in DMEM minus leucine supplemented with either 0.8 mM leucine in the presence or absence of 10 µM Nifedipine. In all cases, proteins were resolved by SDS–PAGE, and Western-blotted using antisera against the proteins indicated. All results are representative of at least three independent experiments.

in which S6K phosphorylates insulin receptor substrate and inhibits insulin signalling (Harrington et al. 2004, Tremblay et al. 2007). Importantly, in the presence of BCH, leucine-stimulated mTORC1 activation was inhibited. Moreover, BCH inhibited PKB and induced autophagy as assessed by the conversion of cytosolic LC3-I to the lipid-bound LC3-II (Fig. 2B).

In order to define the EC50 of l-leucine to activate mTORC1, INS1E cells preincubated in leucine-free media were treated for 30 min with increasing concentrations of l-leucine (Fig. 2C). l-Leucine activated mTORC1 in a dose-dependent manner with EC50s of 267 µM and 227 µM for S6K1 and rpS6 phosphorylation, respectively, with a maximum response at approximately 2 mM (Fig. 2C). Nifedipine, an l-type VGCC inhibitor, is unable to block leucine-stimulated activation of mTORC1 (Fig. 2D) and thus the effects of leucine on mTORC1 are not due to leucine-stimulated insulin secretion.

To investigate the temporal regulation of mTORC1 activation upon leucine re-addition or withdrawal, INS1E cells were either depleted of leucine (Fig. 3A) or leucine added to leucine-starved cells (Fig. 3B) for up to 30 min, and changes in PKB and mTORC1 activity were determined (Fig. 3). The phosphorylation of S6K1 at Thr389 and rpS6 rapidly decreased within 10 min of l-leucine withdrawal, and further decreased to an undetectable level by 15 min (Fig. 3A). The phosphorylation of PKB at Ser473 was also significantly decreased by 5 min of l-leucine withdrawal; however, PKB phosphorylation started to recover by 15 min and returned to basal levels by 30 min (Fig. 3A). In addition, the conversion of LC3-I to LC3-II increased within 5 min of l-leucine withdrawal, indicating the induction of
autophagy (Fig. 3A). The re-addition of leucine to leucine-starved cells caused a rapid (within 5–10 min) increase in mTORC1 activation as determined by the phosphorylation of S6K1 and rpS6 (Fig. 3B). The phosphorylation of PKB at Ser473 also rapidly decreased but returned to basal levels by 30 min (Fig. 3B). In addition, the abundance of LC3-II rapidly decreased, indicating an inhibition of autophagy (Fig. 3B). Given the rapidity of these events, we investigated whether these changes in mTORC1 activity correlated with changes in the intracellular leucine concentration (Fig. 3C). The withdrawal of extracellular leucine for 10 min resulted in the depletion of intracellular leucine whereas within 10 min of its re-addition the intracellular concentration of leucine returned to basal levels (Fig. 3C). Thus under these conditions, changes in the extracellular concentration of leucine are rapidly mirrored by changes in their intracellular concentration, which reflect the activation state of mTORC1.

System-L transporters are required for islet cell function and signalling to mTORC1

Having established the importance of System-L transporters in clonal pancreatic β-cells, we wished to investigate their potential role in primary β-cells (Fig. 4). Therefore, islets of Langerhans were isolated from rats and deprived of leucine before re-addition of the System-L substrate l-leucine in the presence or absence of BCH (Fig. 4A). The re-addition of l-leucine significantly stimulated mTORC1, as determined by the phosphorylation state of S6K1 at Thr389 and rpS6 on S240/244, and reduced the phosphorylation of PKB at Ser473. The co-addition of BCH significantly inhibited leucine-stimulated mTORC1 activation and also induced autophagy as measured by the conversion of LC3-I to LC3-II (Fig. 4A). These results are similar to that observed in INS1E cells (Fig. 2).

**Figure 3**
The temporal kinetics of leucine-dependent mTORC1 activation. INS1E cells were pre-incubated in (A) DMEM containing 0.8 mM l-leucine or (B) DMEM minus leucine for 1 h followed by the incubation in (A) DMEM minus leucine or (B) DMEM containing 0.8 mM l-Leucine for up to 30 min. (A and B) Proteins were resolved by SDS–PAGE and Western blotted using antisera against the proteins indicated. The results are the mean ± s.e.m. for at least three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control (i.e., 0 min time point). (C) Left 2 bars: INS1E cells pre-incubated in DMEM + 0.8 mM Leu for 1 h followed by incubation in DMEM minus Leu for 10 min; right 2 bars: pre-incubated in DMEM minus Leu for 1 h followed by incubation in DMEM + 0.8 mM Leu for 10 min. The cells were harvested and the intracellular concentration of leucine determined by HPLC.
System-L transporters and the pancreatic β-cell

Leucine can also stimulate insulin secretion (Newsholme et al. 2007), therefore, in order to ascertain the role of System-L transporters in leucine-stimulated insulin secretion, rat islets of Langerhans were incubated in leucine-depleted media for 1 h before addition of leucine for 30 min in the presence or absence of BCH. As anticipated, leucine stimulated insulin secretion, but this was significantly inhibited by the presence of BCH (Fig. 4B), showing that leucine-dependent stimulation of insulin secretion is dependent upon System-L transport activity.

As mTORC1 activation promotes β-cell proliferation (Xie et al. 2014), the role of System-L transporters on islet cell proliferation was also investigated. Dispersed islets were incubated either in the absence of leucine or in the presence of BCH, and islet cell proliferation was measured by the incorporation of [3H]thymidine into DNA (Fig. 4C). The addition of BCH or the removal of leucine significantly inhibited islet cell proliferation. Thus, System-L transporter activity is required for islet cell proliferation.

To investigate the effect of BCH or leucine withdrawal on the intracellular leucine concentration, HPLC amino acid analysis was performed (Fig. 4D). The removal of leucine caused the depletion in leucine, whereas its re-addition restored intracellular leucine concentration. The re-addition of leucine in the presence of BCH caused a significant decrease in the intracellular concentration of leucine, indicating that leucine uptake is mediated by System-L transporters. Moreover, [3H]leucine uptake into cells was significantly inhibited by BCH (Fig. 4E), providing further evidence that System-L transporters play a significant role in leucine transport into islets.

System-L transporter expression and function in islets of Langerhans

Having established that System-L transporters play a significant role in β-cell signalling, we wished to investigate which of the System-L transporters were likely responsible. There are four System-L amino acid: LAT1, LAT2, LAT3
LAT1 is highly expressed in primary islets. (A) qPCR analysis of LAT1, LAT2, LAT3 and LAT4 expression in isolated rat islets of Langerhans expressed as fold change in expression of the target genes (LAT1, LAT2, LAT3 and LAT4) relative to the reference gene (18S RNA). The mean normalized expression (MNE) of the target genes was calculated using 2−ΔΔCT method. Expression values were multiplied by 10² for clarity of presentation. The results presented are the mean ± s.e.m of three independent experiments. (B) Rat islets of Langerhans were transfected with siRNAs against: CD98 (Slc3a2) or scrambled siRNA as control. After 72 h the cells were incubated in DMEM minus Leucine for 1 h, followed by the re-addition of 0.8 mM leucine for 30 min. Proteins were resolved by SDS–PAGE, and Western blotted using antisera against the proteins indicated. The results are graphically represented below showing the mean ± s.e.m for three independent experiments. ***P<0.001, **P<0.01, *P<0.05 (A.U. = arbitrary unit).

Both LAT1 and LAT2 are obligate heterodimers that require CD98 for function. Therefore, to inhibit the function of both LAT1 and LAT2, and not LAT3 and LAT4, the expression of CD98 was downregulated using siRNA (Fig. 5B). Knockdown of CD98 caused a significant decrease in the activation of mTORC1 as determined by the phosphorylation of S6K. There was also a trend towards a decrease in both PKB and rpS6 phosphorylation, but these changes proved statistically insignificant. Taken together these data would suggest that LAT1 likely plays a significant role in regulating mTORC1 in β-cells.

**LAT1 plays an important role in islet signalling to mTORC1 in islets of Langerhans**

As LAT1 is the abundantly expressed System-L transporter in the islets and siRNA-mediated knockdown of CD98 inhibits mTORC1 (Fig. 5), we focused on the role of LAT1 in islets. Two distinct siRNAs directed against the mRNA encoding LAT1 (Slc7a5) (labelled LAT1-A and LAT1-B) were transfected into dispersed islet cells (Fig. 6). The efficiency of LAT1 knockdown, as determined by qRT-PCR quantification, was approximately 41% and 59% using siRNAs LAT1-A and LAT1-B, respectively (Fig. 6C and D). The knockdown of LAT1 expression using both siRNAs had no detectable effect on cell morphology (unpublished observations) but caused a significant decrease in 1-leucine-stimulated phosphorylation of S6K1 at Thr389, demonstrating that LAT1 is required for mTORC1 activation (Fig. 6A and B). It also consistently caused a decrease in the phosphorylation of rpS6 at Ser240/244; however, this proved statistically insignificant. Interestingly, the knockdown of LAT1 using siRNA LAT1-B significantly inhibited PKB phosphorylation. Although no statistically significant change in the phosphorylation of PKB was detected using siRNA-A, there was a trend towards a decrease in PKB phosphorylation. In conclusion, the System-L transporter LAT1 plays an important role in signalling to mTORC1 and possibly PKB in islet cells.

**LAT1 plays an important role in islet function and proliferation in islets of Langerhans**

To investigate the role of LAT1 in islet function and proliferation, the expression of LAT1 was knockdown in dispersed islets of Langerhans using siRNA. siRNA-mediated reduction in LAT1 expression significantly inhibited leucine-stimulated insulin secretion (Fig. 7A). Moreover, islet cell proliferation, as determined by the incorporation of [3H]thymidine into DNA, was also significantly inhibited (Fig. 7B). In conclusion, the System-L transporter LAT1 plays an important role in β-cell proliferation and insulin secretion. Although a reduction in LAT1 expression decreases mTORC1 activation, and findings from others and our studies have shown that mTORC1 is required for β-cell proliferation (Balcazar et al. 2009, Xie et al. 2014), it is difficult to confirm whether the effects of reducing LAT1 expression on proliferation is indeed mediated by the inhibition of mTORC1. However, these effects are unlikely due to the potential positive effects of leucine on insulin...
secretion, as these experiments were carried out in nutrient-rich media containing a high concentration of glucose.

**Discussion**

In this report, we provide evidence that System-L transporters, and in particular LAT1, play a critical role in β-cell signalling to mTORC1 and the maintenance of β-cell function and proliferation. LAT1 is highly expressed in rat, mouse and human islets of Langerhans ((Fig. 5) and (Fukushima et al. 2010, Nakada et al. 2014, Zhou et al. 2014)) and is also highly expressed in testicular sertoli cells, ovarian follicular cells and proximal to proliferative zones in the gastrointestinal mucosa (Nakada et al. 2014). In addition, LAT1 is widely expressed in non-epithelial cells showing its highest expression in endothelial cells forming the blood–brain barrier in the brain, indicating that it is also important in transporting amino acids across endothelial–epithelial secretory barriers such as blood–brain barrier (Mastroberardino et al. 1998, Vertrey 2003). As LAT1 expression is upregulated in a range of tumours and is widely expressed in foetal tissue, LAT1 is thought to be particularly important in transporting amino acids into highly proliferating cells (Nakada et al. 2014, Yanagisawa et al. 2014). As LAT1 is an obligate amino acid exchanger, its activity is not only governed by extracellular amino acid concentration but also by the intracellular amino acid concentration controlled by, for example, metabolism and the activity and expression of other amino acid transporters (Meier et al. 2002, Verrey 2003). These include...
incubated for 72 h. 1 µCi/ml 3H-thymidine was added in the last 24 h.

transfected with LAT1 or scrambled siRNA as control and collected after treatments. (B) Dispersed rat islets of Langerhans were incubated in DMEM minus leucine for 1 h followed by the re-addition of (or scrambled siRNA as control. After 72 h the cells were Slc7a5

LAT1 is required for islet cell function and proliferation. Dispersed rat islets of Langerhans were transfected with siRNAs against: (A) LAT1 and experimental conditions. Another possibility is that they could be simply due to differences in cell systems of transport required for the activation of mTORC1, or

ER stress, probably via ATF4 (Harding et al. 2003, Liu et al. 2004, Wang et al. 2011a, Krokowski et al. 2013, Cnop et al. 2014). As β-cells undergo ER stress in the face of obesity and insulin resistance, it is possible that LAT1 expression is increased, which in turn leads to increased mTORC1 activation and a compensatory increase in β-cell function and mass. Therefore, it is important to establish whether there are changes in LAT1 expression in β-cells during both the progression of obesity and type 2 diabetes.

Amino acid transporters have also been proposed to act as receptors that sense changes in the extracellular/intracellular amino acids concentration, possibly by monitoring amino acid flux through the transporter, to transduce an intracellular signal (Hundal & Taylor 2009). For example, SNAT2 has been proposed to act as a ‘transceptor’ and regulate PKB activity (Evans et al. 2007, 2008). Interestingly, BCH inhibited PKB activation. In addition, knockdown of LAT1 also inhibited PKB activity. How System-L transporters influence PKB activity is unknown, although it may be independent of the intracellular concentration of BCAA. Interestingly, the effects of BCH on signalling to mTORC1 and PKB are much stronger than that of amino acid withdrawal, despite the fact that amino acid withdrawal results in a greater effect on the intracellular level of leucine/BCAA. The reason for this is not entirely clear, but a possible explanation is that signalling to mTORC1 and PKB is more responsive to the l-leucine/transceptor complex than it is to the free cytosolic l-Leu concentration (Hundal & Taylor 2009).

As BCAA can activate mTORC1, a kinase that stimulates increased pancreatic β-cell mass and function (for review see (Xie & Herbert 2012)), increased LAT1 expression or activity may be anti-diabetogenic. However, the chronic hyperactivation of mTORC1 inhibits insulin/growth factor signalling mediated by ribosomal protein S6 kinase (S6k) inhibitory phosphorylation of insulin receptor substrate (Um et al. 2004, 2006). Indeed, although β-cell-specific mTORC1 gain-of-function mutant adult mice have initially increased β-cell function and mass (Shigeyama et al. 2008, Hamada et al. 2009) with age, there is a decline in β-cell function and mass mediated by feedback inhibition of mTORC1 and the induction of autophagy (Shigeyama et al. 2008, Bartolomé et al. 2014). Interestingly, obese diabetic humans have increased circulating levels of BCAA, which is predictive of T2DM progression (Newgard et al. 2009, Fiehn et al. 2010, Wang et al. 2011b). Thus, it is plausible that elevated BCAA may cause chronic activation of mTORC1 in β-cells. This could result in a critical switch from mTORC1 stimulating β-cell growth to causing β-cell dysfunction, death and the onset of diabetes. Moreover, it has recently been reported that increased amino acid flux, mediated by increased AA transporter expression, exacerbates ER stress-induced β-cell death and diabetes in the Akita mouse (Krokowski et al. 2013). Hence, although BCAA likely plays an important role in the regulation of mTORC1 activity in islet β-cells, their overall role in metabolic disease remains unclear.
positive role in maintaining glucose homeostasis, chronic increase in circulating BCAA may be a critical determinant in the development of type 2 diabetes. Although this report focuses on the role of LAT1 in β-cell signalling and function, LAT2 and LAT4 were also found to be expressed in both clonal β-cells and islets of Langerhans and thus are also likely to play important roles in the β-cell.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the authors approved the final version of the manuscript.

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Author contributions
TPH conceived and designed the study. AB helped in the design of the study and provided invaluable technical and advice. QC, VDB, JB, SC and TPH conducted the experiments. QC, VDB and TPH analysed the results. TPH wrote the manuscript and all authors approved the final version of the manuscript.

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