Research Article

Iodothyronine Interactions with the System L1 Amino Acid Exchanger in 3T3-L1 Adipocytes

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Thyroid hormones enter isolated white adipocytes largely by a System L1-type amino acid transporter en route to exerting genomic actions. Differentiated 3T3-L1 mouse adipocytes in culture express mRNA for LAT1 (the catalytic subunit of high-affinity System L1). L-[125I]-T3 uptake into 3T3-L1 adipocytes included a substantial saturable component inhibited by leucine. L-[3H]phenylalanine uptake into 3T3-L1 cells was saturable (Km of 31 μM), competitively inhibited by T3 (Ki of 1.2 μM) and blocked by leucine, BCH, and rT3 as expected for substrate interactions of System L1. Efflux of preloaded L-[3H]phenylalanine from 3T3-L1 adipocytes was trans stimulated by external leucine, demonstrating the obligatory exchange mechanism of System L1 transport. T3 (10 μM) did not significantly trans stimulate L-[3H]phenylalanine efflux, but did competitively inhibit the trans stimulatory effect of 10 μM leucine. The results highlight strong competitive interactions between iodothyronines (T3, rT3) and amino acids for transport by System L1 in adipocytes, which may impact cellular iodothyronine exchanges during altered states of protein nutrition.

1. Introduction

The thyroid hormones (THs) L-thyroxine and L-trioiodothyronine are iodothyronines exerting their major physiological effects by regulation of gene expression in target cells, which they must first enter by crossing the plasma membrane [1–3]. Iodothyronines are now known to translocate cell membranes by a variety of mechanisms, which include the sharing of transport systems for large neutral amino acids (LNAA; principally aromatic and branched-chain amino acids) and organic anions (see, e.g., [4–6] for review). TH retain a tyrosine-derived amino acid moiety within the iodothyronine molecular structure, allowing them to be accepted as substrates by LNAA transporters such as System L (notably the “System L” SLC7A5/SLC3A2 heterodimer isoform LAT1) [7, 8] and System T (MCT10; SLC16A10) [9].

White adipose tissue is an important target tissue for TH action, where effects include stimulation of adipogenesis itself [10], modulation of fatty acid synthesis via regulation of expression of lipogenic enzymes [10–12] and modulation of leptin secretion [13, 14]. TH enter rat adipocytes largely by a System L1-type amino acid transporter [15] and LAT1 mRNA are expressed in rat adipocytes [16]. System L1 is also likely to be of major importance for amino acid metabolism in adipose tissue, which is a site of significant BCAA degradation and utilization for fatty acid and sterol synthesis [17, 18]. The System L1 substrate leucine in particular appears to be involved in modulation of the mTOR signalling pathway in adipocytes [19]. The activity and expression of System L1 in adipocytes, and its regulation by TH, may therefore be of quantitative importance for whole body iodothyronine and amino acid turnover. In the present study using differentiated 3T3-L1 mouse adipocytes [20, 21], we investigate how the mechanism of System L1 transport is influenced by iodothyronines.

2. Experimental Procedures

2.1. Materials. L-[2,3,4,5,6-3H]Phenylalanine was obtained from Amersham (GE Healthcare, Slough, U.K.) and L-[125I]-Triiodothyronine from PerkinElmer (Bucks, UK). Cell culture media were obtained from GibcoBRL Life Technologies (Paisley, UK) unless stated otherwise; other chemicals
were obtained from either Sigma Chemicals (Poole, Dorset, UK) or BDH Merck Ltd (Poole, Dorset, UK).

2.2. Cell Culture. 3T3-L1 mouse fibroblasts (ATCC CL-173) were cultured at 37°C, 95% air: 5% CO₂ in Dulbecco’s Modified Eagles Medium (DMEM; high-glucose) with 10% Donor Bovine Serum (DBS) and 1% antibiotic/antimycotic (A/A) solution. Before reaching confluence, cells were resuspended by trypsinisation and reseeded into 6- or 12-well experimental plates. To differentiate into adipocytes [22], the 3T3-L1 fibroblasts were grown to confluence (preadipocytes) and then the medium was changed to DMEM with 10% foetal bovine serum (FBS) and 1% A/A solution over the duration of the differentiation process. For the first two days of differentiation, 1 μg·mL⁻¹ insulin, 5 μM isobutylmethylxanthine, and 100 μM dexamethasone were also added to the media and for the subsequent two days 1 μg·mL⁻¹ insulin alone was added. The adherent cells were allowed to fully differentiate over a further six days, then used for experiments on days 11 or 12.

2.3. ¹²⁵I]-T₃ and ³H]Phenylalanine Uptakes. Immediately prior to an experiment, media was aspirated from the wells and the cells were washed twice in PBS. Transport buffer (121 mM NaCl, 4.9 mM KCl, 2.5 mM MgSO₄, 20 mM Tris-HCl, 1 mM CaCl₂, pH 7.4) containing either L-[¹²⁵I]-T₃ at 30 kBq·mL⁻¹ or L-[³H]phenylalanine at 18.5 kBq·mL⁻¹ and any other necessary compounds (e.g., inhibitors) were then added to the cells. Once the cells had been incubated in the transport buffer for the designated time period, the buffer was aspirated and the cells were washed quickly three times in cold PBS to halt the uptake process. The cells were then lysed in 1.25 mL 50 mM NaOH overnight at room temperature prior to assay of radioactivity by liquid scintillation counting and determination of protein concentration using Bradford reagent (Biorad UK). Preliminary experiments (data not shown) demonstrated that the uptake of 5 μM [³H]phenylalanine was linear for at least 5 minutes and therefore, in all subsequent experiments reported here, phenylalanine uptake was measured over a 5-minute period. L-[¹²⁵I]-T₃ uptakes were measured over a 10-minute period [15]. The specific activity (DPM·pmol⁻¹) of radiotracers was calculated from the radioactivity (DPM) of 10 μL transport buffer measured using liquid scintillation counting; tracer uptake is expressed as pmol·mg protein⁻¹·10 min⁻¹. For trans stimulation studies, 3T3-L1 adipocytes were preincubated in transport buffer containing 2 mM leucine for 15 min prior to the initial PBS washes.

2.4. ³H]Phenylalanine Efflux. 3T3-L1 cells were preloaded with [³H]phenylalanine for a period of 15 minutes according to the uptake protocol described above. The radioactive buffer was then aspirated off and the cells were rapidly washed three times with PBS. The cells were then reincubated in (nonradioactive) transport buffer, half of which was removed at timed intervals and immediately replenished with fresh buffer. At the end of each experiment, the remaining buffer was aspirated and the cells lysed overnight in 1.25 mL 50 mM NaOH. The incubation buffer aliquots and cell lysates were assayed for radioactivity and the lysate also for protein concentration as described above.

2.5. RNA Extraction and RT-PCR. RNA was extracted from cells using TRIzol Reagent (Invitrogen, Poole, UK) according to the manufacturer’s instructions, resuspended in RNase-free H₂O and quantified by UV spectrometry. 1 μg RNA was denatured in the presence of 0.5 μg OligoDT (Oligo Synthesis Service, University of Dundee) at 70°C for 5 min then reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) at 100 U/μg RNA in 25 μL reaction buffer including 500 μM dNTPs (Fermentas) at 42°C for 1 hour. The resulting first-strand cDNA was stored at −20°C prior to use in PCR using gene-specific primers to test for specific mRNA expression. The primer sets used in the PCR program were

LAT1 forward 5’-tctcttgccattgtcacc-3’
LAT1 reverse 5’-atgactcccaggtggtagttcc-3’
LAT2 forward 5’-aagggacagacggaacac-3’
LAT2 reverse 5’-gagggagctgtgaggggtg-3’.

For each PCR, 2 μL of first strand DNA, 1 μM each primer and standard 2x GoTaq Green PCR Master Mix (Promega) were used in a 20 μL total volume. The PCR programme used was 95°C, 3 min; [94°C, 30 s; 55°C, 30 s; 72°C, 1 min] 40 cycles; 72°C, 2 min using a Thermo Scientific Hybaid Px2 thermal cycler.

2.6. Data Analysis. The data are expressed as means ±/− S.E.M for n adipocyte preparations. Statistical significance for uptake measurements was assessed by one-way ANOVA followed by a Dunnet post hoc test. For efflux measurements, Graphpad software (San Diego, CA, USA) was used to determine whether the gradients of the lines of best fit (rate constants) were significantly different from control. Differences were considered significant where P < .05.

3. Results

3.1. L-T₃ Uptake. The uptake of 50 nM L-[¹²⁵I]-T₃ into 3T3-L1 adipocytes included a substantial saturable component, being reduced by around 40% on addition of 10 μM unlabelled T₃ (Figure 1). The magnitude of this saturable component (approximately 1–1.2 pmol·mg protein⁻¹·10 min⁻¹) was similar in both adipocytes and pre-adipocytes, although total uptake was about 50% higher in pre-adipocytes. An excess of the LNAAs leucine (10 mM), inhibited over 80% of saturable T₃ uptake (Figure 1). Pre-incubation of 3T3-L1 adipocytes in transport buffer containing 2 mM leucine for 15 minutes (followed by a rapid wash in PBS) resulted in a minor trans stimulatory increase in saturable 50 nM L-[¹²⁵I]-T₃ uptake (by 20 ± 8.5%, n = 3), although this increase did not reach statistical significance.

3.2. Phenylalanine Uptake. The uptake of 5 μM phenylalanine into 3T3-L1 cells (0.465 ± 0.065 nmol·mg
6–12 measurements. ∗μ minutes in the absence (control) or presence of 10 μM unlabelled T3 or 10 mM leucine in the uptake buffer. Values are expressed ± SEM of 6–12 measurements. ∗∗P < .05; value significantly different from control using Dunnett’s Multiple Comparison Test.

**Figure 1:** Uptake of the iodothyronine L-T3 into 3T3-L1 adipocytes and pre-adipocytes. [125I]-T3 uptake was measured at 50 nM over 10 minutes in the absence (control) or presence of 10 μM iodothyronine or 10 mM amino acid in the uptake buffer. Values are expressed ± SEM of 6–12 measurements. ∗∗P < .05; value significantly different from control using Dunnett’s Multiple Comparison Test.

![Graph showing T3 uptake](image)

**Figure 2:** Effect of amino acids and iodothyronines on uptake of phenylalanine into 3T3-L1 adipocytes. [3H]phenylalanine uptake was measured at 5 μM over 3 minutes in the absence (control) or presence of 10 μM iodothyronine or 10 mM amino acid in the uptake buffer. Values are expressed ± SEM of 3 measurements (except where indicated) as % of the control uptake. **P < .01; value significantly different from control using Dunnett’s Multiple Comparison Test.

![Graph showing phenylalanine uptake](image)

3.3. Phenylalanine Efflux. Semilogarithmic plots of the cumulative loss of preloaded [3H]phenylalanine tracer from 3T3-L1 cells showed that the initial efflux was described by a single rate constant (see Figure 5(a)), indicating that phenylalanine was released largely from a single, freely exchangeable intracellular pool. Efflux of pre-loaded L-[3H]phenylalanine from 3T3-L1 adipocytes was trans stimulated in a concentration-dependent manner by external leucine (Figure 5(a)). Leucine and tryptophan (both System L substrates) were able to generate over 20 times stimulation of basal phenylalanine efflux (Figure 5(b)). T3 is known to be a substrate for System L1 amino acid transport, so we investigated the ability of T3 to trans stimulate phenylalanine efflux. T3 was unable to significantly trans stimulate L-[3H]phenylalanine efflux at 10 μM (a concentration approaching the limit of T3 solubility in culture medium), although it did inhibit the trans stimulatory effect of 10 μM leucine (see Figure 6(a)). rT3 also inhibited the trans stimulatory effect of 10 μM leucine, although T4 did not to any significant degree (Figure 6(a)). The T3 analogue TRIAC (which does not interact with System L1) had no effect on either basal or leucine-stimulated phenylalanine efflux. The effect of T3 upon leucine trans stimulated phenylalanine efflux was found to be concentration dependent (see Figure 6(b)) with 3.2 μM T3 able to inhibit 50% of 10 μM leucine trans stimulated phenylalanine efflux, demonstrating the relatively high affinity of T3 for this interaction.

3.4. Transporter mRNA Expression. We detected mRNA for LAT1, but not LAT2 (the alternative catalytic subunit...
producing System L1-type amino acid transport) in 3T3-L1 cells using RT-PCR (see Figure 7).

4. Discussion

3T3-L1 cells in culture are used extensively as an in vitro model system for studying white adipose tissue [21] and the present results demonstrate that saturable uptake of the thyroid hormone L-T3 in both pre-adipocytes and terminally differentiated 3T3-L1 adipocytes occurs largely by a mechanism inhibited by the LNAA leucine, a substrate of the multifunctional System L1 transport system shared by LNAA and iodothyronines (see [6] for review). Saturable uptake processes appear to be the predominant routes of cellular TH entry for genomic signaling via thyroid receptors in animal cells [7]. There appears to be little change in functional expression of saturable T3 transport during adipocyte differentiation (at least for 3T3-L1 cells), which is perhaps unsurprising given that T3 is important for the differentiation process itself as well as the control of mature adipocyte functions [10].

The nonsaturable “uptake” of T3 into adipocytes includes surface binding (presumably to secreted lipids and lipoproteins) and partitioning into the lipid bilayer as well as passive diffusion into the cytosol [15]. Transport of phenylalanine (a representative LNAA substrate for Systems L1, L2 and T) in 3T3-L1 adipocytes conforms almost exclusively with “classical” System L1-type function as mediated by LAT1 [23], in that (i) phenylalanine influx has a Km in the low micromolar range (31 μM), (ii) it is inhibited by BCH and (iii) phenylalanine efflux is markedly trans-stimulated in a concentration-dependent manner by both leucine and tryptophan (consistent with the obligatory exchange mechanism of System L1 [24]). These features are clearly distinct from those of other likely contributors to phenylalanine transport, specifically because (i) System L2 (LAT3/LAT4) does not recognize tryptophan as a substrate [25, 26], (ii) System T (MCT10, originally named TAT1) does not recognize leucine as a substrate [27, 28], and (iii) LAT3, LAT4, and MCT10 all utilise facilitative diffusion rather than an exchange mechanism [25–27]. Phenylalanine uptake by 3T3-L1 cells was competitively inhibited by T3 (Ki of 1-2 μM) and rT3 in a manner consistent with expected substrate interactions for the LAT1 System L1 transporter [29–32]. This strong competitive interaction between iodothyronines...
and LNAA for transport by System L1 in 3T3-L1 mouse adipocytes is consistent with our previous studies using isolated white adipocytes from rats [15], although the relatively-weak inhibitory effects of T4 in the present study may reflect minor species-specific differences in the relative affinity of different TH for LAT1. The V_{max} for phenylalanine transport in 3T3-L1 cells (2.6 nmol·mg protein^{-1}·min^{-1}) is broadly equivalent to 50 pmol·10^5 cells^{-1}·min^{-1}, a value of the same order of magnitude as that measured for System L1 transport in rat adipocytes [15].

Collectively, our results indicate that the Na^+-independent System L1 transporter contributes substantially to iodothyronine and LNAA transport in 3T3-L1 adipocytes and thus for delivery of TH from the plasma to the cytosol, where ultimately they reach the cell nucleus. System L1 has been shown to be an important effector of T3 transport in tissues including placenta and brain as well as adipose [6] for review). The LAT1 and LAT2 genes encode for alternative catalytic protein subunits of System L1 and these function as heterodimers with 4F2hc/CD988 [23] for review). LAT1 and LAT2 both mediate uptake of iodothyronines as well as LNAA [30, 31] but while both are expressed in rat adipocytes [16], it appears that only LAT1 is expressed at the mRNA level in 3T3-L1 cells. Our transport studies also indicate the predominant functional expression of LAT1 in 3T3-L1 cells. The $K_m$ values obtained for iodothyronine transport by System L1 are substantially lower than those measured for LNAA transport, which help T3 especially to compete effectively with amino acids for transport under physiological conditions (see [6] for review). High-affinity receptors for both T4 and T3 have been identified associated with the plasma membrane of adipocytes [33] and these may also facilitate preferential iodothyronine uptake [15]. To our knowledge, T3 is the most potent natural inhibitor of System L1 in the mammalian body (and easily surpasses the widely-used synthetic inhibitor BCH in terms of $K_i$, if not specificity), although Brasilicardin compounds isolated from bacteria have recently been shown to inhibit with nanomolar potency [34]. Given the therapeutic potential of System L1 inhibitors as immunosuppressants and anticancer drugs [34, 35], it is tempting to suggest that the iodothyronine skeleton might be used as a template for improved design of highly specific, non-TH active, System L1 inhibitors. Reverse T3 but not TRIAC (a T3 analog lacking an amino acid moiety which acts as a potent thyroid receptor agonist) interacts with System L1 in adipocytes, demonstrating structural specificity of System L1 which is clearly distinct from that of nuclear thyroid hormone receptors. TRIAC selectively stimulates the metabolic rate of adipose tissue in vivo, a feature considered as a potential tool to increase whole body energy metabolism [13]. It appears from our results that TRIAC enters adipocytes by a different mechanism to T3, which may help explain why TRIAC administered to rats accumulates in white adipose tissue even when coadministered with high T3 doses [13].

The bulky T3 molecule has a much reduced $V_{max}$ for transport by System L1 compared to LNAA [30], such that its specific interactions with System L1 transport may competitively inhibit not only influx but efflux of
substrate LNAA by retarding conformational changes of the transport cycle (note the inhibitory action of T3 on leucine-induced trans stimulation of phenylalanine efflux in 3T3-L1 cells). Nevertheless, the obligatory exchange mechanism of the System L1 transporter should allow limited LNAA–iodothyronine or T4–T3/rT3 exchanges across the adipocyte plasma membrane and we indeed observed a minor trans stimulation of saturable L-T3 uptake in 3T3-L1 adipocytes preincubated with leucine. It is noteworthy in this respect that adipose tissue in vivo releases small quantities of LNAA [36]. White adipose tissue depots have the capacity to deiodinate iodothyronines (especially rT3) [37, 38], thus it is conceivable that regulation of iodothyronine turnover in fat cells through this pathway would contribute significantly to modulation of whole body T4–rT3 metabolism, perhaps particularly in obese individuals. In this respect, downregulation of iodothyronine uptake by System L1 in adipocytes in the hypothyroid state, as reported previously in adipocytes from hypothyroid rats [15], would tend to reduce the sensitivity of adipose tissue to hormonal activation but...
might help conserve hormone and iodine availability to other tissues by reducing their degradation in adipose tissue. Physiological variations in plasma LNAAs concentrations, as occurs during natural feeding cycles, may impact TH transport and action in adipose (and other tissues where System L1 is the predominant TH transporter).

Abbreviations

BCAA: Branched chain amino acids  
BCh: 2-amino [2,2,1] heptane–2-carboxylic acid  
T₃: L-triiodothyronine  
T₄: L-thyroxine  
rT₃: Reverse L-triiodothyronine  
TH: thyroid hormone  
TRIAC: triiodothyroacetic acid.

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References


