Endocrine (2012) 41:501–511
DOI 10.1007/s12020-012-9594-2

ORIGINAL ARTICLE

Triiodothyronine stimulates glucose transport in bone cells

Evangelos Zoidis · Claudia Ghirlanda-Keller · Christoph Schmid

Received: 5 September 2011 / Accepted: 29 December 2011 / Published online: 19 January 2012

Abstract Thyroid hormones increase energy expenditure and bone turnover in vivo. To study whether 3,3′,5-triiodo-l-thyronine (T_3) stimulates the uptake of glucose in osteoblastic cells, PyMS (a cell line derived from rat bone) cells were kept in serum-free culture medium and treated with T_3. We measured [1-^{14}C]-2-deoxy-D-glucose (2DG) uptake and looked for expression of the high-affinity glucose transporters GLUT1 and GLUT3 by northern and western analysis. T_3 did not influence the cell number but slightly (1.3-fold) increased the protein content of the cell cultures. 2DG uptake was low in serum-deprived cell cultures and was increased by T_3 (up to 2.5-fold at 1 nmol l^{-1} after 4 days) in a dose- and time-dependent manner. Triiodothyronine at 1 nmol l^{-1} increased GLUT1 and GLUT3 abundance in membranes. Therefore, increased glucose uptake induced by T_3 in osteoblasts may be mediated by the known high-affinity glucose transporters GLUT1 and GLUT3.

Keywords Glucose transport · GLUT · Osteoblast · Triiodothyronine

E. Zoidis (✉)
Department of Nutritional Physiology and Feeding, Faculty of Animal Science and Aquaculture, Agricultural University of Athens, 118 55 Athens, Greece
e-mail: ezoidis@aua.gr

C. Ghirlanda-Keller · C. Schmid
Division of Endocrinology and Diabetology, Department of Internal Medicine, University Hospital of Zurich, 8091 Zurich, Switzerland

Introduction

Cellular uptake of glucose is mediated by facilitative glucose transporters; three isoforms are high-affinity transporters: GLUT1 which is found in most tissues (including tumors) and cell lines, GLUT3 (brain/placenta) which has the highest affinity for glucose and is particularly abundant in brain (neurons), and GLUT4 (muscle/fat) which is exclusively expressed in insulin-sensitive tissues such as striated (skeletal and cardiac) muscle and adipose tissue. Both GLUT1 and GLUT3 (mRNA and protein) are expressed in rat osteogenic sarcoma [1] and in rat osteoblastic PyMS cells [2]. We performed studies in PyMS cells, a cell line derived from rat bone (not osteosarcoma) since this cell line is responsive to insulin-like growth factor (IGF) I [3], to calcitriol [4], to an increase in extracellular calcium [5], and to parathyroid hormone [2, 6]. Surprisingly, insulin has been shown to stimulate glucose metabolism and uptake in non-osteosarcoma osteoblastic cells only at relatively high doses [2, 7, 8], although insulin receptors in osteoblasts are considered to play an important role in energy homeostasis (apparently, via an osteocalcin-dependent mechanism; [9, 10]). As opposed to insulin (with predominant effects, concerning overall glucose metabolism on classical target tissues such as liver, adipose tissue, and striated muscles), thyroid hormones (in particular, T_3) may directly affect most tissues, although a recent study has suggested that the well-known effect of thyroid hormones to increase energy expenditure may be predominantly explained via the brain [11]. Such a finding appears to be in contrast to the classical findings according to which local generation and action of T_3 within brown adipose tissue may be responsible for an increased thermogenesis in vivo [12, 13].

It has been found that T_3 directly stimulated glucose uptake in a rat liver cell line [14], in cardiomyocytes in

long-term culture [15] and in 3T3-L1 adipocytes [16], when studied in thyroid hormone-depleted medium.

Osteoblasts are equipped with thyroid hormone receptors (including TRα1) and with type 2 iodothyronine selenodeiodinase, the enzyme to catalyze local formation of T_3 by outer ring deiodination from thyroxine (T_4) [17–19]. A large number of studies have addressed gene expression in response to thyroid hormone receptor activation in bone cells (for review, see [20]), and it had been known for a long time that thyroid hormones and their receptors are essential for skeletal development [21], bone growth, and turnover (both formation and resorption) [22, 23]. However, although bone is a metabolically highly active tissue, requiring a large amount of energy for remodeling, and an important player in coordinating global energy utilization [24], studies addressing glucose uptake regulation in bone cells in vitro by T_3 have not been reported. In view of an increasing number of investigations and the ubiquitous expression of enzymes activating and enzymes inactivating thyroid hormones and of thyroid hormone receptors, there have been several proponents of the relative importance of systemic and local generation, activity and inactivation of thyroid hormones, in the brain, liver, bone, and elsewhere [11, 19, 24, 25].

PyMS cells can be kept in serum-free medium for a prolonged period of time which may be an advantage or even a prerequisite when studying effects of thyroid hormones in a model where the controls should be depleted (at least, to some extent) of thyroid hormones. We therefore used them as a model system to address the question whether T_3 directly enhanced glucose uptake by osteoblastic cells in vitro.

Triiodothyronine increases 2DG uptake in PyMS cells, not within 6 h (i.e., not in short term, as observed with IGF I), but in longer term (after 1 day, and, more markedly, after 4 days); at the same time, T_3 increases the abundance of GLUT1 and GLUT3 in membranes prepared from the cells. Since thyroid hormones increase metabolism [26] and bone turnover, we speculate that the cells meet their increased energy demand by increasing glucose uptake.

Materials and methods

Materials

Fetal calf serum (FCS), cell culture media, gentamicin, glutamine, and trypsin were purchased from Life Technologies (Grand Island, NY). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany) or from Sigma and treated with charcoal (to remove fatty acids, steroid, and thyroid hormones); recombinant human (rh) IGF I was obtained from Ciba-Geigy, Basel. Bovine parathyroid hormone (PTH, 1-34) was purchased from Bachem, Torrence, CA. Triiodothyronine (T_3, 3,3′,5-triiodo-l-thyronine, sodium salt) was purchased from Sigma, (St. Louis MO, USA) and, prior to use, dissolved as a stock solution of 1 mmol l^{-1} in 10 mmol l^{-1} NaOH. 2,4-Dinitrophenol (2-DNP) was from Sigma (St. Louis MO, USA) and dissolved as a stock solution of 0.1 mol l^{-1} in absolute ethanol. 1α,25-Dihydroxyvitamin D (1α,25$(OH)_2D_3$, calcitriol; from Hoffmann-La Roche, Basel) was dissolved (to a 0.1 mmol l^{-1} stock) in absolute ethanol.

Anti-glucose transporter 1 and 3 (rat GLUT1 and GLUT3) antibodies were purchased from FabGennix (Frisco TX, USA), the second antibody, goat anti-rabbit HRP, from Bio-Rad (Reinach, Switzerland).

Cell culture

PyMS cells, an osteoblastic cell line derived from newborn rat calvaria, were kindly provided by Dr. A. Lichtler, University of Connecticut. The cells were passaged in Falcon tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μg/ml), glutamine (2 mmol l^{-1}), and FCS (5% w/v) and kept at 37° C in an atmosphere of 5% v/v CO_2 in air. Cultures between passages 12 and 38 were used.

PyMS cells grown to confluence were detached from the dishes with 0.25% w/v trypsin and replated in Falcon multiwell tissue culture plates (35 mm diameter, 9.6 cm^2 surface area) at a density of 2×10^5 cells per well in DMEM medium containing 5% w/v FCS (day 0). Confluent monolayers formed 3 days after seeding. Cell layers were rinsed with serum-free medium and medium (Ham's F12/α-MEM, 1:1 mixture with gentamicin, 50 μg/ml, glutamine, 2 mmol l^{-1}) supplemented with charcoal-treated BSA, 0.2 g l^{-1} was added (day 3). Two days later (day 5), the media were again replaced with BSA-containing media for two additional (serum-free) days. Cells were rinsed again (day 7) with serum-free medium, and for the next 4 days (i.e., days 7–11 on the culture dishes), cells were exposed to test media, a BSA (0.2 g l^{-1})-containing F12/α-MEM 1:1 mixture to which test agents or corresponding vehicles were added as indicated. Except for some of the data presented in Table 1 and in Fig. 2, all test incubations were stopped 11 days after seeding the cultures, after 8 days without serum, and 4 days after the last (test) medium change. Aliquots of test agents and appropriate vehicle controls were added directly to the media. Treatment protocols are shown in Fig. 1.

Glucose transport studies

After culture (see above) and different treatments, monolayers were washed at room temperature with 1 ml transport

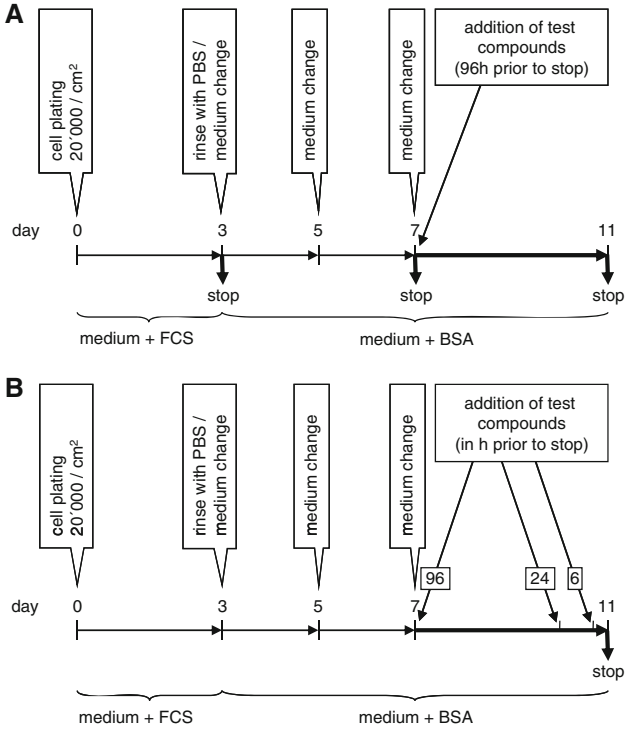

Fig. 1 **a**, **b** Flow diagram of treatment protocols. Cells were plated in serum-containing medium and kept on culture dishes for 11 days, the last 8 days without serum (in albumin-containing medium) and exposed for the last 4 days to serum-free test medium containing T3 as indicated. The protocol shown in the upper panel (**a**) was used only for the experiments shown in Table 1 and in Fig. 2

buffer containing 140 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $CaCl_2$, and 15 mmol l^{-1} N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES, adjusted to pH 7.4 with Tris–HCl). Uptake studies were initiated by adding 1 ml of transport buffer containing 0.1 mmol l^{-1} cold 2DG and 2-deoxy-D-[1-^{14}C] glucose (1 μCi/ml; 58 mCi/mmol, Amersham, UK). Ten minutes later, the buffer was removed and the dishes were quickly rinsed three times with 1 ml of ice-cold transport buffer. The cells were then solubilized with 1 ml of 2% w/v sodium dodecyl sulfate (SDS), and the radioactivity of a 0.5-ml aliquot was counted in a liquid scintillation counter [2].

Determination of protein content and alkaline phosphatase activity

In order to correct for interassay differences in protein content, identically treated dishes were used for protein analysis. After 11 days of culture (8 days in serum-free medium and 4 days in test medium), cells from parallel dishes were lysed into 1 ml of 0.1% w/v Triton X-100 for the determination of protein content by a modification of the Lowry procedure [4] or by the bicinchoninic acid (BCA) method (Pierce, Rockford IL) [6]; alkaline phosphatase activity was also measured, by cleavage of *p*-nitrophenyl phosphate to *p*-nitrophenol at pH of 10.2 [4].

Northern blot analysis

Cells were plated at a density of 1.12 × 10^6/10 cm diameter (58 cm^2 surface area) dish (Falcon) in medium containing 5% w/v FCS and grown for 3 days. Confluent monolayers were then kept in serum-free media for 8 days as above and with hormones added as indicated. Total RNA was obtained after lysis in guanidine isothiocyanate and centrifugation through a cesium chloride cushion and stored at −80°C until assayed; concentrations were determined spectrophotometrically. Comparable amounts of RNA were denatured, electrophoresed, and transferred onto gene screen membranes (DuPont-NEN) and hybridized [27].

cDNA probes of *Glut1* and *Glut3* were constructed using RNA from newborn rat brain and PCR, with the following sets of primers: *Glut1* sense primer, 5′-GCGGGAGAAGAAGGTC-3′; *Glut1* anti-sense primer 5′-AGGAGAGTGGCTGATAAAAA-3′) (GenBank accession no: M13979), and *Glut3* sense primer, 5′-TGGAAGAGCGGTTGGA-3′; *Glut3* anti-sense primer, 5′-GGCAGCGAAGATGATAAAAA-3′) (GenBank accession no: U17978), corresponding to products of 1010 bp (*Glut1*) and 1155 bp (*Glut3*). These cDNA probes have been previously described [2]. β-Tubulin cDNA probe (internal control) was used as described elsewhere [28]. The cDNA probes were labeled by random primer extension using a commercial kit (Boehringer Mannheim, Rotkreuz, Switzerland) and [α-^{32}P] deoxy-CTP (3000 Ci/mmol; Pierce Chemical, Rockford IL, USA) to specific activities of 2–4 × 10^9 cpm/μg DNA.

Western immunoblot analysis for GLUT1 and GLUT3

Cells were plated at a density of 3 × 10^6/15 cm diameter (145 cm^2 surface area) dish (TPP) in medium containing 5% w/v FCS and grown for 3 days. Confluent monolayers were kept in serum-free media for 8 days with hormones added for the test period as indicated. Medium was removed, and the cell layers were rinsed twice with ice-cold PBS; cells were scraped into 4 ml PBS and collected by centrifugation at ∼700 g for 10 min in a tube and the pellet dissolved in 5 ml homogenization buffer (20 mmol l^{-1} HEPES, pH 7.4, 2 mmol l^{-1} EDTA, 255 mmol l^{-1} sucrose, and containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 200 μmol l^{-1} PMSF as proteinase inhibitors), then glass Dounce-homogenized by 15 moves, transferred back to tubes, and centrifuged for 10 min at 700 g and 4°C; the resulting supernatant was then centrifuged at 195,000 g for 75 min, and the pellet (total membranes) redissolved in 0.8 ml. An aliquot was used for determination of the protein concentration by the BCA method, and the tubes were frozen at −80°C. Thirty

microgram samples were mixed with loading buffer, heated to 56°C, applied per lane on SDS-PAGE (10% w/v), and transferred to a 0.2-µm nitrocellulose membrane (Trans-Blot, BioRad 162-0147) for western analysis. Equal loading was confirmed by Ponceau S and actin staining. Filters were incubated overnight at 4°C in (affinity purified) primary antibody; rat GLUT1 (FabGennix Inc; Glut-101 AP) and rat GLUT3 (FabGennix Inc; Glut-301AP)), both at 1:4000, were then washed three times with TBS-T (Tris-buffered saline-Tween-20) and incubated for 1 h in secondary antibody (goat anti-rabbit HRP, BioRad 170-6515, at 1:3000) at room temperature. After washing, proteins were detected using the enhanced chemiluminescence (ECL) detection reagents (Amersham 1059250) applied as recommended by the manufacturer [2].

Densitometry of northern and western blots

Films from northern were analyzed using a BioRad Imaging Densitometer GHS-700, SW: Quantity One 45.2 (BioRad Reinach, Switzerland), and densitometry values given are expressed relative to hormone-free cell cultures on day 11, corrected for β-tubulin mRNA. GLUT1 and GLUT3 westerns were assessed by a luminescent image analyzer, LAS-3000 scanner, SW BioPackage AIDA (Fujifilm Advanced Research Laboratories, Straubenhardt, Germany).

Statistical analysis

Results were obtained by pooling data from a number (3–8) of independent experiments in which exactly the same conditions were tested, as indicated. Data are expressed as means ± SEM. Statistical significance was assessed by paired Student's t test for the data summarized in Tables 1 and 3, and by one-way ANOVA with Bonferroni's multiple comparison correction using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) for the data shown in Table 2 and in Figs. 2, 3, 5, and 6. A P-value <0.05 was considered to be statistically significant. Lineweaver–Burk trend lines were plotted with double inverse value axes for uptake (y-axis) as function of 2DG concentrations (x-axis) and used for estimation of K_m and v_{max}.

Results

Characterization of 2DG uptake stimulation in PyMS cells by IGF I and PTH following longer term culture (serum deprivation); effects of triiodothyronine and dexamethasone

Both IGF I and PTH stimulate 2DG uptake in PyMS cells (Tables 1, 2). To see whether serum withdrawal (to decrease proliferation and to get rid of serum-derived hormones) and long-term hormone pretreatment with dexamethasone or T_3 influenced 2DG uptake and the subsequent response to PTH, serum was withdrawn and cells were exposed to PTH for the final 6 h either on day 3 or on day 11 of culture (8 days serum-deprived, Fig. 1, flow diagram). 2DG uptake was significantly lower after 11 days than after 3 days of culture (Table 1). IGF I was somewhat more and PTH was much more effective with the long-term serum deprivation protocol (Tables 1, 2). T_3 did not specifically enhance the responsiveness to subsequent stimulation of 2DG uptake by IGF I or PTH, but T_3 significantly increased 2DG uptake per se (Table 2). A stimulatory effect of T_3 on protein content was also significant but much less pronounced than that on 2DG uptake (Table 3). Dexamethasone decreased 2DG uptake and apparently thereby increased the relative subsequent stimulation by IGF I and PTH (Table 2). Other striking findings included a significant decrease in protein content and 2DG uptake, and an increase in alkaline phosphatase activity, apart from the markedly increased PTH response (Table 1)

Table 1 Protein content, alkaline phosphatase activity, 2DG uptake, and responsiveness to IGF I and PTH of PyMS cells after 3 and 11 days of culture

	Day 3	Day 11
Protein content (mg/dish)	0.16 ± 0.01*	0.11 ± 0.01
Alkaline phosphatase activity (µmol/mg protein × h)	2.06 ± 0.21*	8.12 ± 0.73
2DG uptake (basal) (pmol/mg protein × 10 min)	3342 ± 152*	746 ± 34
Fold stimulation by IGF I	1.67 ± 0.06*	4.11 ± 0.25
Fold stimulation by PTH	1.16 ± 0.03*	3.57 ± 0.20

Cells were kept on 35 mm diameter multiwell dishes for 3 or 11 days, respectively

Data (means ± SEM) are given of $n = 32$ (from eight experiments) for protein content, alkaline phosphatase activity, and (basal) 2DG uptake; $n = 12$ (four experiments in triplicate) for 6 h stimulation by 1 nmol l^{-1} IGF I and by 10 nmol l^{-1} PTH

* $P < 0.05$ for comparison of 3-day culture versus 11-day culture

Table 2 Effects of long-term treatment with dexamethasone and triiodothyronine and of short-term treatment with IGF I (A) and PTH (B) on 2-deoxyglucose (2DG)-uptake in rat PyMS cells

Long term (4 days) Short term (final 6 h)	Control 2DG uptake (nmol/mg protein × 10 min)	Dex, 100 nmol l^{-1} 2DG uptake	T_3, 1 nmol l^{-1} 2DG uptake
A: IGF I			
Control	0.85 ± 0.09	0.35 ± 0.03*	2.27 ± 0.20*
IGF I, 1 nmol l^{-1}	3.26 ± 0.14*	2.67 ± 0.11**	4.12 ± 0.18**
B: PTH			
Control	0.71 ± 0.07	0.45 ± 0.04	3.27 ± 0.15*
PTH, 10 nmol l^{-1}	2.43 ± 0.13*	2.16 ± 0.18*	4.15 ± 0.15**

Cells were grown for 11 days; the culture medium contained Dex or T_3 for the last 4 days and IGF I or PTH for the final 6 h as indicated, and 2DG uptake was measured. Data (means ± SEM) from four separate experiments each (carried out in triplicate) are given, for stimulation with IGF I (A) and with PTH (B), respectively

* $P < 0.05$ for comparison of hormone treated versus control

** $P < 0.05$ for comparing combined treatment versus IGF I- or PTH-treatment alone

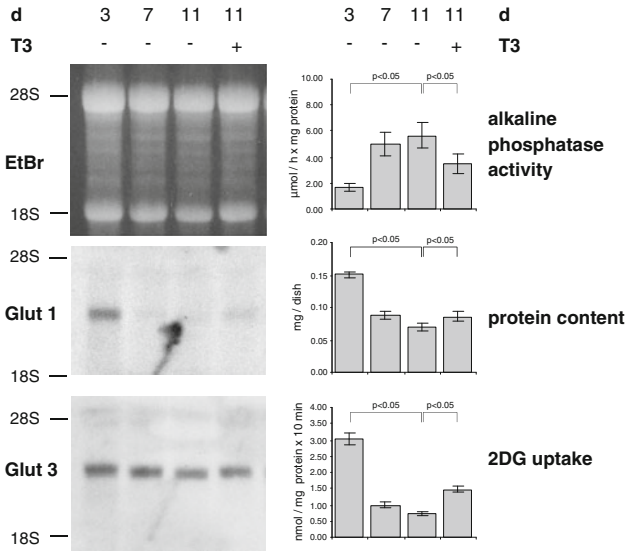

Fig. 2 Effects of serum withdrawal and of T_3 on Glut1 and Glut3 mRNA, alkaline phosphatase activity, protein content, and 2DG uptake. Northern blot analysis for Glut1 and Glut3 mRNA. Total RNA was prepared from PyMS cells (cultured as outlined in Fig. 1a, serum withdrawn on day 3 and treated for the last 4 days with hormone-free control or T_3-containing test medium) and subjected to northern. Twenty microgram RNA were loaded per lane, *bars* indicate the position of ribosomal RNA. Corrected densitometry signals found with this blot were for Glut1 mRNA 5.1 (day 3), 0.7 (day 7), 1 (by definition, day 11, control), 1.9 (day 11, T_3); for Glut3 mRNA 1.7 (day 3), 1.5 (day 7), 1 (day 11, control), 1.1 (day 11, T_3). *Columns on the right panel* (from four separate experiments carried out with the same culture protocol) show alkaline phosphatase activity, protein content, and 2DG uptake (means ± SEM)

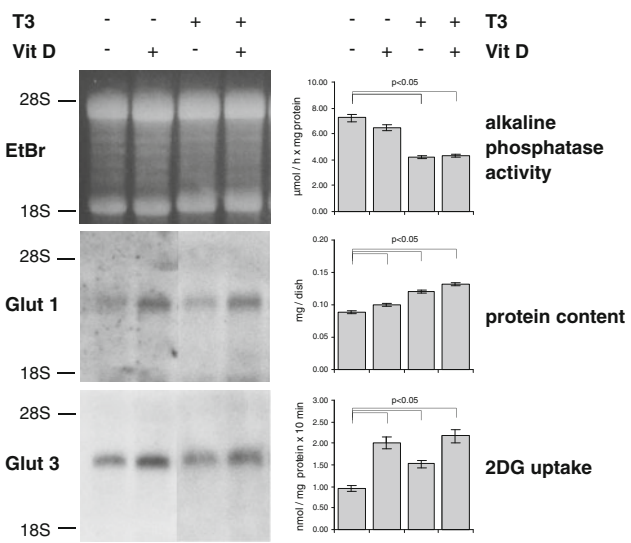

Fig. 3 Effects of T_3 and/or calcitriol on Glut1 and Glut3 mRNA, alkaline phosphatase activity, protein content, and 2DG uptake. Cells were kept on culture dishes for 11 days, the last 8 days without serum and exposed for the last 4 days to serum-free test medium containing calcitriol and/or T_3 at a concentration of 1 nmol l^{-1}. RNA was prepared for northern analysis. Corrected densitometry signals found with this blot were for Glut1 mRNA 1 (by definition, control), 1.4 (Vit D), 0.5 (T_3), 1.2 (T_3 + Vit D); for Glut3 mRNA 1 (control), 1.8 (Vit D), 1.5 (T_3), 1.9 (T_3 + Vit D). *Columns on the right panel* (from five separate experiments carried out in triplicate with the same culture protocol) show alkaline phosphatase activity, protein content, and 2DG uptake (means ± SEM)

over time when cells were kept in serum-free culture (Table 1; Fig. 2). We therefore checked both Glut1 and Glut3 mRNA expression and, in parallel, 2DG uptake over time when serum was withdrawn to characterize the condition where we wished to test the effects of T_3.

Glut1 and Glut3 mRNA

RNA was analyzed for Glut-specific transcripts by northern blot hybridization; Glut1 and Glut3 mRNAs were detected (Fig. 2). Glut1 but not Glut3 mRNA decreased markedly following serum deprivation between days 3 and 11; Glut1 mRNA became difficult to detect by northern hybridization

Table 3 Effects of T3 on cell number, protein content, 2DG uptake, Glut1 and Glut3 mRNA abundance, membrane protein yield, and relative abundance of GLUT1 and GLUT3 in rat PyMS cells

	Control	T_3
Cell number (per 9.6 cm^2 dish)	322,192 ± 13,982	323,731 ± 12,697
Protein content (mg/9.6 cm^2 dish)	0.16 ± 0.01	0.21 ± 0.01*
(treated/control)	(1)	1.34 ± 0.01*
2DG uptake (nmol/mg protein ×10 min)	1.30 ± 0.04	3.21 ± 0.15*
(treated/control)	(1)	2.46 ± 0.09*
Glut1 mRNA abundance by northern		
(treated to control)	(1)	2.2 ± 1.0
(median; range)		1.8; 0.4–4.9
Glut3 mRNA abundance by northern		
(treated to control)	(1)	1.0 ± 0.1
(median; range)		1.0; 0.4–2.7
Membrane protein yield (μg/145 cm^2 dish)	421 ± 90	542 ± 80
(treated/control)	(1)	1.26 ± 0.10*
(median; range)		1.32; 1.09–2.11
GLUT1 abundance by immunoblot		
(treated/control)	(1)	1.64 ± 0.08*
(median; range)		1.67; 1.27–1.90
GLUT3 abundance by immunoblot		
(treated/control)	(1)	2.11 ± 0.27*
(median; range)		1.77; 1.54–3.20

Cells were kept on culture dishes for 11 days. Cell numbers were counted, and 2DG uptake, alkaline phosphatase activity, and protein content were assessed from parallel (35 mm diameter, multiwell) dishes. RNA and total membranes were isolated from larger dishes. RNA was analyzed for Glut1 and Glut3 mRNA by northern and densitometry; data are given relative to controls, corrected for β-tubulin. Membrane preparations were analyzed by western analysis; the relative abundance of GLUT1 and GLUT3 is given in arbitrary densitometric units (hormone-free control defined as 1). $n = 13$ (three experiments in triplicate and two in duplicate) for cell number; $n = 23$ exp ($n = 74$) for protein content and glucose uptake; $n = 4$ for Glut1 mRNA, $n = 17$ for Glut3 mRNA; $n = 6$ for membrane preparations and westerns; the latter performed with equal amounts of TM protein, analyzed in duplicate, twice

* $P < 0.05$ for comparison of hormone treated versus control

in 11-day cultures, while Glut3 mRNA signals remained prominent (Fig. 2). Both mRNAs were barely affected by T_3 treatment (minor, inconsistent effects) for (6, 24, or) 96 h of the cells; a significant effect on Glut1 and Glut3 mRNA could not be detected, although 2DG uptake was clearly enhanced under the same conditions (Figs. 2, 3, 4, and 5; Tables 2, 3). We also tested 1α,25-dihydroxyvitamin D (1α,25$(OH)_2D_3$; calcitriol); at 1 nmol l^{-1}, it enhanced Glut1 and Glut3 mRNA as well as 2DG uptake (Fig. 3).

Characterization of PyMS cell responses to T_3 and comparison to effects of IGF I and calcitriol, and of 2,4-dinitrophenol (2-DNP)

T_3 stimulated 2DG uptake dose dependently, significantly at 0.1 nmol l^{-1} and maximally at 1–10 nmol l^{-1}; half-maximal stimulation was achieved at ~0.12 nmol l^{-1} (Fig. 4). A comparable (but much flatter) curve was seen for protein content (Fig. 4). IGF I had no effect on 2DG uptake when tested at 1 nmol l^{-1} under the same conditions as in Fig. 4, i.e., 4 days after the addition of the hormone (Fig. 5). T_3 and IGF I stimulated 2DG uptake with a quite distinct time course; IGF I stimulated 2DG uptake after 6 h (Tables 1, 2; Figs. 5, 6) but no longer after 4 days, whereas the reverse was true for T_3 which had no effect after 6 h and a marked effect after 4 days (Figs. 5, 6). Calcitriol significantly increased 2DG uptake after 4 days of exposure (Fig. 3). T_3 did not increase cell number within these 4 days (Table 3) but increased protein content (per dish) and decreased alkaline phosphatase activity per well and (more markedly) per protein (Figs. 3, 5). For comparison, the effects of T_3, IGF I, and calcitriol on 2DG uptake and protein content are given in Figs. 3 and 5.

To disrupt the oxidative chain in mitochondria, PyMS cells were treated with 2,4-dinitrophenol (2-DNP); uncoupling electron transport from ATP production. The compound could be tested at 0.1 mmol l^{-1} for incubations over the last 4 days of serum-free cultures (as in Fig. 1) and was well tolerated by the cells without obvious signs of toxicity; there were no major changes in cell morphology and no

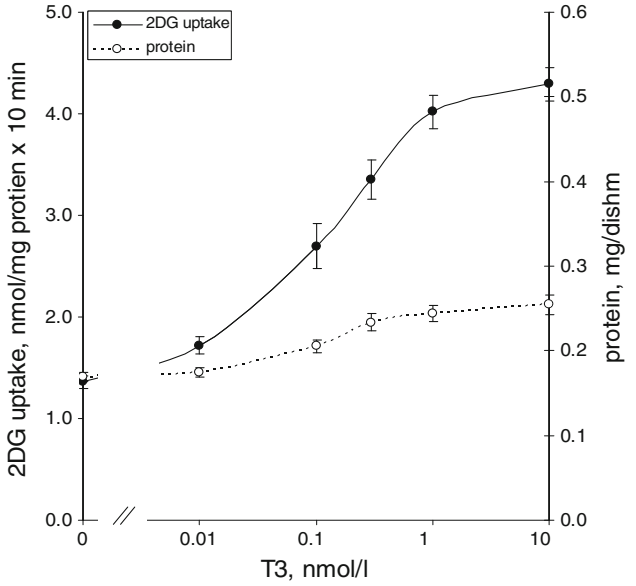

Fig. 4 T_3 dose dependency of 2-deoxyglucose (2DG) uptake and protein content. Cells were kept on culture dishes for 11 days, the last 8 days without serum and exposed for the last 4 days to serum-free test medium containing T_3 at a concentration as indicated on the abscissa. Data are given as means ± SEM from six separate experiments carried out in triplicate

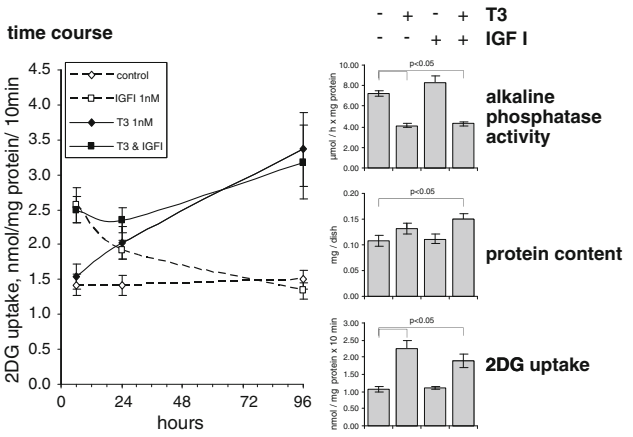

Fig. 5 Time course comparing stimulation of 2-deoxyglucose (2DG) uptake by T_3 and by IGF I, and effects of T_3 and IGF I on alkaline phosphatase activity and protein content after 4 days. Cells were tested as shown in Fig. 1b. T_3 and/or IGF I (or vehicle, control; each hormone to a final concentration of 1 nmol l^{-1}) were added as indicated 96, 24, or 6 h before assessing 2DG uptake on day 11 (four experiments carried out in triplicate). *Columns on the right panel* show alkaline phosphatase activity, protein content, and 2DG uptake for the 96 h (4 days) point from seven experiments carried out in triplicate (means ± SEM)

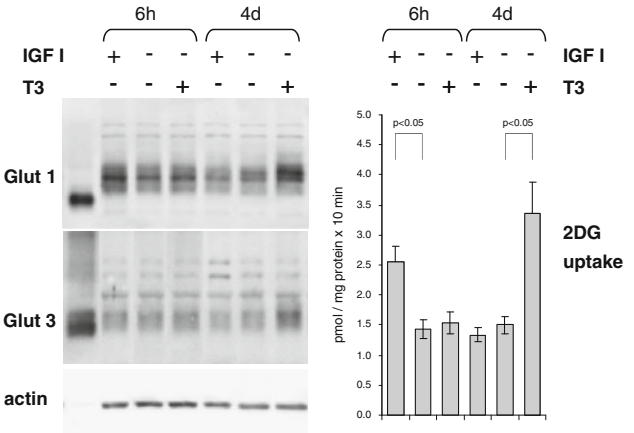

Fig. 6 Effects of IGF I and T_3 on GLUT1 and GLUT3 protein and 2DG uptake. Total membranes were prepared from rat brain and from PyMS cells (treated for the last 4 days or 6 h with T_3 or IGF I) and subjected to western. Thirty microgram protein were loaded per lane (only 3 µg in case of the first lane, rat brain); *bars* indicate the position of marker proteins. Densitometry signals found with this blot were for GLUT1 1.86 (IGF I, 6 h), 1.22 (control, 6 h), 1.32 (T_3, 6 h), 0.80 (IGF I, 4 days), 1 (by definition, control, 4 days), 1.89 (T_3, 4 days); for GLUT3 1.30 (IGF I, 6 h), 1.21 (control, 6 h), 1.32 (T_3, 6 h), 0.71 (IGF I, 4 days), 1 (by definition, control, 4 days), and 1.88 (T_3, 4 days). Corresponding 2DG uptake data from four experiments (means ± SEM) are given in columns on the right panel. Statistical evaluation of 4-day T_3 tests (including summary of westerns) in Table 3

decrease in protein content. DNP (tested at 0.1 mmol l^{-1}) was quite potent in stimulating 2DG uptake, to 3.66 ± 0.37 nmol/mg protein ×10 min, as compared to 0.60 ± 0.04 nmol/mg protein ×10 min in vehicle-treated controls (5 exp in triplicate).

Characterization of 2-deoxyglucose transport

2DG uptake was linear over time and Na independent. Kinetic parameters of the glucose transport system were obtained from Lineweaver–Burk plots for which experiments with 2DG concentrations ranging from 0.02 to 2 mmol l^{-1} were carried out. The apparent affinity constant (K_M) of the glucose transport as estimated from curves was similar in cells kept for the last 4 days in hormone-free control medium (∼0.9 mmol l^{-1}) and in cells exposed for the last 4 days to 1 nmol l^{-1} T_3 (∼ 1.3 mmol l^{-1}, mean of 4 exp.); thus, T_3 did not alter the K_M of the transport system but increased v_{max} of the glucose transport system when assessed in 4-day tests (as in Fig. 4; Tables 2, 3).

GLUT1 and GLUT3

Total membranes were analyzed for GLUT1 and GLUT3 protein by western blot; for comparison, membranes were prepared from rat brain, serving as positive control for GLUT1 and GLUT3 (Fig. 6). The strongest signals with GLUT1 and GLUT3 antibodies were found in membranes prepared from brain (at ∼44 and at ∼45−48 kDa), but bands were also detected in membranes prepared from PyMS cells, at apparent molecular masses of ∼48−50 kDa

for GLUT1 and of 45−49 kDa for GLUT3. T_3 treatment of the cells tended to increase the yield in membranes (to one-third above that of control), which were obtained per dish (Table 3), and resulted in an increased abundance of both GLUT1 and GLUT3 in the membrane preparations, not within 6 h but consistently after 4 days, corresponding to a mean stimulation by a factor of 1.6 (GLUT1) and 2.1 (GLUT3), respectively (Fig. 6; Table 3). Short-term (6 h) treatment with IGF I increased abundance of GLUT1 and GLUT3 proteins in TM prepared from the cells, and concomitantly, 2DG uptake (Fig. 6, right panel; Fig. 5).

Discussion

Triiodothyronine stimulates cells in vitro to increase 2DG uptake. Growing the cells in serum-free medium offers two advantages: (a) it decreases (presumably growth-related) 2DG uptake and (b) it deprives the cells of thyroid hormones, allowing observation of the effects of T_3 at low concentrations. The condition of longer term serum deprivation resulted in a marked decrease in protein content and glucose uptake, and in an increased alkaline phosphatase activity and PTH responsiveness (in terms of 2DG uptake) of the cells, as compared to shorter term culture in the presence of serum (Table 1; Fig. 2). In the case of PTH stimulation, it remains unclear whether an increased response may be related to the state of differentiation of the cells or simply due to the fact that growth-related glucose uptake becomes less important in the longer term serum deprivation setting [2]. Osteoblasts are not unique in constitutively taking up glucose and they are not unique with regard to responsiveness to thyroid hormones. T_3 increased glucose uptake also in liver, muscle, and fat (i.e., in classical insulin target) cells [14–16, 29].

In vivo, T_3 stimulates metabolic rate with a concomitant increase in fuel (lipids as well as carbohydrates) oxidation. T_3 stimulates numerous genes and proteins involved in substrate/energy metabolism, including proteins involved in glycolysis and fatty acid oxidation, and uncoupling proteins [30–32]. Considering overall (whole body) metabolism, liver and skeletal muscles are particularly important target tissues of T_3. In skeletal muscle, T_3 increases the phosphorylation of both AMP-activated protein kinase (AMPK, an enzyme central for cellular energy status, maintaining the balance between ATP production and consumption) and of Akt/PKB and, by activating these signal pathways, stimulates both fatty acid and glucose metabolism. In vivo, T_3 also increases serum-free fatty acids [33].

Connective tissue cells grown in vitro are distinct from skeletal muscle in vivo: there is less supply with fuels (especially fatty acids) and with oxygen, and their energy requirement is lower. The cultured cells will depend much more on glycolysis than on mitochondrial ATP production so that increased glucose uptake becomes especially important in case the cells need more energy.

Glucose uptake by rat osteoblastic cells is a Na-independent process with an apparent affinity constant (K_M) for 2DG uptake in PyMS cells of ~ 1 mmol l^{-1}, i.e., close to the published K_M of the high-affinity glucose transporters, particularly GLUT3 [34]. K_M values for the high-affinity GLUTs vary according to the literature, being lowest for GLUT3 (1.4 mmol l^{-1}) and higher for GLUT1 (6.9 mmol l^{-1}) [35]. Our analysis with osteoblastic cells suggests a dominant role of high-affinity glucose transporters but cannot give an accurate figure since it is likely that several GLUTs account for overall glucose uptake by the cells. Previous studies indicated that rat osteogenic osteosarcoma cells and the (non-tumorigenic) PyMS cell line express both GLUT1 and GLUT3 [1, 2]. Glut1 mRNA encoding the isoform that is ubiquitously found in proliferating cells decreased over time (Fig. 2). Glucose uptake was also highest in serum-primed cells and down-regulated when PyMS cells were kept for a prolonged period of time in serum-free medium. In contrast, under these conditions, alkaline phosphatase activity increased and so did PyMS cells responsiveness (in terms of 2DG uptake stimulation) to PTH (and to IGF I) (Fig. 2; Table 1). In order to avoid different degrees of residual serum effects, the time course experiments presented in Fig. 5 share a common culture period including the same time in serum-free medium. Glucose transport and 2DG uptake in osteoblasts and PyMS cells cannot be attributed to a specific GLUT isoform, but our findings suggest that GLUT3 (and possibly GLUT1) remains the best candidate. GLUT3 and GLUT1 could be identified in PyMS cells, and high-affinity glucose transport would be consistent with GLUT3 playing a central role [2]. According to the data summarized in Table 3, an increased GLUT1 and GLUT3 number (per cell and per well) might account to a large extent for the increased 2DG uptake by the cells in response to a 4-day treatment with T_3.

Hypoxia or inhibition of mitochondrial respiration requires and results in an increased glucose uptake which has been particularly well studied with regard to brain where increased expression of GLUT1 and/or of GLUT3 has been implicated and can be considered to be neuroprotective and of particular relevance when both oxygen and glucose supply are low (hypoxemia and hypoglycemia) or when inflammation increases energy demand [36–39]. It has remained difficult, however, to reconcile mRNA expression and GLUT protein expression data, and it has remained impossible to assign a change in function (glucose transport) to specific changes in GLUT number abundance and activity of all the GLUTs in plasma membranes. Our study shares these limitations although the data

(Table 3) are compatible with the notion that increased availability of GLUTs 1 and 3 in the PyMS cells is related to an increased 2DG uptake by the same cells.

GLUT3 has been reported to play a role in L6 muscle cells adapting to treatment with 2,4-dinitrophenol (2-DNP); uncoupling electron transport (from NADH to O_2) from ADP phosphorylation (to ATP) by DNP increases energy demand [40]. DNP disrupts the oxidative chain in mitochondria and thereby imposes a metabolic stress to the cells. DNP increased glucose uptake in L6 muscle cells and GLUT3 (protein) levels, presumably by prolonging the half-life of glucose transporters, without affecting Glut3 mRNA. Our observations in bone cells (where DNP at 0.1 mmol l^{-1} also dramatically increased 2DG uptake when tested under the conditions of the current study) treated with T_3 are reminiscent of those findings; increased glucose uptake was accompanied by increased (GLUT1 and) GLUT3 content in membranes prepared from the cells, without a corresponding increase in Glut3 mRNA (Table 3) as might have been expected for a hormone known to act on target cells predominantly by affecting transcription. We did not try to further address the discrepancy between mRNA and protein expression but it may well be that an increased half-life of the glucose transporters may be at least part of the explanation, as described by Khayat et al. [40] for DNP effects (which are much stronger than those of T_3 and therefore more suitable for mechanistic analysis). The stimulation of 2DG uptake induced by T_3 is considerable and not paralleled by an increase in cell number (Table 3). Since we did not include more detailed analysis on DNA synthesis, however, minor or transient effects of T_3 on proliferation are not formally excluded with certainty in our study. We speculate that T_3 may target mitochondria and act on uncoupling proteins (UCP) [26, 30]; increased uptake of glucose would be required to meet the elevated energy demand. Proteins uncoupling oxygen consumption from ATP generation were first discovered and their role (catecholamine and thyroid hormone dependency) characterized in more detail in brown adipose tissue (UCP-1, dissipating energy as heat), but T_3 has been found to regulate UCP-2 expression in a variety of tissues including adipose tissue, heart and skeletal muscle, liver, and lung [41]. T_3 may also increase UCP expression in bone. We have not addressed this question in our current study.

Positron emission tomography−computed tomography (PET−CT) using fluoro-deoxyglucose (FDG) is an imaging technology allowing visualization of rapidly growing tumors. Concerning bone, it is striking that osteosarcoma cells (rapidly growing in vitro) take up much more glucose (and phosphate) than their non-transformed osteoblastic counterpart, as previously discussed ([27], unpublished). It has been noticed more recently that F-18 FDG PET imaging visualizes not only rapidly growing tumors but also (particularly brown adipose) tissues exhibiting high-energy expenditure and heat generation/thermogenesis, as modulated by cold exposure, the autonomous nervous system and thyroid hormones [42–49]. In that situation, increased FDG uptake has been speculated to reflect increased energy expenditure. We propose that in our setting, we are dealing with an in vitro counterpart, i.e., stimulation of glucose uptake in quasi-quiescent cells may result from increased UCP expression and less effective energy use when cells are exposed to thyroid hormones. Such an explanation appears likely in the case of DNP and a possibility in the case of T_3.

T_3 stimulated glucose uptake markedly while increasing protein content moderately but did not increase cell number, and decreased alkaline phosphatase activity in PyMS cells under the conditions of the current study. A lack of stimulatory effects of T_3 treatment on osteoblast number proliferation along with an increased RNA and protein content of the cells is in line with findings in primary rat calvaria osteoblasts where T_3 increased ornithine decarboxylase activity but not cell number [50]. In our cell culture system, T_3 did not appear to have major effects on cell replication or apoptosis (the rates of both are quite low in PyMS cells studied in our setting; not shown), and at the doses tested, T_3 did not appear to be toxic to the cells. We do not know, however, the fate of the glucose taken up by the cells and whether T_3 increased heat production. It should be acknowledged as a limitation of our study that we did not study metabolic effects of T_3 in our cell culture system. Additional studies are required to understand for which purpose the cells use glucose increasingly after exposure to T3.

Irrespective of the relative importance of several mechanisms potentially underlying an increased energy requirement in the cells in response to thyroid hormone exposure, our findings show for the first time that T_3 at low doses stimulates glucose uptake by osteoblastic cells in vitro, supporting the view that thyroid hormones directly influence bone cells in a manner that includes increased provision of an important energy source.

Acknowledgments We thank Martina Gosteli for advice with the preparation of the GLUT cDNA probes, Oliver Tschopp for help with the statistical analysis, and Michèle Rothfuchs for help with the preparation of the manuscript. This work has been supported by the Swiss National Science Foundation (grant 32-46808.96).

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. D.M. Thomas, F. Maher, S.D. Rogers, J.D. Best, Expression and regulation by insulin of GLUT3 in UMR 106–01, a clonal rat osteosarcoma cell line. Biochem. Biophys. Res. Commun. **218**, 789–793 (1996)
2. E. Zoidis, C. Ghirlanda-Keller, C. Schmid, Stimulation of glucose transport in osteoblastic cells by parathyroid hormone and insulin-like growth factor I. Mol. Cell. Biochem. **348**, 33–42 (2011)
3. C. Schmid, J. Zapf, E.R. Froesch, Production of carrier proteins for insulin-like growth factors (IGFs) by rat osteoblastic cells. FEBS Lett. **244**, 328–332 (1989)
4. C.M. Veldman, I. Schläpfer, C. Schmid, 1α,25-dihydroxyvitamin D3 stimulates sodium-dependent phosphate transport in osteoblast-like cells. Bone **21**, 41–47 (1997)
5. C. Schmid, C. Keller, I. Schläpfer, C. Veldman, J. Zapf, Calcium and insulin-like growth factor I stimulation of sodium-dependent phosphate transport and proliferation of cultured rat osteoblasts. Biochem. Biophys. Res. Commun. **245**, 220–225 (1998)
6. C. Schmid, C. Ghirlanda-Keller, M. Gosteli-Peter, Ascorbic acid decreases neutral endopeptidase activity in cultured osteoblastic cells. Regul. Pept. **130**, 57–66 (2005)
7. C. Schmid, T. Steiner, E.R. Froesch, Insulin-like growth factors stimulate synthesis of nucleic acids and glycogen in cultured calvaria cells. Calcif. Tissue Int. **35**, 578–585 (1983)
8. E.R. Froesch, C. Schmid, J. Schwander, J. Zapf, Actions of insulin-like growth factors. Annu. Rev. Physiol. **47**, 443–467 (1985)
9. M. Ferron, J. Wei, T. Yoshizawa, A. Del Fattore, R.A. DePinho, A. Teti, P. Ducy, G. Karsenty, Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell **142**, 296–308 (2010)
10. K. Fulzele, R.C. Riddle, D.J. DiGirolamo, X. Cao, C. Wan, D. Chen, M.-C. Faugere, S. Aja, M.A. Hussain, J.C. Brüning, T.L. Clemens, Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. Cell **142**, 309–319 (2010)
11. M. López, L. Varela, M.J. Vázquez, S. Rodríguez-Cuenca, C.R. González, V.R. Velagapudi, D.A. Morgan, E. Schoenmakers, K. Agassandian, R. Lage, P.B. Martínez de Morentin, S. Tovar, R. Nogueiras, D. Carling, C. Lelliott, R. Gallego, M. Oresic, K. Chatterjee, A.K. Saha, K. Rahmouni, C. Diéguez, A. Vidal-Puig, Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. Nat. Med. **16**, 1001–1008 (2010)
12. J.E. Silva, P.R. Larsen, Adrenergic activation of triiodothyronine production in brown adipose tissue. Nature **305**, 712–713 (1983)
13. J.E. Silva, P.R. Larsen, Potential of brown adipose tissue type II thyroxine 5′-deiodinase as a local and systemic source of triiodothyronine in rats. J. Clin. Invest. **76**, 2296–2305 (1985)
14. S.P. Weinstein, J. Watts, P.N. Graves, R.S. Haber, Stimulation of glucose transport by thyroid hormone in ARL 15 cells: increased abundance of glucose transporter protein and messenger ribonucleic acid. Endocrinology **126**, 1421–1429 (1990)
15. M.A. Gosteli-Peter, C. Schmid, J. Zapf, Triiodothyronine increases glucose transporter isotype 4 mRNA expression, glucose transport, and glycogen synthesis in adult rat cardiomyocytes in long-term culture. Biochem. Biophys. Res. Commun. **221**, 521–524 (1996)
16. R. Romero, B. Casanova, N. Pulido, A.I. Suarez, E. Rodriguez, A. Rovira, Stimulation of glucose transport by thyroid hormone in 3T3-L1 adipocytes: increased abundance of GLUT1 and GLUT4 glucose transporter proteins. J. Endocrinol. **164**, 187–195 (2000)
17. C.H.A. Gouveia, M.A. Christoffolete, C.R. Zaitune, J.M. Dora, J.W. Harney, A.L. Maia, A.C. Bianco, Type 2 iodothyronine selenodeiodinase is expressed throughout the mouse skeleton and in the MC3T3-E1 mouse osteoblastic cell line during differentiation. Endocrinology **146**, 195–200 (2005)
18. J.H.D. Bassett, G.R. Williams, The skeletal phenotypes of TR and TRβ mutant mice. J. Mol. Endocrinol. **42**, 269–282 (2009)
19. J.H.D. Bassett, A. Boyde, P.G.T. Howell, R.H. Bassett, T.M. Galliford, M. Archanco, H. Evans, M.A. Lawson, P. Croucher, D.L. St. Germain, V.A. Galton, G.R. Williams, Optimal bone strength and mineralization requires the type 2 iodothyronine deiodinase in osteoblasts. Proc. Nat. Acad. Sci **107**, 7604–7609 (2010)
20. A.I. Gogakos, J.H.D. Bassett, G.R. Williams, Thyroid and bone. Arch. Biochem. Biophys. **503**, 129–136 (2010)
21. A. Fraichard, O. Chassande, M. Plateroti, J.P. Roux, J. Trouillas, C. Dehay, C. Legrand, K. Gauthier, M. Kedinger, L. Malaval, B. Rousset, J. Samarut, The T3Ra gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. EMBO J. **16**, 4412–4420 (1997)
22. S.M. Krane, G.L. Brownell, J.B. Stanbury, H. Corrigan, The effect of thyroid disease on calcium metabolism in man. J. Clin. Invest. **35**, 874–887 (1956)
23. P. Charles, J.W. Poser, L. Mosekilde, F.T. Jensen, Estimation of bone turnover evaluated by ^{47}calcium-kinetics: efficiency of serum bone gamma-carboxyglutamic acid-containing protein, serum alkaline phosphatase and urinary hydroxyproline excretion. J. Clin. Invest. **76**, 2254–2258 (1985)
24. C.B. Confavreux, R.L. Levine, G. Karsenty, A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. Mol. Cell. Endocrinol. **310**, 21–29 (2009)
25. W.S. Simonides, M.A. Mulcahey, E.M. Redout, A. Muller, M.J. Zuidwijk, T.J. Visser, F.W. Wassen, A. Crescenzi, W.S. da-Silva, J. Harney, F.B. Engel, M.J. Obregon, P.R. Larsen, A.C. Bianco, S.A. Huang, Hypoxia-inducible factor induces local thyroid hormone inactivation during hypoxic-ischemic disease in rats. J. Clin. Invest. **118**, 975–983 (2008)
26. A. Lanni, M. Moreno, A. Lombardi, F. Goglia, Thyroid hormones and uncoupling proteins. FEBS Lett. **543**, 5–10 (2003)
27. E. Zoidis, C. Ghirlanda-Keller, M. Gosteli-Peter, J. Zapf, C. Schmid, Regulation of phosphate (Pi) transport and NaPi-III transporter (Pit-1) mRNA in rat osteoblasts. J. Endocrinol. **181**, 531–540 (2004)
28. C. Schmid, I. Schlapfer, M. Peter, M. Boni-Schnetzler, J. Schwander, J. Zapf, E.R. Froesch, Growth hormone and parathyroid hormone stimulate IGFBP-3 in rat osteoblasts. Am. J. Physiol. **267**, 226–233 (1994)
29. Zorzano, A., Palacín, M., Gumà, A.: Mechanisms regulating GLUT4 glucose transporter expression and glucose transport in skeletal muscle. Acta Physiol. Scand. **183**, 43–58 (2005)
30. V. Lebon, S. Dufour, K.F. Petersen, J. Ren, B.M. Jucker, L.A. Slezak, G.W. Cline, D.L. Rothman, G.I. Shulman, Effect of triiodothyronine on mitochondrial energy coupling in human skeletal muscle. J. Clin. Invest. **108**, 733–737 (2001)
31. K. Clement, N. Viguerie, M. Diehn, A. Alizadeh, P. Barbe, C. Thalamas, J.D. Storey, P.O. Brown, G.S. Barsh, D. Langin, In vivo regulation of human skeletal muscle gene expression by thyroid hormone. Genome Res. **12**, 281–291 (2002)
32. W.E. Visser, K.A. Heemstra, S.M. Swagemakers, Z. Ozgür, E.P. Corssmit, J. Burggraaf, W.F. van Ijcken, P.J. van der Spek, J.W. Smit, T.J. Visser, Physiological thyroid hormone levels regulate numerous skeletal muscle transcripts. J. Clin. Endocrinol. Metab. **94**, 3487–3496 (2009)
33. P. De Lange, R. Senese, F. Cioffi, M. Moreno, A. Lombardi, E. Silvestri, F. Goglia, A. Lanni, Rapid activation by 3,5,3′-L-triiodothyronine of adenosine 5′-monophosphate-activated protein kinase/acetyl-coenzyme A carboxylase and Akt/protein kinase B signaling pathways: relation to changes in fuel metabolism and

myosin heavy-chain protein content in rat gastrocnemius muscle in vivo. Endocrinology **149**, 6462–6470 (2008)
34. G.I. Bell, C.F. Burant, J. Takeda, G.W. Gould, Structure and function of mammalian facilitative sugar transporters. J. Biol. Chem. **268**, 19161–19164 (1993)
35. Simpson, I.A., Dwyer, D., Malide, D., Moley, K.H., Travis, A., Vannucci, S.J.: (2008) The facilitative glucose transporter GLUT3: 20 years of distinction. Am. J. Physiol. **295**, E242–E253 (2008)
36. B.L. Ebert, J.D. Firth, P.J. Ratcliffe, Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct cis-acting sequences. J. Biol. Chem. **270**, 29083–29089 (1995)
37. D.H. Lee, M.Y. Chung, J.U. Lee, D.G. Kang, J.W. Paek, Changes of glucose transporters in the cerebral adaptation to hypoglycaemia. Diabetes Res. Clin. Pract. **47**, 15–23 (2000)
38. P. Cidad, P. Garcia-Nogales, A. Almeida, J.P. Bolanos, Expression of glucose transporter GLUT3 by endotoxin in cultured rat astrocytes: the role of nitric oxide. J. Neurochem. **79**, 17–24 (2001)
39. M.H. Maurer, H.K. Geomor, H.F. Bürgers, D.W. Schelshorn, W. Kuschinsky, Adult neural stem cells express glucose transporters GLUT1 and GLUT3 and regulate GLUT3 expression. FEBS Lett. **580**, 4430–4434 (2006)
40. Z.A. Khayat, A. McCall, A. Klip, Unique mechanism of GLUT3 glucose transporter regulation by prolonged energy demand: increased protein half-life. Biochem. J. **333**, 713–718 (1998)
41. Xiao, H., Massaro, D., DeCarlo Massaro, G., Biadasz Clerch, L.: Expression of lung uncoupling protein-2 mRNA is modulated developmentally and by caloric intake. Exp. Biol. Med. **229**, 479–485 (2004)
42. C. Cohade, M. Osman, H.K. Pannu, R.L. Wahl, Uptake in supraclavicular area fat ("USA-Fat"): description on 18F-FDG PET/CT. J. Nucl. Med. **44**, 170–176 (2003)
43. J.E. Silva, Thermogenic mechanisms and their hormonal regulation. Physiol. Rev. **86**, 435–464 (2006)
44. J.R. Clarke, S. Brglevska, E.W. Lau, S. Ramdave, R.J. Hicks, A typical brown fat distribution in young males demonstrated on PET/CT. Clin. Nucl. Med. **32**, 679–682 (2007)
45. A.M. Cypess, S. Lehman, G. Williams, I. Tal, D. Rodman, A.B. Goldfine, F.C. Kuo, E.L. Palmer, Y.H. Tseng, A. Doria, G.M. Kolodny, C.R. Kahn, Identification and importance of brown adipose tissue in adult humans. N. Engl. J. Med. **360**, 1509–1517 (2009)
46. W.D. van Marken Lichtenbelt, J.W. Vanhommerig, N.M. Smulders, J.M. Drossaerts, G.J. Kemerink, N.D. Bouvy, P. Schrauwen, G.J.J. Teule, Cold-activated brown adipose tissue in healthy men. N. Engl. J. Med. **360**, 1500–1508 (2009)
47. K.A. Virtanen, M.E. Lidell, J. Orava, M. Heglind, R. Westergren, T. Niemi, M. Taittonen, J. Laine, N.J. Savisto, S. Enerbäck, P. Nuutila, Functional brown adipose tissue in healthy adults. N. Engl. J. Med. **360**, 1518–1525 (2009)
48. W.E. Visser, E.C.H. Friesema, T.J. Visser, Transport of thyroxine and 3,3′,5-triiodothyronine in human umbilical vein endothelial cells. Endocrinology **150**, 1552–1557 (2009)
49. M.C. Skarulis, F.S. Celi, E. Mueller, M. Zemskova, R. Malek, L. Hugendubler, C. Cochran, J. Solomon, C. Chen, P. Gorden, Thyroid hormone induced brown adipose tissue and amelioration of diabetes in a patient with extreme insulin resistance. J. Clin. Endocrinol. Metab. **95**, 256–262 (2010)
50. C. Schmid, T. Steiner, E.R. Froesch, Triiodothyronine increases responsiveness of cultured rat bone cells to parathyroid hormone. Acta Endocrinol. **111**, 213–216 (1986)