Ontogenetic profile and thyroid hormone regulation of type-1 and type-8 glucose transporters in rat Sertoli cells

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Summary

The glucose transporters (GLUTs) gene encode glycoproteins responsible for facilitating transfer of glucose across plasma membrane. In testis, different members of this family are present. In particular the main GLUT mRNA expression within the adult testis is the type 8, while type 1 is more expressed in prepubertal testis. Thyroid hormone, which receptors and function have been characterized in the testis, plays a crucial role in the cellular energetic metabolism. In fact, in the immature Sertoli cells, GLUT1 is up regulated by L-triiodothyronine (T3). The aim of this paper is to investigate the expression profile of GLUT1 and GLUT8 in the testis during development and in adulthood and analyse the role of T3 on their expression. To analyse the expression of GLUT8 and GLUT1 we performed Northern blot and RT-PCR experiments in the whole testis and in Sertoli cells from rats of different ages. Treatments in vivo and in vitro with T3 were used to study the effect of thyroid hormones on GLUT1 and GLUT8 expression. The activity of the rat GLUT1 promoter and its regulation by T3 was studied with transient transfections in gonadal and non-gonadal cell lines and in primary Sertoli cell cultures. GLUT8 is expressed at a low level in the prepubertal testis and Sertoli cells and does not appear to be under T3 control. GLUT1 is the predominant form in immature Sertoli cells. The effect of T3 on its mRNA accumulation was quantified and confirmed by RT-PCR (control: 0.65 ± 0.17; T3: 1.23 ± 0.04, arbitrary units, p < 0.05). However, transfection experiments showed that T3 does not directly regulate GLUT1 promoter in any cell line tested. This is confirmed by the evidence that, upon extensive analysis, the rat GLUT1 promoter and the first intron sequence do not show any thyroid responsive elements. Our data demonstrate that GLUT1 and GLUT8 are both expressed in prepubertal testis, but only GLUT1 is regulated by T3. In addition, we found that the effect of T3 cannot be attributed to its action on GLUT1 promoter.

Keywords: glucose, glucose transporter, Sertoli cell, thyroid hormone

Introduction

Growing evidence both in animals and humans support the role of thyroid hormones and thyroid hormone nuclear receptors (TRs) in the development of male gonadal tissues. As germ cell maturation requires lactate (Jutte et al., 1982)
transporter proteins (GLUTs) are a super-family of transport facilitators with 13 family members, distributed in a wide variety of species (Joost & Thorens, 2001), which help the diffusion of hexoses into mammalian cells (Bell et al., 1990). Different members of this protein family are expressed in the testis: GLUT1 (Ulisse et al., 1992), GLUT3 (Burant & Davidson, 1994), GLUT5 (Burant et al., 1992) and GLUT8 (Doege et al., 2000; Ibberson et al., 2000). In particular, GLUT1 is expressed in both adult and immature rat Sertoli cells, while GLUT8 is the predominant form in the adult testis (Doege et al., 2000; Ibberson et al., 2000) and is prevalently located in the head of spermatocytes (Schurmann et al., 2002).

The prepubertal testis is a target organ for the thyroid hormone (Jannini et al., 1995). In fact, immature Sertoli cell (but not Leydig and peritubular cell) nuclei bind thyroid hormone with high affinity and specificity (Jannini et al., 1990). This binding is the result of the expression of the isoform α1 of the nuclear thyroid hormone receptor, being the isoform β virtually absent (Jannini et al., 1994). A critical window of TR expression and T3 action, localized in the foetal, neonatal and prepubertal ages, has been demonstrated (Jannini et al., 1995). Interestingly, a similar expression pattern has been recently demonstrated in the human testis (Jannini et al., 2000).

In Sertoli cells, follicle-stimulating hormone (FSH) is the main energy metabolism regulator (Hall & Mita, 1984), but GLUT1 mRNA expression (Ulisse et al., 1992) and lactate production (Palmero et al., 1989) were stimulated by T3 in a process requiring de novo protein synthesis, suggesting an effect at transcriptional level.

Thus, in Sertoli cells, FSH and thyroid hormone regulate glucose uptake at different levels, through both a fast membrane signalling mechanism and a delayed action through the nuclear level, so that energy requirements of the developing germ cells can be met (Jannini et al., 1995). In fact, neonatal testis fragments cultured in vitro with T3 show a significant increase in the size of seminiferous cords and in the number of gonocytes, concomitant with a decreased percentage of degenerating germ cells (Jannini et al., 1993).

While the regulation of GLUT8 by thyroid hormone has never been studied, it is interesting to note the cell-specificity of the T3 effect on the expression of GLUT1. Thyroid hormone increases GLUT1 protein in adipose cells (Matthaei et al., 1995; Romero et al., 2000), but not in other tissues. In fact, a lack of increase of GLUT1 in skeletal muscle and brain (Weinstein et al., 1991), its decrease in cardiac muscle from hyperthyroid rats (Weinstein & Haber, 1992) and its increase in hypothyroid animals (Castello et al., 1994) have been demonstrated. The reasons for the discrepancy between the observations on the role of thyroid hormone on GLUT1 expression obtained from different tissues remain unexplained. In fact, the action mechanism of GLUT1 regulation by T3 is largely unknown.

In the present study, the expression profiles of two glucose carriers, GLUT1 and GLUT8, were investigated in the testis during development and in adulthood, and the effect of T3 on their expression was analysed. In addition, the activity of rat GLUT1 promoter was studied in transfection experiments using the monkey COS7 kidney cell line, and in Sertoli cell primary and immortalized cultures, before and after stimulation with T3.

**Materials and methods**

**Cell cultures**

Primary cultures enriched in Sertoli cells were prepared from 8 to 20-day-old Wistar rats as previously described (Galdieri et al., 1981). The cells were plated in MEM containing 0.1% bovine serum albumin (Sigma-Aldrich, St Louis, MI, USA) and incubated in 5% CO2 at 32°C. After 48 h culture, the Sertoli cell monolayer was treated with a hypotonic solution (20 mM Tris–HCl, pH 7.4) for 3 min to remove germ cells contamination. In preliminary experiments, the purity of Sertoli cell preparations was assessed after 2 days of culture by staining for peritubular cells with alkaline phosphatase (Palombi & Di Carlo, 1988). In these preparations, Sertoli cells accounted for approximately 90% of the cell population, as judged by phase-contrast examination, with the main contaminant being germ and peritubular cells. The monkey COS7 kidney cell line (Gluzman, 1981) was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich). The adult mouse Sertoli cell lines TM4 (Mather, 1980) were grown in DMEM supplemented with 2.5% FBS and 5% horse serum (Sigma-Aldrich). Preliminary experiments performed by RNase protection assay (Jannini et al., 1994) and RT-PCR demonstrated that this cellular line does not express any TRs (not shown).

**Animal treatment**

To induce hyperthyroidism, prepubertal and adult male Wistar rats received by intraperitoneal injection 1 μg/g BW/day of T3 (dissolved in 0.04 N NaOH) for 3 days (Jannini et al., 1992). The control animals received the appropriate dilution of the stock solution of 0.04 N NaOH without thyroid hormone. Hypothyroidism was provoked in adult rats by surgical thyroidectomy followed by feeding with a low iodine test diet and 1% calcium lactate for 1 month (Jannini et al., 1992). The animals were then killed at the indicated times and their testes removed for RNA extraction.
**Extraction and analysis of RNA**

The Trizol (Invitrogen, Carlsbad, CA, USA) method was used to prepare total RNA from Sertoli cells and TM4 cells. The purity and integrity of RNA preparations were checked spectrophotometrically and by gel electrophoresis before carrying out the analytical procedure. Total mRNA extracted from primary cultures of Sertoli cells was used in a semiquantitative RT-PCR analysis. First strand complementary DNA was made using, for each sample, 2 μg of total RNA in the presence of M-MuLV reverse transcriptase (Invitrogen) and Poli d(T)12-18 primer (Invitrogen). The obtained cDNA was used as a template for the PCR amplification of GLUT1 (655 bp) and rat GAPDH (983 bp) using the following primers: rGLUT1up: 5′-CAA ACA TGG AAC CAC CGC TAT GGA-3′; rGLUT1do: 5′-GGA ACA GCT CCA AGA TGG TGA CC-3′; ratGAPDHup: 5′-TGA AGC GTG TGA ACG GAT TTT GC-3′; ratGAPDHDlo: 5′-CAT GTA GGC CAT GTC GTC CAC CAC-3′.

GAPDH was used as an internal control. The PCR was performed with REDTaq DNA polymerase (Sigma-Aldrich; 2.5 units for each reaction), 10 μM of both upstream and downstream primers, and 1.5 mM magnesium chloride. Amplification conditions were 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 35 cycles in the DNA thermal cycler (PCR Cetus; Perkin-Elmer Corp., Rome, Italy; ~20 cycles for GAPDH amplification). The genomic contamination was excluded by running the PCR without RT. Furthermore, the primers were chosen in different exons.

**Northern blot**

The RNA species were separated by electrophoresis on a 1% agarose, 3% formaldehyde gel and then blotted onto nylon filters (Hybond-N; Amersham, Uppsala, Sweden). The blots were hybridized with a 419-bp fragment of GLUT8 cDNA obtained by RT-PCR (oligos used: MGL-U 5′-TCC TCA CTC AAC ACC AGT ACC AGG AG-3′; MGL-D 5′-TGG GGA GGC TCT GGG TCA GTT GGA AG-3′). The hybridization was carried out at 65 °C for 1 h in QuickHyb solution (Stratagene, La Jolla, CA, USA). The blots were washed once with 2 × SSC and 1% SDS at room temperature for 15 min, once with 1 × SSC and 1% SDS at room temperature for 15 min, and twice with 0.5 × SSC and 1% SDS at 65 °C for 30 min. The hybridized blots were exposed to X-ray film at −80 °C with two intensifying screens. Where indicated the filters were hybridized under the same conditions with a probe against GLUT1 gene. The GLUT1 probe (655 bp) was obtained by RT-PCR using previously mentioned oligos: rGLUT1up and rGLUT1do. Finally, the filters were normalized using a probe against β-actin gene, obtained by RT-PCR (oligos used: β-actin1: 5′-GAC GAC ATG GAG AAG ATC TGG-3′; β-actin2: 5′-GAG GAT GGC GCA GTG GCC AT-3′).

**COS7 and TM4 cell lines transfections**

One day before the transfection, cells were plated out in DMEM containing serum that was stripped of T3 by ion-exchange (Samuels et al., 1979). Cells were transfected by calcium phosphate co-precipitation with 3 μg of reporter plasmid pGL2-GLUT1, 100 ng of pRL-SV40 containing Renilla (Sea pansy) luciferase (Promega, Madison, WI, USA) as an internal control, and 1 μg of pGSScTHRα (TRα) (Carosa et al., 1998). Three μg of 3PAL-Luc, a vector containing a specific T3 responder element (TRE) (Mangelsdorf & Evans, 1995) was used as a positive control.

The vector pGL2-GLUT1, which contains the 5′-flanking region of the rat GLUT1 gene spanning from −2104 to +138 of the transcription start site cloned in front of luciferase gene in the vector pGL2-basic (Promega), was kindly provided by Dr Ismail-Beigi (Case Western Reserve University, Cleveland, OH, USA) (Behrooz & Ismail-Beigi, 1997).

The total pGS5 vector quantity, with or without the receptor insert, was kept constant in all experiments. The precipitate DNA mixture was left on the cells for 16 h. T3 (Sigma-Aldrich) was added at the indicate concentrations, and vehicle (ethanol 100% in the first dilution) added to controls. After 48 h culture the cells were lysed with LucLite Substrate Buffer Solution (Packard, Groningen, The Netherlands).

**Prepubertal Sertoli cell transfections**

Sertoli cells from the primary cultures were transfected in 35 mm plastic tissue culture dishes with the Fugene6 method (Boehringer, Mannheim, Germany) using 1.8 μg of reporter plasmid pGL2-GLUT1, 100 ng of pRL-SV40 containing Renilla (Sea pansy) luciferase (Promega) as an internal control, and 1 μg of pGSScTHRα (TRα) (Carosa et al., 1998). The total pGS5 vector quantity, with or without the receptor insert, was kept constant in all experiments. T3 (Sigma-Aldrich) was added at the concentration 10^{-7} M, and vehicle (ethanol 100% in the first dilution) added to controls. After 48 h culture the cells were lysed with LucLite Substrate Buffer Solution (Packard).

**Luciferase activity measurement**

Firefly luciferase (LAA) and Renilla luciferase (LAR) activities were measured sequentially using a FireLighte Kit (Packard) and a model Lumi Count (Packard). The relative luciferase activity (RLA) was calculated as: RLA = LAA/LAR. Transfections were performed in duplicate and the average values from at least three experiments are shown.

**Statistical analysis**

Results are expressed as the mean ± SE of at least three experiments and values were statistically compared using the Student’s t-test. Results were determined to be significantly different if p values were <0.05.
Results

Ontogenetic profile and in vivo thyroid hormone regulation of GLUTs

Type 1 and type 8 GLUT are both expressed in the testis, but with different ontogenetic control (Fig. 1). Type 8 is the predominant form found in the adult testis (Ibberson et al., 2000; Scheepers et al., 2001). In testes from 1-day-old animals, a low level of GLUT8 was detectable only after 10 days of film exposure (Fig. 1B). Its expression remained low during development and showed a large increase in adulthood.

In contrast, GLUT1 was expressed at a low level in the 1-day-old testis, but increased rapidly, reaching almost adult levels 5 days after birth (Fig. 1A). In the adult testis, GLUT8 was approximately 40 times more expressed than in the prepubertal testis and was prevalent with respect to GLUT1 (Fig. 1D and E). As expected, T3 treatment, in the adult testis, did not modify the expression of GLUT1 and 8 (Fig. 1D and E), in fact, at this age, TRs are absent and the testis is unresponsive to thyroid hormones.

Expression and in vivo thyroid hormone regulation of GLUT1

The expression of GLUT1 and GLUT8 in prepubertal Sertoli cells was evaluated by RT-PCR (Fig. 2). Both GLUT mRNAs were expressed in prepubertal Sertoli cells, being, at this stage, GLUT1 prevalent with respect to GLUT8. Figure 2 confirms and quantifies, in prepubertal Sertoli cell primary cultures, the increase of GLUT1 mRNA upon in vitro T3 treatment (10^{-7} M), detected by RT-PCR analysis. Densitometric analysis of three independent PCR experiments demonstrates the 100% increase in GLUT1 after T3 treatment (0.65 ± 0.17 vs. 1.23 ± 0.04 AU (arbitrary units), p < 0.05; Fig. 2). On the contrary, expression of GLUT8 did

Figure 1. Expression of GLUT1 and GLUT8 mRNA in developing and adult testis. Representative Northern blot analysis of 10 μg/lane of total RNA hybridized with rat GLUT1 and GLUT8 cDNAs. (A) an autoradiograph of RNAs extracted from the whole testis of 1, 5, 15 day post natum (dpn) euthyroid rats. Samples were hybridized with GLUT1 probe (A). The film is shown after overnight exposure. The same blot (B) was hybridized with rat GLUT8 probe and exposed for 10 days and then rehybridized with β-actin probe (6-h exposure) (C). (D) A representative autoradiograph of total RNA obtained from the whole tests of adult hypothyroid, euthyroid and hyperthyroid rats hybridized with GLUT1 (6-day exposure); the same blot was hybridized with GLUT8 and exposed overnight (E). It was then normalized with β-actin (3-day exposure) (F). Autoradiographic bands from three separate experiments were scanned using a two-dimensional scanning densitometer and optical densities for each band were obtained. GLUT1 and GLUT8 density were normalized for the optical density obtained by rehybridizing the same filter with β-actin cDNA probe.

Figure 2. In vitro expression of GLUT1 and GLUT8 mRNAs. Total RNA obtained from prepubertal Sertoli cells before and after T_{3} treatment (10^{-7} M; 48 h) was analysed by semiquantitative RT-PCR. The cDNA transcribed from 2.5 μg of total RNA from Sertoli cells was used as a template for PCR amplification of GLUT1, GLUT8, GAPDH was used as loading control of RNA concentration and integrity. Densitometric values represent the mean ± SE of three different experiments; *p < 0.05.
not appear to be under T3 control. The presence of GLUT8 in prepubertal Sertoli cell and the absence of regulation by T3 was also confirmed by Northern blot, to avoid the interference of germ cell contamination (Fig. 3).

Action mechanism of GLUT1 regulation by thyroid hormone

To determine whether this effect was caused by augmented transcription induced by GLUT1 promoter, transient transfections were performed with pGL2-GLUT1 in COS7 cell lines in the presence or absence of TRα1, with or without T3 (Fig. 4). No changes in luciferase expression before and after T3 treatment were observed at any concentration of T3 used (from 10^{-7} M to 10^{-9} M; Table 1). Transfection with a vector containing a specific TRE, 3PAL-Luc, was used as a positive control (Fig. 4).

To understand if somatic cells of the seminiferous epithelium have different GLUT1 control mechanisms with respect to other cells, pGL2-GLUT1 was transfected in the adult murine Sertoli cell line TM4 and in the primary culture of immature rat Sertoli cells. As expected, in the TM4 cell lines (Fig. 5), which do not express thyroid receptors, no significant change in luciferase activity was observed in the absence or presence of TRα1 before and after T3 treatment. In the prepubertal Sertoli cell, no activation of GLUT1 promoter, in either the control or the T3 treated cell was found (Fig. 5). In the same cells, co-transfection with TRα1 demonstrated activation only in absence of T3. In contrast, reduction of promoter activity was seen in the presence of T3.

**Discussion**

We have demonstrated that GLUT types 1 and 8 are both expressed in the testis. However, while GLUT8 is the predominant form in the adult testis, GLUT1 is the main type in the developing testis. GLUT8 expression remains low during development and is high at 60 days. This is in agreement with other findings indicating that GLUT8 is expressed in the acrosomal region of mature spermatozoa, the most abundant cell type in the adult testis (Schurmann et al., 2002). However, we also found GLUT8 present in immature Sertoli cells, although at a lower level than GLUT1. GLUT8 do not appear to be regulated by in vitro T3 treatment in prepubertal Sertoli cells.

We found that GLUT1 follows a different pattern of expression and regulation. In fact, GLUT1 increases during development and reaches adult levels before puberty is completed. In prepubertal Sertoli cells, GLUT1 increases by

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**Figure 3.** Expression of GLUT8 in prepubertal Sertoli cells before and after T3 treatment. A 10 μg of total RNA extract of prepubertal Sertoli cells was separated by electrophoresis and hybridized with GLUT8. The concentration and the integrity of RNA was tested with β-actin.

**Table 1.** Analysis of rat GLUT1 promoter (pGL2-GLUT1) activity in COS7 cells

<table>
<thead>
<tr>
<th>Treatment (24 h)</th>
<th>Folds of activation (arbitrary units)</th>
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<tbody>
<tr>
<td>pGL2-GLUT1</td>
<td>1</td>
</tr>
<tr>
<td>pGL2-GLUT1 + T3 (10^{-7} M)</td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>pGL2-GLUT1 + TRα1</td>
<td>0.89 ± 0.09</td>
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<tr>
<td>pGL2-GLUT1 + TRα1 + T3 (10^{-9} M)</td>
<td>0.84 ± 0.05</td>
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<tr>
<td>pGL2-GLUT1 + TRα1</td>
<td>0.99 ± 0.08</td>
</tr>
<tr>
<td>pGL2-GLUT1 + TRα1 + T3 (10^{-7} M)</td>
<td>0.64 ± 0.03</td>
</tr>
<tr>
<td>pGL2-GLUT1 + TRα1</td>
<td>0.78 ± 0.06</td>
</tr>
<tr>
<td>pGL2-GLUT1 + TRα1 + T3 (10^{-6} M)</td>
<td>0.73 ± 0.05</td>
</tr>
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Thyroid receptor α1 (TRα1); l-triiodothyronine (T3)
hypothesized that the stimulation of GLUT1 by T3 may be mediated by other factors not present in all cells, but only in the testis. For this reason, in transfection experiments we used both primary cultures of cells expressing GLUT1 and TRα1, such as immature Sertoli cells, and a Sertoli cell line derived from an adult murine source, not expressing TRs, such as TM4. In these cells, the GLUT1 promoter was not regulated by T3 either in presence or absence of TRα1. In fact, the increase the GLUT1 promoter activity, observed in both cell lines only in presence of TRα1 and in absence of T3, depend on the recruitment of corepressor by unliganded TR and on the subsequent withdrawal of factors from the promoter. Therefore, it merely depends on the excessive presence of co-transfected TRα1 (Tagami et al., 1999).

The possibility that the regulatory region of GLUT1 gene under thyroid hormone control is placed in a different site of the promoter, away from the 2242 bp studied, cannot be currently ruled out. However, at the end of our study, when the complete rat GLUT1 gene sequence became available, we analysed the 5′ promoter flanking region and first intron with sequence scan for transcription factors binding sites and did not find any classical TRE. The result of the in vitro GLUT1 2242 pb promoter analysis, the absence of putative TRE in 5′ flanking region of promoter and first intron of GLUT1 gene and the variable results obtained in different tissues of effect of T3 stimulation on GLUT1 expression (Weinstein et al., 1991; Weinstein & Haber, 1992; Castello et al., 1994) may support the hypothesis that GLUT1 gene is not the direct target of T3.

Finally, the increase of GLUT1 after T3 stimulation in Sertoli cells may be the result of a translation action on mRNA stabilization. In fact, studies from several laboratories have demonstrated that GLUT1 is specifically regulated at post-transcriptional level (Jain et al., 1995; Boado & Pardridge, 1998; Qi & Pekala, 1999) by a group of proteins which can bind a cis-acting element located at the 5′- untranslated region of its mRNA. The embryonic lethal abnormal vision (ELAV)/Hu proteins bind to a class of mRNAs containing an AU-rich sequence, stabilizing and/or activating translation of target RNA (Keene, 1999; Brennan & Steitz, 2001). Studies with 3T3-L1 adipocyte ectopically expressed Hel-N1 (human ELAV-like neuronal protein 1) demonstrate an increase in GLUT1 protein (eight to ninefolds) and a twofold increase in GLUT1 mRNA (Jain et al., 1997). Expression of ELAV homologue proteins is ubiquitous, and in humans and mice some of them are expressed in the testis (Atasoy et al., 1998). The increase in GLUT1 mRNA observed in Sertoli cells after T3 treatment may be a consequence of its binding with an ELAV/Hu protein and therefore the major stability of its mRNA. We are currently exploring both these possibilities.

In summary, we confirmed the presence of type 1 and 8 GLUT mRNAs in the rodent testis, and demonstrated their ontogenetic profile. The expression of GLUT1, but not of GLUT8, is increased by the thyroid hormone treatment and this effect is observed only before puberty. Furthermore, we demonstrated that the increase of GLUT1 expression could not be ascribed to a transcriptional action of T3 on its promoter.
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