Adipose thyrotrophin receptor expression is elevated in Graves’ and thyroid eye diseases ex vivo and indicates adipogenesis in progress in vivo

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Abstract

The thyrotrophin receptor (TSHR) provides an autoantigenic link between the thyroid and orbit in Graves’ (GD) and thyroid eye diseases (TED). We measured TSHR transcripts in different fat depots to determine whether TSHR expression levels are influenced by the autoimmune/inflammatory process and/or thyroid hormone status, using quantitative real-time PCR. Nine intact or fractionated adipose samples, from patients with GD and/or TED, were analysed ex vivo. Eight expressed the TSHR, at levels approaching the thyroid, and one was at the limit of detection. Thirteen/fifteen orbital and abdominal fat samples from patients free of GD and TED, measured ex vivo, were negative for TSHR transcripts and two were at the limit of detection. All preadipocyte samples induced to differentiate in vitro expressed the TSHR. To investigate the influence of thyroid hormone status on adipose TSHR expression, we induced hyper- and hypothyroidism in BALBc mice by administering tri-iodothyronine and propylthiouracil respectively. In euthyroid animals, whole fat samples were at the limit of detection and were not altered by thyroid hormone status. The results show that adipose TSHR expression ex vivo indicates adipogenesis in progress in vivo and is associated with the autoimmune/inflammatory process in GD and TED but is not restricted to the orbit or influenced by thyroid hormone status.

Journal of Molecular Endocrinology (2003) 30, 369–380

Introduction

Thyroid eye disease (TED) is a frequent complication of Graves’ disease (GD). The orbital tissues in TED and the thyroids in GD display lymphocytic infiltration, indicating the autoimmune etiology of these disorders (Ludgate & Baker 2002). In GD, the thyrotrophin receptor (TSHR) is the autoantigenic target of thyroid-stimulating antibodies (TSAB) which mimic the action of thyrotrophin (TSH) and cause hyperthyroidism and goitre (Paschke & Ludgate 1997). The pathogenesis of TED is more complex and a variety of mechanisms contribute to the increase in orbital contents leading to proptosis, which accounts for most of the signs and symptoms (Heufelder et al. 2000). The extra-ocular muscles (EOM) can be enlarged in TED, primarily due to oedema, but the myocytes themselves are relatively unaffected (Hufnagel et al. 1987). Two further processes increase the contents of the orbit, production of glycosaminoglycans by orbital fibroblasts (Hansen et al. 1997) and hypertrophy and hyperplasia of the adipose tissue (Sorisky et al. 1996). The increase in fat cell number is the consequence of adipogenesis, in which preadipocytes differentiate into mature adipocytes. The process involves several distinct phases including growth arrest and the induction of the central transcriptional regulators of adipogenesis (Rosen & Spiegelman 2000), peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding-protein-α (C/EBPα).

The adipose tissue has also received considerable attention as the source of a putative autoantigenic
link between the thyroid and the orbit, the TSHR. The majority of fat depots in rats (Endo et al. 1995) and guinea pigs (Roselli-Rehfuss et al. 1992) are considered to express a functional TSHR, as demonstrated by RT-PCR, TSH binding and TSH-induced cAMP accumulation (Haraguchi et al. 1996a). In addition, in vitro models revealed that TSHR expression was highest in rat preadipocytes undergoing adipogenesis to mature adipocytes (Haraguchi et al. 1996b).

TSHR expression in human fat is more controversial. In neonatal adipose tissue, TSH and TSAB mediate lipolysis in vitro (Marcus et al. 1988), indicating a functional TSHR on mature adipocytes. This in vitro effect declines with age, as does serum TSH which eventually attains adult levels following the TSH surge at birth.

In adult fat, the complete spectrum for TSH-binding sites, from absent to low and high affinity, has been reported (Mullin et al. 1976, Davies et al. 1978, Perros & Kendall-Taylor 1994). TSHR transcripts in orbital fat were first inferred from RT-PCR studies of orbital contents which yielded a product, whereas the individual components, connective tissue and EOM did not and orbital fat was not tested (Feliciello et al. 1993). We demonstrated TSHR transcripts in TED orbital fat, using Northern blot analysis in which transcripts in normal orbital and abdominal fat were at the limit of detection (Crisp et al. 1997). Subsequently, this has been confirmed by RT-PCR and liquid hybridization analysis, the latter also indicating that, in common with rodent fat, TSHR expression in man is highest during adipogenesis (Valyasevi et al. 1999).

TSHR protein, detected by immunocytochemistry, has been reported by ourselves (Crisp et al. 2000) and others, on TED orbital fat tissues ex vivo and at all stages of the preadipocyte to mature adipocyte differentiation pathway in vitro (Bahn et al. 1998). Furthermore, although preadipocytes are unresponsive to TSH, 10 mU/ml of the hormone stimulates cAMP production in differentiating cells and mature adipocytes, irrespective of the depot (Crisp et al. 2000).

The situation in TED has become more complex since TSHR expression is clearly not restricted to the orbit, even though the receptor is most abundant in TED orbits (Crisp et al. 1997). At least two explanations could account for this: (1) a further autoantigen, with restricted orbital- or disease-associated expression, remains to be identified and (2) the extra-thyroidal changes are more systemic than previously appreciated. There is evidence from another extra-thyroidal manifestation, pretibial myxoedema (PM) to support both points of view. Immunoglobulins from GD patients stimulate the production of intercellular adhesion molecule-1 from GD but not normal fibroblasts, highlighting a difference in these two populations of cells (Heufelder & Bahn 1992) and TSHR immunoreactivity has been demonstrated in PM and normal skin biopsies (Rapoport et al. 2000, Daumerie et al. 2002).

Further support for the role of the TSHR in TED derives from the success in modelling the disease using TSHR primed T cells or genetic immunization (Many et al. 1999, Costagliola et al. 2000).

The aim of the present study was to investigate whether TSHR transcript levels vary in fat from different depots and/or is influenced by the autoimmune process in GD or thyroid hormone status. These studies required a quantitative method suitable for analysis of small samples. We opted for quantitative real-time RT-PCR (QRT-PCR) using SYBR green dye, which intercalates into the DNA backbone, and is measured at the end of each PCR cycle during the exponential phase of the reaction, using a light cycler.

Materials and methods

Patients studied and sample collection

Thyroid tissue was collected from five patients with GD and seven patients with multi-nodular goitre (MNG), two of which were toxic. Adipose tissues were collected from three locations: (1) orbital, seven TED patients undergoing decompression and three donors with non-inflammatory eye disease, (2) cervical, eight patients undergoing thyroidectomy for GD or MNG and (3) abdominal, eleven patients undergoing a range of surgical procedures.

Samples were either snap frozen and stored at −80 °C prior to RNA extraction or separated into preadipocyte and adipocyte fractions as described below.

All samples were obtained with informed consent and with the approval of the local Bro Taf ethics committee.

Journal of Molecular Endocrinology (2003) 30, 369–380

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Adipose disaggregation and separation
Preadipocytes and mature adipocytes were obtained by collagenase digest followed by centrifugation through phthalic acid dionyl ester, as previously described (Crisp et al. 2000). The preadipocytes and mature adipocytes were either used immediately for RNA extraction or resuspended in complete medium and transferred to tissue culture flasks.

Culture conditions and differentiation protocol
The preadipocytes were grown in complete medium (Dulbecco’s modified Eagles’ medium/Ham’s F12 1:1 media) containing 10% foetal calf serum (FCS), until confluent, when they were trypsinized, frozen in 10% dimethyl sulphoxide in FCS and stored in liquid nitrogen. The cells were rapidly thawed, resuspended in complete medium, seeded in 12-well plates and maintained at 37°C until ~75% confluent, when they were transferred to serum-free medium alone or containing differentiation reagents: biotin 33 µM, panthothenic acid 17 µM, apotransferrin 10 µg/ml, tri-iodothyronine (T₃) 0·2 nM, cortisol 100 nM, insulin 500 nM and 0·25 mM IBMX for the first 3 days. Differentiation continued for a further 8–11 days in differentiation medium without isobutyl methylxanthine (IBMX), when cells were examined for morphological evidence of differentiation and/or oil red O staining as previously described (Crisp et al. 2000) and RNA was extracted.

RNA extraction, reverse transcription and QRT-PCR
Solid tissues were weighed prior to pulverization and 900 µl solution D (Chomczynski & Sacchi 1987) added per 100 mg tissue or 5 × 10⁶ cells and the RNA pellets were re-suspended in 20–50 µl water and their absorbance at 260 nm measured. The resulting total RNA was reverse transcribed using 0·1 units MMLV reverse transcriptase and 40 pmol oligo dT, using a maximum of 1 µg RNA or all of the RNA extracted in samples too small for accurate quantification. Real-time PCR was performed, using SYBR green to monitor the kinetics of PCR amplification in a light cycler (Idaho Technology, Salt Lake City, Utah, USA or Roche, Indianapolis, IN, USA), to quantitate transcripts of the housekeeping gene, adenine phosphoribosyl transferase (APRT) and the TSHR. In addition, transcripts of PPARγ were quantified as an indicator of adipogenesis in preadipocytes cultured in either serum-containing medium (SCM) or differentiation medium.

The APRT reactions were performed in the presence of Taqstart antibody (Sigma, St Louis, MO, USA) to produce a ‘hot-start’. Results are expressed as transcript copy numbers (TCN) obtained from the crossing points on standard curves established using cloned cDNA for all three genes. The melting curves of the amplicons were compared with that of the standards and the PCR products were retained for gel (and initially sequence) analysis to confirm their identities. Intron/exon spanning primers used, optimized conditions and amplicon sizes are shown in Table 1.

Table 1 Primers and conditions for human QRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Mg²⁺/annealing</th>
<th>Amplicon/melting</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRT</td>
<td>Exons 3 and 5</td>
<td>2·5 mM/55 °C 2 s</td>
<td>247 bp/91 °C</td>
</tr>
<tr>
<td></td>
<td>f5’gctgcgtgcctctcatcgcgaag r5’tcttaaggggagggtctc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSHR</td>
<td>Exons 7 and 9</td>
<td>3·5 mM/60 °C 2 s</td>
<td>287 bp/85 °C</td>
</tr>
<tr>
<td></td>
<td>f5’caatgggacaaagtccgatg r5’tctgattttctctgtattc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>Exons 3 and 5</td>
<td>3 mM/55 °C 2 s</td>
<td>390 bp/89 °C</td>
</tr>
<tr>
<td></td>
<td>f5’cagtgaggtgtctcataa r5’ccttgggcatactctgtgat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

f, Forward; r, reverse; Mg²⁺, final magnesium concentration; annealing, temperature and time for primer annealing; amplicon, size of the PCR product; melting, melting point of the PCR product.
from experiments in which the CHO-expressing TSHR gave a value of $1-5 \times 10^2$ TSHR TCN and negative controls in the absence of any template were zero.

### Induction of hyper- and hypothyroidism in BALBc mice

Three groups of five 6-week-old male and female BALBc mice were obtained from the breeding colony at the University of Wales College of Medicine (UWCM) and maintained on standard chow and water ad libitum. One group was untreated, a second received 0.1% (w/v) propylthiouracil (PTU) and the third 12 µg/ml T₃, in both cases administered in the drinking water. After 15 days, the animals were killed, blood was obtained for serum and their thyroids, orbital contents, abdominal and epidydimal fat were removed and snap frozen.

All procedures were performed with Home Office approval (project grant number 40/2052).

To confirm that the PTU and T₃ treatments had induced hypo- and hyperthyroidism respectively, total thyroxine (T₄; Gamma coat Incstar Corporation, Stillwater, MN, USA) and T₃ levels (Biomerica Inc, Newport Beach, CA, USA) were measured by immunoassay according to the manufacturer’s instructions.

RNA from all of the snap-frozen tissues was extracted and reverse transcribed, as described above. Real-time PCR was used to quantitate transcripts for the housekeeping gene, β actin, and the TSHR using standard curves established with cloned murine cDNA for both genes. The identities of the amplicons were confirmed by melting curve, gel and sequence analysis. Intron/exon spanning primers used, optimized conditions and amplicon sizes are shown in Table 2.

### Results

**TSHR TCN is increased in GD thyroid tissue compared with MNG**

In human thyroid tissue, a wide range of TSHR TCN was obtained irrespective of whether results are reported as a ratio with the housekeeping gene, APRT or as an absolute value per 25 ng input RNA. In GD and MNG, the mean TSHR TCN per 25 ng RNA was $9.8 \times 10^3$ (s.d. $3.7 \times 10^3$) and $1.2 \times 10^3$ (s.d. $1.0 \times 10^3$) and mean TSHR TCN per 100 APRT was $2.7 \times 10^3$ (s.d. $1.1 \times 10^3$) and $5.7 \times 10^2$ (s.d. $1.5 \times 10^2$) respectively. Results for the individual samples are shown in Fig. 1.

**The majority of adipose samples do not express the TSHR; the exceptions being GD and TED**

None of the whole adipose tissues sampled, from the neck or abdomen of patients without GD or TED ($n=11$), expressed TSHR transcripts, despite APRT TCN being in the range of 100–5000/25 ng input RNA, indicating intact RNA and adequate reverse transcription. In contrast, receptor expression in adipose samples from two out of two TED orbital and three out of three GD neck fats approached the levels found in the thyroid ($\sim 100$ TSHR TCN/100 APRT) and, in a single normal orbital fat tested, it was at the limit of detection (<10 TSHR/100 APRT). Results for the individual samples are shown in Fig. 2.

**A similar pattern emerges when testing preadipocytes and mature adipocytes**

Five samples of orbital (four TED and one normal) and four samples of abdominal fat were fractionated into preadipocytes and adipocytes.
and analysed for TSHR TCN directly from the gradient, i.e. with no period in culture. All of the preadipocytes were negative with the exception of the single TED orbital tested, which expressed 10 TSHR TCN per 100 APRT. The situation in the mature adipocytes was more complex with one TED orbital and one abdominal sample at the limit of detection (<10 TSHR/100 APRT), two TED orbital samples positive (>10 TSHR/100 APRT) and two abdominal samples negative. Results for the individual samples are shown in Fig. 3.

**Short-term culture in FCS or hormone-induced differentiation increases TSHR TCN in preadipocytes**

Three samples of orbital (two TED and one normal) and five samples of abdominal fat were analysed for TSHR TCN following 5 days culture in SCM and/or up to 14 days in differentiation medium. A further two abdominal fat samples were analysed for PPARγ TCN in SCM and differentiation medium. In four of the seven preadipocyte samples cultured in SCM, TSHR transcripts were positive or at the limit of detection irrespective of whether they were derived from patients with GD/TED or not. All differentiating samples expressed >10 TSHR TCN/100 APRT. Results for the individual samples are shown in Fig. 4.

Preadipocytes in SCM have none of the characteristic signs of adipogenesis whereas those in the differentiation protocol are rounded and have lipid droplets, which are oil red O positive, as shown in Fig. 4. Furthermore, PPARγ transcripts in preadipocytes maintained in SCM are below the limit of detection. In contrast, in the differentiating preadipocytes, levels of this transcription factor, whose expression coincides with that of C/EBPα
and thus provides a good marker of adipogenesis (Rosen & Spiegelman 2000), are increased as summarized in Fig. 5.

In summary, a total of nine adipose samples have been analysed from patients with GD and/or TED ex vivo, either as whole fat samples, preadipocytes or mature adipocytes without a period in culture. Eight of these were positive for TSHR expression, i.e. the TSHR TCN per 100 APRT was \( >10 \), the remaining sample was at the limit of detection. Conversely, of fifteen fat samples from patients not suffering from GD or TED analysed ex vivo, thirteen were negative for TSHR transcripts and two were at the limit of detection.

At the time of the analysis, all of the patients, even those with GD and/or TED would have been rendered euthyroid as a prerequisite for surgery. To investigate the influence of thyroid hormone status on adipose TSHR expression, we have used a murine model.

**Thyroid hormone status does not influence TSHR TCN in murine adipose tissues**

In euthyroid BALBc mice, TSHR TCN in the thyroids ranged from 1 to \( 3 \times 10^3 / 25 \text{ ng input RNA} \) or 200 to 5000 TSHR/100 \( \beta \) actin transcripts and is comparable with the values obtained from diseased (GD or MNG) human thyroid tissues.

We have measured TSHR TCN in the orbital contents, pooled visceral and epididymal adipose depots. All samples were at the limit of detection, i.e. below the \( 10^1 \) standard using QRT-PCR, although an amplicon could be detected when the products from 25 PCR cycles were visualized on an ethidium bromide-stained agarose gel, as shown in Fig. 6.

To ensure that the treatment with PTU and T3 had induced hypo- and hyperthyroidism respectively, the total T4 and T3 levels were measured in
the mice. In euthyroid mice, mean total $T_4$ was 4.5 (s.d. 0.3) and in PTU-treated mice it was 2.4 (S.D. 0.35) µg/dl. In euthyroid mice, mean total $T_3$ was 1.1 (S.D. 0.01) and in $T_3$-treated mice it was 6.3 (S.D. 0.7) ng/ml.

When the orbital contents, visceral and epididy- mal fat pads from the PTU- and $T_3$-treated BALBc mice were analysed by QRT-PCR, the values obtained were at the limit of detection, as in the untreated euthyroid animals, indicating no major influence of thyroid hormone status on adipose TSHR transcript levels. The entire experiment was repeated with similar results.

**Discussion**

The present work has confirmed and extended our previous studies and indicates that the TSHR is expressed *ex vivo*, not only in TED orbital adipose tissues but also in other fat depots from patients with GD. This suggests that the adipose changes present in the orbit, which result in TSHR expression, may be systemic rather than localized and supports the concept that patients with TED (and/or PM) have a severe form of GD. What are those changes likely to be? Previously we have reported oil red O staining in samples of TED orbital preadipocytes following short-term culture but not in these cells when isolated from patients free of GD and TED (Crisp et al. 2000). Preadipocytes, from any patient and fat depot, which can be induced to differentiate *in vitro*, express TSHR detectable by immunocytochemistry and have oil red O-positive cells. In the current work, we have found TSHR transcripts only in freshly isolated preadipocytes from TED orbits and in all samples undergoing hormone-induced differentiation. These results indicate that TSHR expression is maximal during adipogenesis and that this process must be ongoing *in vivo* in any fat sample in which TSHR transcripts can be detected *ex vivo*, i.e. without *in vitro* culture.
Figure 4 QRT-PCR measurement, reported as TCN on the y axis, of TSHR in fractionated preadipocytes cultured in SCM (PreC1–7) for up to 5 days or following the 14-day differentiation protocol (Diff1–3). Samples with an asterisk are from TED orbital fat and the remaining samples are from normal abdominal or orbital fat. TSHR transcripts per 100 transcripts of APRT (solid bars), TSHR transcripts per 25 ng input RNA (open bars) and APRT transcripts per 25 ng input RNA (shaded bars). In samples in which only APRT data are presented, no TSHR transcripts were detected. The micrographs demonstrate that the preadipocytes in SCM (A) have none of the signs of adipogenesis whilst those in differentiation medium are rounded and have lipid-containing vacuoles (B) which are oil red O positive. All results are the mean of duplicates which vary by <20%.
Although most authors would agree that TSHR expression is increased during adipogenesis of rodent and human preadipocytes (Haraguchi et al. 1996a,b, Valyasevi et al. 1999) it is not a universal finding. A recent study suggests that TSHR transcript and protein expression are reduced as differentiation proceeds and are maximal in cultured preadipocytes (Bell et al. 2000). Furthermore these cells responded to TSH with an increase in activity of p70S6 kinase, a downstream component of TSH signalling identified in a rat thyroid cell line.

It seems clear that culture conditions can affect gene expression and previously it has been reported that TSHR expression is upregulated by serum in FRTL5 cells and differentiating 3T3 LI (Shimura et al. 1997), with insulin or insulin-like growth factor-I identified as the active component. The current work demonstrates that TSHR transcripts are present in early passage preadipocytes in short-term culture, when the equivalent population analysed ex vivo were negative. However, this suggests that studies, including our own, showing TSHR immunocytochemistry or functional TSHR, both of which require a period in culture, may be misleading. It is on this basis that functional TSHR expression has been suggested in adipose depots outside the orbit and from individuals having no GD or TED. A limited number of ex vivo Northern analyses (Crisp et al. 1997, Janson et al. 1998) and immunocytochemistry (ICC) studies (Crisp et al. 2000) have shown that such fat tissues can express TSHR at the limit of detection. From our results we would suggest that the TSHR in these samples

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Figure 5 QRT-PCR measurement, reported as TCN on the y axis, of PPARγ in fractionated preadipocytes cultured in SCM (PreC1–3) for up to 5 days or following the 14-day differentiation protocol (Diff1–3). Samples are from normal abdominal fat. APRT transcripts per 25 ng input RNA (shaded bars), PPARγ transcripts per 25 ng input RNA (open bars) and PPARγ transcripts per 100 transcripts of APRT (solid bars). In samples in which only APRT data are presented, no PPARγ transcripts were detected.
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is restricted to mature adipocytes, which are largely refractory to TSH. Conversely, if preadipocytes express the TSHR, they have the capacity to undergo adipogenesis and contribute to pathogenesis via three routes: (1) expanded volume, (2) increased autoantigen expression and (3) elaboration of inflammatory adipocytokines. Whether TSH ligands can trigger this process in human primary preadipocytes is still not clear although in rat preadipocytes TSH stimulates growth but inhibits differentiation (Haraguchi et al. 1999).

One might reason that our results are based on the measurement of transcripts and this may not equate with protein. In initial experiments to validate the technique we have found that TSHR TCN correlates with functional receptor at the cell surface, at least in CHO cells transfected with the human TSHR (Evans et al. 1999 data not shown). Furthermore, the results we obtained from preadipocytes in culture showing increased TSHR transcripts (the current study) and TSHR immunoreactivity (Crisp et al. 2000) imply that transcript also correlates with protein in cultured primary cells. The higher levels of TSHR transcript in GD thyroid tissue compared with MNG mirrors our previous demonstration of receptor protein in these glands using ICC (Sequeira et al. 2002).

The results we obtained in the murine adipose tissues are surprising considering the numerous reports of TSHR transcripts in rodent fats and differentiating cells (Roselli-Rehfuss et al. 1992, Endo et al. 1995, Haraguchi et al. 1996a) and of high-affinity TSH binding to rodent epidydimal fat (Iida et al. 1987). The difference may simply reflect the methodology used to detect transcripts since previous studies have analysed the final PCR product to yield a qualitative rather than quantitative result. Even though the BALBc fat samples were at the limit of detection, we were still able to visualize the final PCR product and would have reported these tissues as positive using conventional PCR. Furthermore, we investigated TSHR transcript levels in the adipose tissues of female, euthyroid NOD mice, a strain in which destructive thyroiditis but no orbital pathology can be induced using syngeneic TSHR primed T cells (Many et al. 1999). In common with the BALBc, NOD adipose TSHR transcripts were at the limit of detection (data not shown). We are not aware of any other studies investigating TSHR expression,
either as transcripts or via TSH binding or signal transduction, in primary murine fat tissues. However, our results are in agreement with previous experiments from one of us showing an absence of TSHR immunoreactivity in BALBc orbital contents, unless a TED-like pathology has been induced in the animal using TSHR primed T cells (Many et al. 1999).

The absence of TSHR expression in cervical fats from TMNG patients and the results obtained in the T3-treated mice suggest that thyroid hormone status does not influence adipogenesis, and thus TSHR expression. However, we are aware that subtle changes may not have been observed in the murine samples when the euthyroid values were at the limit of detection. In human fat, TSHR expression is an early marker of adipogenesis, since preadipocytes in short-term culture express receptor protein and transcripts but not PPARα and are oil red O negative (unless they are derived from TED orbits). If hyperthyroidism per se does not trigger differentiation, it could be caused by inflammatory or autoimmune processes. Recent reports have suggested that interleukin-6 may be implicated (Jyonouchi et al. 2001) but since in vitro manipulation can alter TSHR expression it seems that in vivo experiments will be required to resolve this issue.

In conclusion, we have shown, for the first time, that adipose TSHR expression is associated with the autoimmune or inflammatory process in GD and TED and indicates in vivo adipogenesis in progress but it is not restricted to the orbit or influenced by thyroid hormone status.

Acknowledgements

We are indebted to Mrs Carol Lane (Department of Ophthalmology, UWCM) and Professor Malcolm Wheeler (Department of Surgery, UWCM) for providing surgical samples and to Dr Mike Christie (Guy’s, King’s and St Thomas’ Medical School, London, UK) for supplying adipose tissue from NOD mice. We thank Professor Gilbert Vassart and Dr Sabine Costagliola (ULB, Brussels, Belgium) for providing cDNAs for the human and mouse thyrotrophin receptors and Brahms Diagnostica GmbH (Hennigsdorf, Germany) for kindly supplying us with 125I-TSH. This work was supported by grants from the Division of Medicine at UWCM and the Wales Office for Research and Development. There are no conflicts of interest that would compromise the impartiality of the authors.

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Journal of Molecular Endocrinology (2003) 30, 369–380

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Received 4 December 2002

Accepted 20 December 2002