Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts

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1Departments of Medicine and Biochemistry, Microbiology, and Immunology, Loeb Health Research Institute at the Ottawa Hospital, University of Ottawa, Ottawa, Canada K1Y 4E9; 2Departments of Medicine, Biochemistry, and Molecular Biology, Albany Medical College and the Samuel S. Stratton Veterans Affairs Medical Center, Albany, New York, 12208; and 3Division of Molecular Medicine, University of California at Los Angeles School of Medicine, Harbor-University of California at Los Angeles Medical Center, Torrance, California 90502

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Bell, Andrea, AnneMarie Gagnon, Laura Grunder, Sonia J. Parikh, Terry J. Smith, and Alexander Sorisky. Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. Am J Physiol Cell Physiol 279: C335–C340, 2000.—Controversy continues about whether, and to what levels of abundance, thyroid-stimulating hormone receptors (TSHR) are found in human tissues other than the thyroid gland. Restricted expression to the thyroid and orbit would suggest that TSHR represents the target autoantigen in thyroid-associated ophthalmopathy. A more generalized pattern of tissue expression would be inconsistent with TSHR acting as the autoantigen that is solely responsible for selectively targeting the immune system to the orbit. We have detected TSHR mRNA in human abdominal adipose tissue by Northern blot analysis. TSHR protein was also detected, by immunoblotting with two different antibodies, in preadipocytes isolated from human abdominal subcutaneous and omental adipose tissue and in derivative adipocytes differentiated in primary culture. Preadipocytes treated with thyroid-stimulating hormone (TSH) exhibited a sevenfold increase in the activity of p70 S6 kinase, a serine/threonine kinase recently recognized as a downstream target of TSHR in thyroid cells. Activation of p70 S6 kinase by TSH was also observed in orbital fibroblasts. Thus TSHR protein expression is found in fibroblasts from several anatomic locations, suggesting that factors other than site-limited TSHR expression must be involved in restricting the distribution of Graves’ disease manifestations. Furthermore, the presence of functional TSHR in preadipocytes raises the possibility of a novel role for TSHR signaling in adipose tissue development.

p70 S6 kinase; thyroid-associated ophthalmopathy

THYROID-ASSOCIATED OPHTHALMOPATHY (TAO) is a well-described but poorly understood component of Graves’ disease. It is characterized by cell-mediated autoimmune interactions with orbital tissues. This process leads to the accumulation of hyaluronan, a glycosaminoglycan, and to often intense inflammation (28). Fatty tissue expansion also occurs in TAO, and we have previously demonstrated that a subpopulation of orbital fibroblasts from TAO and control patients can differentiate into adipocytes in culture (30). Therefore, some orbital fibroblasts are preadipocytes, that is, specialized fibroblasts committed to the adipocyte lineage.

The initiation of TAO has been postulated to depend on expression of thyroid-stimulating hormone receptor (TSHR) in orbital preadipocytes (1, 20). Evidence of TSHR expression in orbital tissue was first documented by the RT-PCR (11, 16), but concerns were raised about the reliability of these data (6, 20). Northern blotting of TSHR mRNA in orbital tissue was subsequently reported (9), as was the presence of TSHR protein in orbital fibroblasts, by indirect immunofluorescence (1). Expression of TSHR restricted to orbital fibroblasts and thyrocytes would be consistent with its proposed role as the critical autoantigen directing immunocompetent cells to the orbit, thereby contributing to the pathogenesis of TAO.

However, TSHR appears to be expressed in other tissues that are not ordinarily targeted by the autoimmune process associated with Graves’ disease. Studies on guinea pig, mouse, and rat adipocytes have suggested the presence of TSHR by binding studies and mRNA analysis (10, 14, 27). TSH cell-surface binding, TSH-stimulated cAMP responses, and the presence of TSHR mRNA in nonorbital human adipose tissue have also been described (3, 9, 17, 23). Here, we report the expression of TSHR mRNA and protein in human preadipocytes from abdominal subcutaneous and omental tissue. Preadipocytes were isolated, and their TSHR protein levels were compared with adipocytes differentiated in vitro. The preadipocyte TSHR is functional, as indicated by the ability of TSH to stimulate p70 S6 kinase (p70 S6K) in these cells.

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MATERIALS AND METHODS

Tissue preparation and cell culture. Adipose tissue from the abdominal subcutaneous and omental regions were obtained from patients undergoing elective surgery (approved by the research ethics committee of the Loeb Health Research Institute). After removal of connective tissue and capillaries by dissection, adipose tissue either underwent RNA extraction or digestion with collagenase (1.5 mg/ml; Boehringer Mannheim) as described by Hauner et al. (15). To isolate the preadipocytes, the collagenase-treated tissue was then subjected to several rounds of size filtration and centrifugation (15). Preadipocytes were seeded at $5 \times 10^4$ cells/cm$^2$ and allowed to adhere overnight in DMEM (GIBCO BRL) supplemented with 10% FBS. Medium contained penicillin and allowed to adhere overnight in DMEM-F-12 (1:1) supplemented with 33 M biotin, 17 M pantothenate, 10 M transfersin, 0.2 nM triiodothyronine, 1 M insulin, 0.2 M carbachol, 0.2 M carbachol, 0.2 M triiodothyronine, and 1 M dexamethasone (Stereoloids) and 0.1 M 3-isobutyl-1-methylxanthine (IBMX). Cells were maintained in this differentiation medium for ~18 days. Control cultures were treated in parallel, with the omission of cPGI, dexamethasone, and IBMX and with a lower insulin concentration (0.1 M). When indicated, preadipocytes were grown to confluence in DMEM supplemented with 20% FBS. Orbital fibroblasts were grown out from tissue explants, obtained from patients with TAO, in Eagle’s medium supplemented with 10% FBS, glutamine, and antibiotics, as previously described (30). Thyroid tissue was obtained from surgical patients according to protocols approved by the Albany Medical College institutional review board. KAT-50 human thyroid epithelial cells were kindly provided by K. Ain (Lexington, KY). CHO-K1 Chinese hamster ovarian cells, McA-RH7777 rat hepatoma cells, and J774 mouse macrophage cells were generous gifts from Y. Marcel (Ottawa, ON). Jurkat human leukemia cells were from R. Haché (Ottawa, ON).

Northern blot analysis of mRNA. Human adipose and thyroid tissue and KAT-50 cells were flash-frozen, RNA was isolated (7), and Northern analysis was carried out (8) as described previously (33). Briefly, RNA was electrophoresed on denaturing 1% agarose-formaldehyde gels. Samples were transferred to a Zeta probe membrane (Bio-Rad). Hybridization was performed with a TSHR probe, generated from human TSHR cDNA, in 5 X saline sodium citrate, 50% formamide, 5 X Denhardt’s solution, 50 mM phosphate buffer (pH 6.5), 1% SDS, and 0.25 mg/ml salmon sperm DNA at 48 °C overnight. After high-stringency washes, membranes were exposed to X-Omat film (Kodak) to visualize radioactive hybridization. The manufacturer’s instructions and reprinted with a commercial monoclonal anti-TSHR antibody from Novocastra (NCL-TSH-R2) that was diluted to 1:60. This antibody recognizes TSHR amino acid residues within region 125–369. A monoclonal antibody directed against the influenza hemagglutinin antigen (gift from J. Liu, Ottawa, ON) was used at the same dilution as a negative control primary antibody. Detection was accomplished by ECL after incubation with peroxidase-conjugated anti-mouse secondary antibody.

Activation of p70 S6K. Confluent preadipocyte and orbital fibroblast cultures were placed in serum-free medium overnight; the medium was made with DMEM-F-12, 33 M biotin, 17 M pantothenate, 0.2 nM triiodothyronine, and 0.5% BSA. TSH (either purified from bovine pituitary; 2 IU/mg protein or human recombinant TSH, 7.3 IU/mg protein; both from Sigma) was added for 1 h at 37°C, and cells were then lysed in ice-cold PBS containing 1% Nonidet P-40, 0.2 mM sodium vanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 mg/ml benzamidine, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 μM microcystin, and 1 mM β-glycerophosphate. When indicated, 100 nM wortmannin or vehicle was added 15 min before TSH stimulation. Unless otherwise indicated, TSH refers to purified bovine TSH. Lysates were incubated with anti-p70 S6K (Santa Cruz Biotechnology) coupled to Protein A-Sepharose for 90 min at 4°C. After centrifugation, the supernatant lysate was removed, and the activity of the immunoprecipitated p70 S6K was measured using an in vitro kinase assay kit (Upstate Biotechnology). After this assay, Laemmli buffer was added to the immunoprecipitated p70 S6K, and SDS-PAGE and Western blot analysis was performed to detect gel mobility shifts. Jurkat cells and human preadipocytes were treated with TSH as described above, and activation of p70 S6K was also measured by Western analysis of total cell lysates using a polyclonal phosphospecific p70 S6K antibody at a 1:250 dilution (Thr389, New England Biolabs).

RESULTS

Subcutaneous adipose tissue was subjected to Northern blot analysis for TSHR mRNA levels. As seen in Fig. 1, a distinct band of 4.6 kb was clearly detected after 7 days of exposure to X-ray film. For comparison, RNA from thyroid tissue (1-day exposure) and from KAT-50 thyroid epithelial cells (7-day exposure) was analyzed. A band of similar size was observed comparable to that seen in the RNA from the abdominal fat. Isolated subcutaneous preadipocytes and their differentiated adipocyte counterparts KAT-50 cells and orbital fibroblasts were analyzed by Western blotting for TSHR protein expression. Figure 2A shows the immunoblot probed with the anti-TSHR-(352—366) antibody. Blots from all four cell types exhibit the 230-, 180-, and 100-kDa bands at variable intensities that are characteristi-
cally recognized with this antibody. The 230- and 180-kDa bands are thought to represent preprocessed forms of TSHR, as described by Ban and colleagues (2) in the FRTL-5 cell line. An additional less-intense 100-kDa band was observed by these authors in COS-7 cells over-expressing transfected human TSHR, and this may possibly represent the relatively less abundant mature and functional form of the receptor (2). They were unable to detect this band in FRTL-5 cells and postulated that this was most likely due to the considerably lower level of TSHR protein expressed in these cells when compared with the transfected COS-7 cells. Alternately, the anti-TSHR-(352—366) antibody may preferentially recognize these putative precursor forms.

To confirm the specificity of the anti-TSHR-(352—366) antibody, the membrane was stripped and reprobed with another primary anti-TSHR antibody, NCL-TSH-R2 (Fig. 2B). The prominent band at 100 kDa is consistent with that expected for the mature TSHR protein and is similar to what was observed for a chimeric TSHR protein produced by a cDNA sequence encoding the NH₂-terminal human TSHR fused to the COOH-terminal domain of the biotin carboxyl carrier protein of Escherichia coli acetyl-CoA carboxylase (21). For KAT-50 and orbital fibroblast cells, the 100-kDa band appears as a doublet, with the upper band corresponding to the 100-kDa band seen with the anti-TSHR-(352—366) immunoblot in Fig. 2A. Although posttranslational processing of TSHR may yield multimeric intermediates, the mature functional form is thought to be the ~100-kDa holoreceptor (2, 12, 19, 22, 25, 31). The 100-kDa band that we observed is specific for the TSHR in that this band was not detected using a control primary monoclonal antibody and because cells that do not express TSHR failed to exhibit this band (Fig. 2C).

Preadipocytes and adipocytes differentiated in primary culture from abdominal subcutaneous adipose tissue samples from four subjects were compared by immunoblot analysis for TSHR protein expression using the NCL-TSH-R2 antibody (Fig. 3). For two subjects, parallel samples of abdominal omental adipose tissue were also studied. The TSHR bands are present in all samples. Omental preadipocyte samples appear expressing transfected human TSHR, and this may possibly represent the relatively less abundant mature and functional form of the receptor (2). They were unable to detect this band in FRTL-5 cells and postulated that this was most likely due to the considerably lower level of TSHR protein expressed in these cells when compared with the transfected COS-7 cells. Alternately, the anti-TSHR-(352—366) antibody may preferentially recognize these putative precursor forms.

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to have higher levels of TSHR that decrease during differentiation.

To determine whether the TSHR protein observed in preadipocytes is functional, we investigated whether treatment with TSH (20 μM) could activate p70 S6K. This serine/threonine kinase is normally regulated by receptor tyrosine kinases and has been implicated in transcriptional and translational control (26). Recently, p70 S6K was reported to be stimulated by TSH in a rat thyroid cell line in which it plays an important role in mitogenesis (5). Activation of p70 S6K can be measured through its in vitro kinase activity on a specific peptide substrate based on the relevant residues of its substrate, S6. The activation of p70 S6K, resulting from its own serine/threonine phosphorylation, is also associated with an upward shift on SDS-PAGE (5, 26). As shown in Fig. 4A, TSH stimulated p70 S6K in subcutaneous preadipocytes by sevenfold. The immunoprecipitated enzyme was then subjected to SDS-PAGE and Western analysis with anti-p70 S6K antibody (Fig. 4B). TSH treatment of preadipocytes induced a clear upward shift in migration of the p70 S6K. Orbital fibroblasts exposed to TSH also exhibited an upward gel shift of p70 S6K indicative of activation. As expected, TSH-treated Jurkat cells, which do not express TSHR (see Fig. 2C), showed no evidence of phosphorylated p70 S6K (Fig. 4C). To rule out any effect of possible contaminants in the bovine TSH (>90% pure by SDS-PAGE; Sigma), we treated human preadipocytes with 3.6 μM human recombinant TSH and confirmed the activation of p70 S6K. To explore the possible upstream regulators of p70 S6K activation by TSH, we treated human preadipocytes with 100 nM wortmannin, a phosphatidylinositol 3-kinase inhibitor (24). Figure 4D demonstrates that wortmannin completely abrogates the p70 S6K response to TSH. Therefore, both abdominal preadipocytes and orbital fibroblasts express TSHR protein that is competent to activate p70 S6K in a ligand-dependent fashion.

**DISCUSSION**

The association of TAO with goitrous hyperthyroidism in Graves’ disease has led many investigators to propose the existence of a shared target autoantigen in the thyroid and orbit. TSHR was considered a good candidate, given its integral role in mediating the hyperthyroidism and glandular growth of Graves’ disease. The presence of TSHR in orbital tissue and orbital fibroblasts was demonstrated using RT-PCR in 1993 (11, 16), but the possibility of illegitimate transcription was raised (6, 9, 20). Subsequently, TSHR in orbital cells was detected, albeit very faintly, by Northern blot analysis (9). Bahn et al. (1), using indirect immunofluorescence, described the presence of TSHR protein in orbital fibroblasts/preadipocytes. The levels of TSHR that they observed were reduced with cell passage.

If TSHR were the critical link between hyperthyroidism with TAO, and if its autoantigenicity were solely responsible for limiting disease manifestations to the orbit and thyroid, then its expression ought to be restricted to those two tissues. Our immunoblot analysis does demonstrate TSHR protein in orbital fibroblasts, in agreement with Bahn et al. (1). However, we have
detected TSHR mRNA in abdominal adipose tissue, as have others (9, 17). Using two different antibodies, we have also demonstrated TSHR protein expression for the first time in preadipocytes and adipocytes differentiated in vitro, isolated from subcutaneous and omental depots. TSHR protein is therefore present in abdominal fat.

Very recently, it was reported that differentiation of orbital preadipocyte fibroblasts into adipocytes in vitro resulted in increased levels of TSHR and enhanced TSHR-dependent cAMP responses (32). However, no data were provided to document the degree of adipocyte differentiation, which was stated to occur in 10–20% of cells. It is quite possible that nondifferentiating orbital fibroblasts exposed to the differentiation medium (containing, among other factors, the cAMP-elevating agents cPGl3 and IBMX) were responsible for the enhanced cAMP responses to TSH observed under the culture conditions employed in those studies. These same authors were unable to detect TSHR mRNA in abdominal adipose tissue using a RNase protection assay (RPA). However, because we (in this report) and others (9, 17) have demonstrated the presence of TSHR mRNA in adipose tissue/adipocytes by Northern analysis and because we have shown that preadipocytes express functional TSHR protein, it would appear that technical issues related to RPA methodology might have been problematic in that study. It is also likely that the strategy employed by those authors using a cadaveric source of adipose tissue was suboptimal compared with the fresh adipose tissue we obtained at surgery.

Our data argue against the concept that a restricted expression of TSHR in orbital connective tissue accounts for the anatomic selectivity exhibited in Graves’ disease. Instead, we suggest that TSHR may be expressed in fibroblasts/preadipocytes of many different tissues, where it could function as a key cell-signaling molecule. This view does not speak against TSHR having a potentially important role in the pathogenesis of the extrathyroidal manifestations of Graves’ disease but suggests rather that the anatomic site-restricted manifestations are a consequence of other attributes of the orbital fibroblast phenotype. We have defined a number of candidates recently. These include a particular susceptibility of orbital fibroblasts to activation by proinflammatory signals such as cytokines and CD40/CD40 ligand engagement (4, 29, 33). Moreover, we have identified proteins that appear to be expressed at high levels in orbital and pretilial fibroblasts but at considerably lower levels in fibroblasts from irrelevant regions of the body (34).

The TSHR protein expressed in abdominal preadipocytes and orbital fibroblasts is functional, as indicated by its ability to stimulate p70 S6K, a recently recognized downstream target of this receptor in thyroid cells. The stimulation that we describe here was stronger than that reported in the thyrocyte cell line, perhaps due to the higher concentration of, and longer exposure to, TSH. Importantly, this is the first evidence that preadipocytes possess a competent TSHR capable of activating p70 S6K, suggesting a potential role for TSHR in adipose tissue development. Others have proposed a role for TSHR in the regulation of lipolysis in neonatal human adipocytes (17). Very recently, TSH was found to induce proliferation and inhibit differentiation of cultured rat preadipocytes (13). The precise cellular responses arising from TSHR ligation in human preadipocytes remain to be elucidated and could include proliferation, differentiation, or apoptosis. Because excess abdominal fat is a major risk factor for type 2 diabetes mellitus and cardiovascular disease, further investigation into the role of TSHR in preadipocyte cell biology seems warranted.

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