0013-7227/96/$03.00/0
Endocrinology
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Vol. 137, No. 8
Printed in U.S.A.

Differentiation of Rat Preadipocytes Is Accompanied by Expression of Thyrotropin Receptors

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ABSTRACT

To investigate the regulation of expression of the TSH receptor (TSHR) in extrathyroidal tissues, the level of TSHR messenger RNA (mRNA) and TSH-dependent signal transduction were determined in isolated rat adipocytes and cultured preadipocytes. The epididymal, sc, and perirenal, but not the interscapular brown adipose tissues, possessed TSHR mRNA and increased cAMP responses to TSH and were thus used as the source of preadipocytes.

Morphological analysis revealed that the combination of insulin and T_3 most effectively caused the differentiation of rat preadipocytes. These differentiated preadipocytes exhibited increased cAMP production in response to TSH. The addition of FCS to the culture medium inhibited the differentiation of rat preadipocytes as well as TSH-stimulated production of cAMP. The stimulation of differentiation was associated with an increased expression of TSHR mRNA levels, whereas the inhibition of differentiation was associated with a decreased expression of TSHR mRNA, as detected by Northern blot analysis.

The results indicate that the expression and function of the TSHR in cultured rat preadipocytes are closely related to cellular differentiation. Cultured rat preadipocytes appear to provide a useful system for studying the mechanism of extrathyroidal expression of TSHR. (*Endocrinology* **137:** 3200–3205, 1996)

TSH EXERTS its biological effects by binding to TSH receptor (TSHR). TSHR expression is regulated by various transcription factors, such as thyroid-specific transcription factor-1, cAMP-responsive element-binding protein, and single stranded DNA-binding proteins (1–4). These factors work cooperatively to ensure the thyroid-specific expression of TSHR. That TSH stimulates lipolysis (5) and binds to fat cell membrane from guinea pigs (6, 7) and humans (8–10) has long been known. Therefore, the existence of TSHR in these tissues was thought to be related to the extrathyroidal manifestations of Graves' disease. Recently, reverse transcription-PCR revealed the existence of TSHR messenger RNA (mRNA) in such extrathyroidal tissues as fibroblasts, lymphocytes, and cardiomyocytes (11–14). More recently, we cloned a full-length TSHR complementary DNA (cDNA) from rat fat cells (15). This clone differs by one amino acid relative to rat thyroid TSHR cDNA. In addition, the fat TSHR cDNA, when transfected into Chinese hamster ovary cells, is functionally identical to thyroid TSHR in Chinese hamster ovary cells. These observations, including ours, indicate the importance of TSHRs in extrathyroidal tissues. However, the mechanism of their expression and their functional roles have not been clarified. It is partially because of the lack of nonthyroidal cultured cells that express TSHR.

We attempted to evaluate how extrathyroidal TSHRs are regulated to better explain the physiological roles of these receptors using isolated rat adipocytes and cultured preadipocytes.

Received December 17, 1995.

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Materials and Methods

Rat preadipocyte preparation and cell culture

Male SD rats (4–6 weeks old) were fed, then killed by cervical dissection. Rat preadipocytes were prepared as described by Deslex *et al.* (16). Briefly, epididymal, perirenal, and sc fat from the inguinal regions were resected under sterile conditions and bathed in Krebs-Ringer buffer containing 10 mM HEPES pH 7.4, and 1% BSA fraction V. Fat tissue was subsequently chopped into pieces and digested in the above buffer containing 1 mg/ml collagenase for 1 h at 37 C. Cells were filtered through 25-μm nylon mesh. The filtrate was centrifuged at 600 × *g* for 5 min to isolate the first pellet of preadipocytes. Next, the floating adipocytes were resuspended and centrifuged at 600 × *g* for 5 min to obtain the second pellet. The first and second pellets were suspended in Ham's F-12 medium-DMEM (1:1) containing 10% FCS and antibiotics (50 U/ml penicillin G, 50 μg/ml streptomycin, and 1 μg/ml amphotericin B) and plated into culture plates for 18 h. Attached cells were washed twice with Ham's F-12 medium-DMEM (1:1) and then incubated in Ham's F-12 medium-DMEM (1:1) containing antibiotics, 10 μg/ml insulin, 200 pM T_3, and 10 μg/ml transferrin for 6–8 days before the experiments. Culture media were changed every 3 days. Cultured fat cells were fixed and stained with hematoxylin and oil-red O.

Bovine TSH (bTSH), insulin, T_3, and transferrin were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human TSH (rhTSH) was purchased from Genzyme Co. (Cambridge, MA), and hCG was obtained from Teikoku Zouki Co. Life Science Laboratory (Tokyo, Japan). BRL37344 was kindly donated by Asahikasei Co. (Shizuoka, Japan).

FRTL-5 cell culture

FRTL-5 cells were kindly donated by Dr. L. D. Kohn (NIH, Bethesda, MD). The cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a six-hormone mixture (6H) containing insulin (10 μg/ml), somatostatin (10 ng/ml), hydrocortisone (10^{-8} M), transferrin (5 μg/ml), glycyl-L-histidyl-lysine acetate (10 ng/ml), and bTSH (10 mU/ml) (17). Insulin, somatostatin, hydrocortisone, transferrin, and glycyl-L-histidyl-lysine acetate were obtained from Sigma. Cells were grown in 48-well plates in 6H up to confluency, followed by 10 days in the same medium devoid of bTSH.

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Northern blot analysis

Total RNA was isolated from cells by the guanidine isothiocyanate extraction method (18). Twenty micrograms of total RNA were electrophoresed in each lane in 1.0% formaldehyde-agarose gel and blotted onto a nitrocellulose filter as previously described (15). A rat thyroid TSHR cDNA and rat β-actin cDNA, kindly donated by Dr. L. D. Kohn (NIH), were labeled with [α-^{32}P]deoxy-CTP by using a random primer labeling kit (Takara Shuzo Co., Kyoto, Japan). Blots were hybridized in 50% formamide, 2.5 × Denhardt's solution, 5 × SSPE (0.6 M NaCl, 40 mM sodium phosphate, and 4 mM EDTA, pH 7.4), 0.1% SDS, 0.1 mg/ml heat-denatured salmon sperm DNA, and 5% dextran sulfate. Hybridization was performed at 42 C for 12 h with radiolabeled probes. The filters were then washed three times at room temperature in 2 × SSC (0.3 M NaCl and 30 mM sodium acetate, pH 7.0) containing 0.1% SDS, followed by washing three times at 53 C in 0.1 × SSC containing 0.1% SDS. The filters were exposed to an imaging plate and analyzed using Bas 2000 image analyzer (Fuji Film Co., Tokyo, Japan).

cAMP measurement

Isolated rat adipocytes. Isolated rat adipocytes were prepared by the method of Rodbell (5). Cells were incubated in Krebs-Ringer buffer containing 10 mM HEPES (pH 7.4), 2 U/ml adenosine deaminase, 1 mM isobutylmethylxanthine, and 4% BSA for 45 min. After incubation, the cells were sonicated in the presence of 10 mM EDTA and immediately transferred to the filtration centrifuge tubes (C3LGC, Millipore, Tokyo, Japan) on ice. After centrifuging the tubes, the filtrate was heated at 70 C for 5 min. Subsequently, cAMP in the filtrate was measured.

Cultured rat preadipocytes and FRTL-5 cells. Cells in 24- or 48-well culture plates (Corning, Iwaki Glass Co., Tokyo, Japan) were washed once with the cAMP assay buffer (DMEM containing 10 mM HEPES, pH 7.4, and 1 mM isobutylmethylxanthine) and preincubated in cAMP assay buffer for 30 min at 37 C. The cells then were incubated with additives in 0.5 ml cAMP assay buffer for 30 min at 37 C. Incubation was stopped by adding 0.5 ml 10 mM EDTA, pH 7.4, and boiling the cells immediately, as described previously (19). cAMP was measured using commercially available kits (Yamasa-Shoyu Co., Choushi, Japan).

Results

TSHR mRNA in various fat tissues

To select the most suitable source of preadipocytes, we compared the levels of bTSH-dependent cAMP production and TSHR mRNA from rat epididymal, sc, and perirenal fat tissue as well as the interscapular brown adipose tissue. Northern blot analysis revealed that TSHR mRNA was abundant in all fat tissues except brown adipose tissue (Fig. 1A). The sizes of TSHR mRNA bands were the same as those in FRTL-5 cells (data not shown). Heavy exposure of nitrocellulose paper was required to reveal a small amount of TSHR in brown adipose tissue (data not shown). Densitometric data normalized by that of β-actin showed that perirenal and sc fat tissues had 54% and 19% TSHR mRNA compared to epididymal fat tissue, respectively.

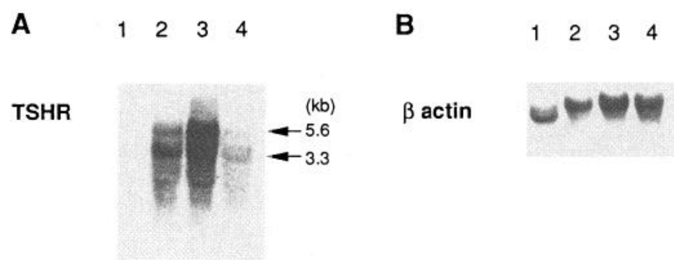

FIG. 1. Expression of TSHR mRNA (A) and β-actin mRNA (B) in various rat fat tissues. Northern blot analysis of total RNA (20 μg/lane) from rat interscapular brown adipose tissue (lane 1), perirenal fat tissue (lane 2), epididymal fat tissue (lane 3), and sc fat tissue (lane 4) was performed with a ^{32}P-labeled rat thyroid TSHR cDNA probe.

bTSH-dependent cAMP production in isolated rat adipocytes

No bTSH-dependent production of cAMP was observed in brown adipose tissue. However, when 30 mU/ml bTSH were added to the adipocytes prepared from epididymal, sc, and perirenal fat tissues, cAMP was increased by 2100%, 1430%, and 910%, respectively (Fig. 2). The epididymal, sc and perirenal fat tissues were, therefore, chosen as the source for culturing of preadipocytes.

bTSH-dependent cAMP production in cultured preadipocytes

To determine the relationship between rat preadipocyte differentiation and TSH-dependent cAMP production, cultured rat preadipocytes under different conditions were challenged with bTSH (Fig. 3). When the cells were cultured in the presence of insulin, T_3, and transferrin, they achieved full differentiation. The increase in cAMP production was observed in the presence of 1 mU/ml bTSH. Maximal cAMP production was observed in the presence of 10 mU/ml TSH to a level as high as 310% of the baseline. The amount of

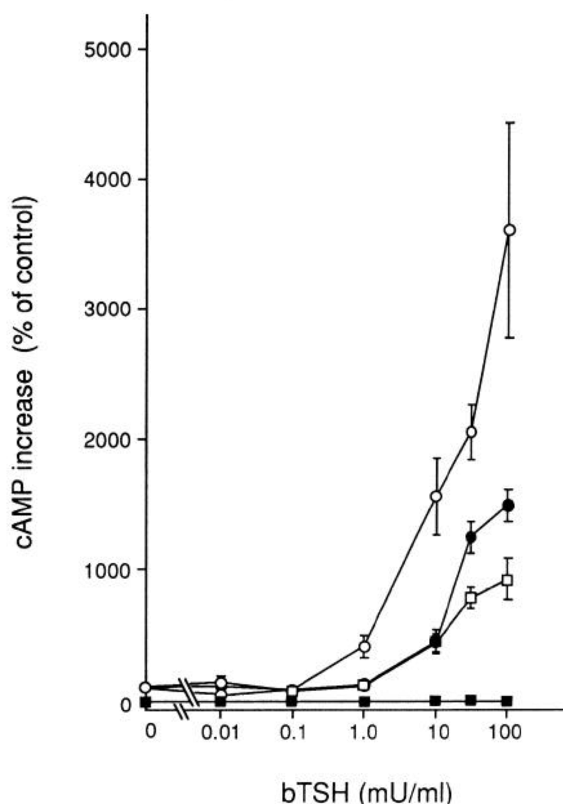

FIG. 2. bTSH-dependent cAMP production by various rat adipocytes. Rat adipocytes were prepared from rat perirenal (●), epididymal (○), sc (□), and brown adipose (■) fat tissues. cAMP production was measured as described in *Materials and Methods*. cAMP production without bTSH was expressed as 100% for each group of cells.

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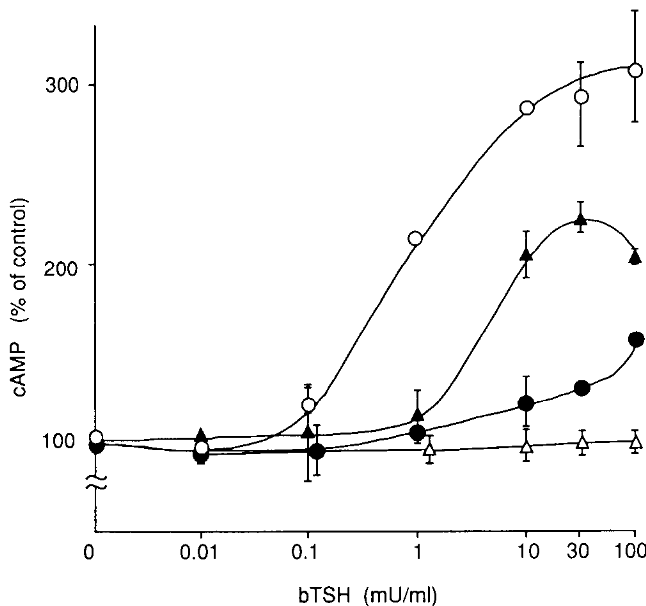

FIG. 3. Differentiation-dependent cAMP response of cultured preadipocyte to bTSH. Rat preadipocytes were prepared from rat epididymal, perirenal, and sc fat tissues and cultured in Ham's F-12 medium-DMEM containing 10% FCS for 1 day (▲) or 6 days (△), in Ham's F-12 medium-DMEM containing 10% FCS for 3 days, then in Ham's F-12 medium-DMEM without serum but with 10 μg/ml insulin and 200 pM T_3 for 3 days (●), and in Ham's F-12 medium-DMEM containing 10% FCS for 18 h, then in Ham's F-12 medium-DMEM without FCS but with insulin and T_3 for 5 days (○). The cells were incubated with increasing concentrations of bTSH for 30 min, and total cAMP was measured. Values are the mean ± SEM (n = 3).

glycerol released into the medium was 34.6 μg/well·120 min (157% increase over basal) in the presence of 10 mU/ml bTSH. Conversely, when the cells were cultured in the presence of 10% FCS for 6 days without hormones, these cells lost bTSH responsiveness. The cells cultured in the presence of 10% FCS for 6 days grew rapidly to cover the whole surface of culture wells. They had a fibroblast-like appearance and no lipid droplet (data not shown). The cells cultured without FCS and hormones also lost TSH responsiveness (data not shown). Undifferentiated rat preadipocytes, cultured in the presence of 10% FCS for 1 day to allow attachment of cells, increased cAMP production in the presence of 10 mU/ml TSH to a lesser degree than fully differentiated cells.

To further elucidate the importance and specificity of bTSH-dependent activation of adenylyl cyclase, the responses of the cells to other hormones and additives were studied. hCG concentrations as high as 10^4 mIU/ml did not stimulate cAMP production by differentiated rat preadipocytes. Isoproterenol (1 μM), which is a good stimulus for rat adipocytes, did not stimulate cAMP production by differentiated rat preadipocytes, whereas 1 μM BRL 37344, a β_3-adrenergic agonist, increased cAMP production by differentiated preadipocytes by 380%. BRL37344 did not increase the cAMP level in undifferentiated (cultured in the presence of 10% FCS for 6 days) cells. Finally, forskolin (50 μM) increased cellular cAMP in both undifferentiated and differentiated preadipocytes by more than 34,000% over the basal value. This ruled out the possibility that the low expression of adenylyl cyclase was the cause of the insensitivity of undifferentiated rat preadipocytes to bTSH.

Comparison of cAMP production by rat preadipocytes and FRTL-5 cells using bTSH and rhTSH

TSH-dependent cAMP production by rat preadipocytes was compared with that by FRTL-5 cells. When bTSH was used to stimulate FRTL-5 cells, cAMP production was observed in the presence of 0.01 mU/ml bTSH (Fig. 4A). Maximal cAMP production was observed in the presence of 10–30 mU/ml TSH. However, when rat preadipocytes were stimulated with bTSH, a dose-response curve shifted to the right. A concentration of 1 mU/ml bTSH was required to stimulate cAMP production. Next, rhTSH was used to stimulate FRTL-5 cells and differentiated rat preadipocytes (Fig. 4B), to avoid problems that could arise from the impurity of bTSH. rhTSH stimulated FRTL-5 cells at a concentration higher than 3×10^{-11} M. The concentrations of rhTSH required to stimulate differentiated preadipocytes were higher than those needed to stimulate FRTL-5 cells. The cells cultured in the presence of 10% FCS for 6 days did not respond to rhTSH (data not shown).

Northern blot analysis

A full-length rat TSHR cDNA was used as the probe to detect the differentiation-dependent changes in TSHR mRNA. Rat preadipocytes cultured in Ham's F-12 medium-DMEM containing 10% FCS for 1 day had a small amount of TSHR mRNA (Fig. 5A, lane 1). Conversely, there was a large increase in TSHR mRNA in differentiated preadipocytes (Fig. 5A, lane 3). Densitometric data normalized by that of β-actin showed a 3.9-fold increase in the TSHR mRNA level in differentiated *vs.* undifferentiated cells (Fig. 5A, lane 1 *vs.* lane 3). Cells cultured in the absence of hormones but in the presence of 10% FCS (Fig. 5A, lane 2) demonstrated a decrease in TSHR mRNA compared with that in differentiated cells. The cells cultured without any additives (Fig. 5A, lane 4) had little TSHR mRNA. These changes in the amount of

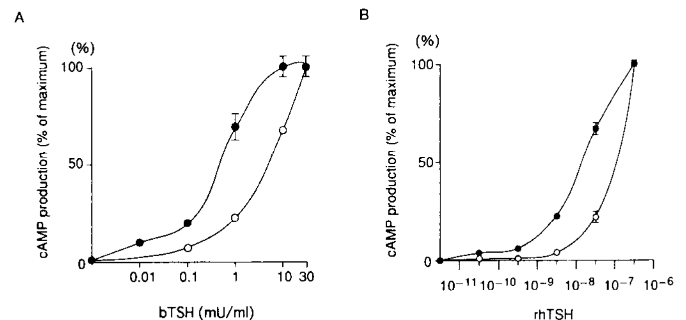

FIG. 4. Comparison of TSH-dependent cAMP production by rat preadipocytes and FRTL-5 cells. Rat preadipocytes were cultured in 48-well plates in the presence of 10% FCS for 18 h, followed by culture with 10 μg/ml insulin and 200 pM T_3 without FCS. FRTL-5 cells were cultured in 48-well plates as described in *Materials and Methods.* FRTL-5 cells (●) and rat preadipocytes (○) were incubated in the presence of various concentrations of bTSH (A) or rhTSH (B) for 30 min, and total cAMP was measured. The result is expressed as the percentage of the response obtained at 30 mU/ml bTSH (A) or 3×10^{-7} M rhTSH (B).

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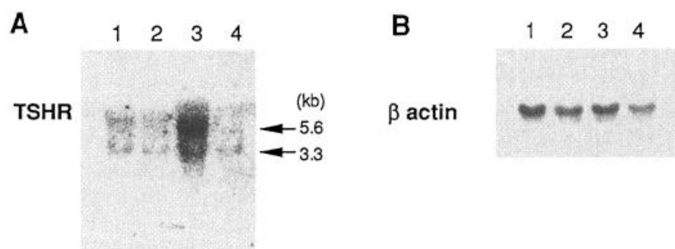

FIG. 5. Expression of TSHR mRNA (A) and β-actin mRNA (B) in cultured preadipocytes at different stages of differentiation. Total RNA was prepared from rat preadipocytes cultured in Ham's F-12 medium-DMEM containing 10% FCS for 1 day (lane 1), in Ham's F-12 medium-DMEM containing 10% FCS for 6 days (lane 2), in Ham's F-12 medium-DMEM with 10% FCS for 18 h, then in Ham's F-12 medium-DMEM without FCS but with 10 μg/ml insulin and 200 pM T_3 for 5 days (lane 3), and in Ham's F-12 medium-DMEM without any additives for 5 days (lane 4). Twenty micrograms of total RNA were applied to each lane and subjected to Northern blot analysis as described in *Materials and Methods*.

mRNA compared favorably to those in bTSH-dependent cAMP responsiveness. mRNA for β-actin is shown in Fig. 5B.

bTSH-dependent cAMP production and cell culture conditions

Cells were cultured under the conditions shown in Fig. 6 to evaluate the effects of insulin, T_3, and transferrin on bTSH-dependent production of cAMP. Insulin alone was sufficient to elicit a small increase in TSH-dependent cAMP production. The largest increase in cAMP was obtained when cells were cultured in the presence of insulin and T_3. The addition of transferrin to insulin and T_3 decreased bTSH-dependent cAMP production.

Oil-red O staining

Lipid-specific staining (oil-red O staining) identified the cells that had lipid-containing vacuoles. Cells cultured for 1 day had a few small vacuoles less than 5 μm in diameter. When the cells were cultured in the presence of 10% FCS for 6 days, they spread out to cover the surface of the dish, and all vacuoles disappeared. Cells cultured in the presence of either insulin or T_3 were moderately differentiated. The percentage of cells that had small and medium-sized lipid-containing vacuoles was 78% for the insulin-treated cells and 75% for the T_3-treated cells (Fig. 7).

Fully differentiated preadipocytes were obtained in the presence of insulin and T_3. They had large (>20 μm) as well as medium-sized and small vacuoles. The percentage of lipid-containing cells in this group increased up to 93%. When transferrin was added to insulin and T_3, the numbers of both large and small vacuoles decreased.

Discussion

Cellular differentiation is characterized by complex patterns of gene expression that take place in a precise chronological sequence. During this process, tissue-specific factors bind to the promoter regions of target genes to regulate the expression of specific proteins. In the case of the thyroid gland, thyroglobulin (1), thyroid peroxidase (2), and TSHR (3) are so-called thyroid-specific proteins. The molecular mechanisms of expression of these proteins have been extensively studied recently. However, since Rodbell (5) first reported that TSH stimulated lipolysis by rat adipocytes, TSHR in fat cells has been the subject of study in the field. TSH-binding activity was reported in guinea pig (6, 7) and human (8–10) fat cells. Binding of Graves' IgG to fat cell membrane from guinea pig was also reported (20, 21). A lipolytic effect of TSH on human adipocytes had been known (22, 23). Recently, we cloned a full-length TSHR cDNA from

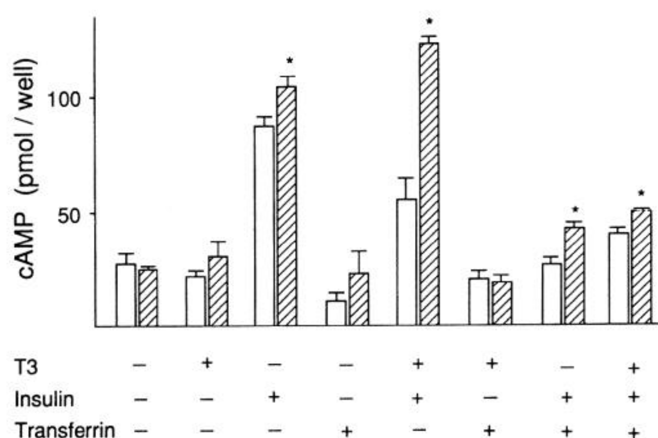

FIG. 6. bTSH-dependent cAMP production by rat preadipocytes under different culture conditions. Rat preadipocytes were cultured in Ham's F-12 medium-DMEM containing 10% FCS for 18 h to allow attachment to the 24-well plates. The cells were then incubated in the medium with or without 10 μg/ml insulin, 200 pM T_3, and 10 μg/ml transferrin as described in the figure. Subsequently, after 30-min preincubation without bTSH, the cells were incubated with 0 mU/ml (□) or 30 mU/ml (▨) bTSH for 30 min. Values are expressed as the mean ± SEM (n = 3). *, $P < 0.01$.

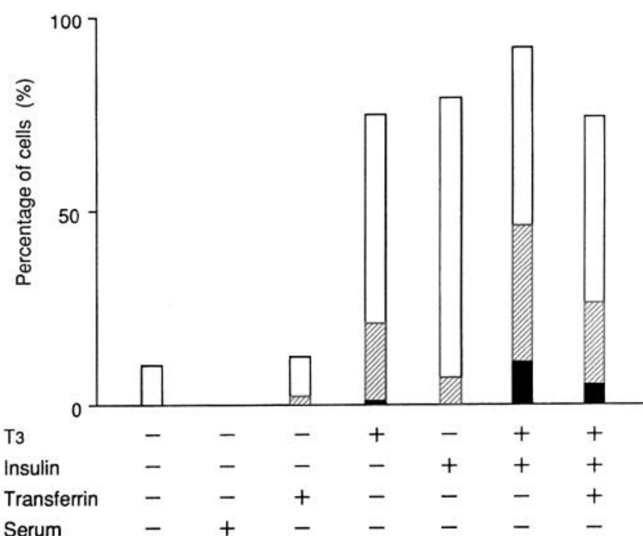

FIG. 7. Histochemical study of cultured rat preadipocytes. The cells were cultured in 12-well plates in the presence of 10% FCS for 18 h, then under the conditions described in the figure. The cells were stained with oil-red O and hematoxylin. From each of the different culture conditions, 300 cells were randomly evaluated and classified according to the diameters of lipid droplets. Another identical experiment was performed, yielding similar results. The diameters of the lipid droplets were less than 5 μm (□), more than 5 μm but less than 10 μm (▨), and more than 10 μm (■).

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rat fat cells (15). These findings, including ours, are contrary to the idea that TSHR is thyroid specific. Despite these studies, little is known about the molecular mechanism of expression of TSHR in fat cells. Fat tissue has specialized functions, *i.e.* it stores lipid and releases glycerol in response to lipolytic hormones. The expression of the fat-specific proteins that are required for lipolysis is regulated by several transcription factors (24–26). Thyroid-specific transcription factor-1 is absent in rat adipocytes and preadipocytes (data not shown). To carry out a detailed study to understand the mechanism of expression of TSHR in extrathyroidal tissues, we need nonthyroidal cells that could be handled easily and whose level of TSHR mRNA could be manipulated. Thus, rat preadipocyte is the only cultured cell system to date to be used for that purpose.

TSHR mRNA was abundant in all rat fat tissues tested, except interscapular brown adipose tissue. In addition, bTSH did not stimulate cAMP production in brown adipose tissue. These results differ from previous data obtained in guinea pigs (27). This may be due to species difference. We used cultured rat preadipocytes from white adipose tissues. When cells were cultured in the presence of both insulin and T_3 without FCS, the number of differentiated cells increased to more than 90% of the total. This differentiation was accompanied by an increase in the TSHR mRNA level. The sizes of TSHR mRNA bands were the same as those in FRTL-5 cells (3). When the cells were cultured in the presence of either insulin or T_3, each of them stimulated cell differentiation and bTSH-dependent cAMP production. Stimulatory effects of T_3 and insulin on norepinephrine-induced lipolysis have been previously reported (28, 29). In contrast, when FCS was added to the medium, the proportion of differentiated cells was decreased, which confirmed the finding of Deslex *et al.* (16). This suppression of differentiation was accompanied by the decrease in TSHR mRNA and bTSH-stimulated production of cAMP. The expression of TSHR mRNA and TSHR function are, therefore, closely related to the differentiation phenotype. TSHR should be classified as one of the receptors whose expression is differentiation-dependent in the rat preadipocyte culture system along with the adenosine receptor (30), the PG receptor (31), and the β_3-adrenergic receptor.

The low sensitivity of TSHR to bTSH was reported in isolated rat epididymal fat cells (15). Cultured preadipocytes also had low sensitivities to bTSH and rhTSH compared to those of FRTL-5 cells. The concentrations of bTSH (1–30 mU/ml) needed to stimulate cultured preadipocytes are far above the physiological concentrations *in vivo*. This may be needed to segregate thyroidal tissue as a target organ of TSH from extrathyroidal tissues, where their metabolism would be minimally influenced by physiological levels of TSH. It must be clarified whether the low sensitivity to TSH was derived from a low binding affinity to TSH in rat preadipocytes. Unfortunately, preadipocytes seeded at a high cellular density, sufficient to cover the bottom surface of the wells at the end of culturing, did not differentiate (data not shown) for unknown reasons. Therefore, under conditions that allowed full differentiation of the cells, a large area at the bottom of the wells that was not covered with cells remained. This probably gave a high background TSH binding (data not shown) and prevented us from measuring specific TSH binding using cultured preadipocytes. hCG at a concentration of 10^4 mIU/ml caused the maximal cAMP production through LH/CG receptor, but did not stimulate cAMP production through TSHR (32). hCG (10^4 mIU/ml) had no effect on rat cultured preadipocytes. This showed that contaminants such as LH or FSH in the bTSH preparation were not affecting the results.

The presence of TSHR in fat cells suggests that it may be involved in the pathogenesis of extrathyroidal manifestations, especially exophthalmos, of Graves' disease. There are several reports that describe the enlargement of retrobulbar adipose tissue in Graves' patients, as evaluated by computer tomography (33, 34). If TSHR serves as a common antigen in thyroid gland and retroorbital tissue, it could cause hyperfunction of the thyroid gland and trigger an inflammatory process in retroorbital tissue. Actually, involvement of an immune reaction in the pathogenesis of Graves' ophthalmopathy has been suggested (35, 36). The successful amplification of TSHR mRNA from retroorbital tissue by PCR (11, 12, 37) and the detection of immunoreactive TSHR protein (38) have been reported, whereas an attempt to detect TSHR in fat cells as a functional receptor protein was not successful (39). The limited access to retroorbital tissue from the patients makes detailed biochemical studies difficult. However, it should be noted that none of the research performed on this issue has focused on preadipocytes. A project using human preadipocyte culture is underway in our laboratory to establish functional expression of TSHR in orbital fat tissue in humans.

In summary, the functional expression of TSHR in rat preadipocytes is closely related to differentiation of the cells. The rat preadipocyte culture is a good system for evaluating the regulation of TSHR gene expression in extrathyroidal cells.

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