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Thyrotropin Receptors in Brown Adipose Tissue: Thyrotropin Stimulates Type II Iodothyronine Deiodinase and Uncoupling Protein-1 in Brown Adipocytes*

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ABSTRACT

It has been demonstrated that TSH receptors are expressed not only in thyroid gland but also in extrathyroidal tissues. Brown adipose tissue of guinea pig has been reported to express TSH receptor messenger RNA (mRNA), but the physiological roles of TSH receptors in brown adipose tissue have not been understood. We studied the expression and function of TSH receptors in rat brown adipose tissue and cultured rat brown adipocytes. Northern analysis demonstrated the expression of TSH receptor mRNA in rat brown adipose tissue and cultured rat brown adipocytes. TSH receptor mRNA in rat brown adipose tissue was decreased by cold exposure of the rat, and its mRNA in cultured rat brown adipocytes was also decreased by incubation with TSH or $(Bu)_2$cAMP. TSH increased the intracellular cAMP concentration in cultured rat brown adipocytes in a dose-dependent manner. Type II iodothyronine deiodinase mRNA, its activity, and uncoupling protein-1 mRNA in cultured rat brown adipocytes were significantly increased by incubation with TSH in a dose-dependent manner. These results suggest the expression of functional TSH receptors in brown adipose tissue, which may be involved in regulation of the expression of type II iodothyronine deiodinase and uncoupling protein-1. (*Endocrinology* **142:** 1195–1201, 2001)

TSH, WHICH is secreted from adenohypophyseal thyrotrophs, binds TSH receptors on the plasma membrane of thyroid follicular cells to stimulate adenylate cyclase, resulting in the elevation of intracellular cAMP levels, which activates follicular cell growth and thyroid hormone synthesis (1). TSH receptors are present not only in thyroid gland, but also in white adipose tissue (WAT), and TSH has been reported to stimulate lipolysis in white adipocytes (2–4). Although expression of TSH receptor messenger RNA (mRNA) has been demonstrated in brown adipose tissue (BAT) as well as WAT in the guinea pig (5), little attention has been directed to the physiological roles of TSH receptors in BAT.

Although WAT is considered energy-storing adipose tissue, BAT is known as energy-dissipating adipose tissue. BAT has important roles in nonshivering thermogenesis observed in small mammals arousing from hibernation, in small rodents undergoing acclimation to cold, and in newborn mammals, including humans (6). BAT is also known to be involved in diet-induced thermogenesis (6). Thermogenesis is achieved by uncoupling protein-1 (UCP-1), which is present in the inner mitochondrial membrane of BAT, but not in WAT (7). The synthesis of UCP-1 is transcriptionally stimulated mainly via β_3-adrenergic receptors by norepinephrine (NE), which is released from sympathetic nerve endings in response to decreased environmental temperature or food intake (7). T_3 is reported to be required for the optimal expression of UCP-1 in rat BAT (8). A substantial fraction of T_3 found in BAT is produced by local conversion from prohormone T_4, which is accomplished by type II iodothyronine deiodinase (DII) (8). DII is present in a limited number of tissues, including central nervous system, pituitary, pineal gland, and BAT, but not in WAT. DII activity is insensitive to inhibition by 6-propyl-2-thiouracil (PTU) or increases in hypothyroidism (9). Importantly, BAT DII activity is markedly stimulated by cold exposure through the adrenergic mechanism (10). The adrenergic stimulation of BAT DII activity appears to be transcriptionally regulated and requires protein synthesis (11), and DII mRNA in rat BAT has been demonstrated to be increased by cold exposure (12).

In the present study we studied the possible expression of TSH receptors in rat BAT and the possible physiological roles of TSH receptors in brown adipocytes by analyses of intracellular cAMP concentrations and the expression of BAT-specific proteins, namely DII and UCP-1, which play pivotal roles in thermogenesis.

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Materials and Methods

Materials

[α-^{32}P]UTP and [^{125}I]T_4 were purchased from NEN Life Science Products (Boston, MA). The RIA kit for cAMP was obtained from Yamasa Co.

(Chosi, Japan). AG 50W-X2 resin and protein assay kit were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). All other chemicals were of the highest quality and were obtained from Life Technologies, Inc. (Gaithersburg, MD), Sigma (St. Louis, MO), or Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless otherwise indicated.

Animals and experimental procedures

Male Wistar rats, weighing approximately 150 g, were maintained individually on a 12-h light, 12-h dark schedule (lights on at 0600 h) at 25 ± 1 C and fed standard laboratory chow and tap water *ad libitum*. The rats were acclimated to this condition for at least 1 week before the experiment. Rats were killed at room temperature or after exposure to cold (4 C) for 1, 6, and 24 h. All of the experimental procedures were approved by animal care and experimentation committee, Gunma University, Showa Campus.

Isolation and cell culture of rat brown adipocytes

Precursor cells were isolated from the interscapular BAT of 20- to 21-day-old male Wistar rats according to the method described by Néchad (13), Forest (14), and Hernandez (15) with minor modifications. Interscapular BAT was removed, cut into small pieces, and digested by shaking in 2 ml/g wet wt tissue of digestion medium (DMEM supplemented with 2 mg/ml collagenase type I, 0.2% BSA, 3 nM H_2SeO_3, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 25 U/ml nystatin) at 37 C for 60 min. Subsequently, the digested tissue was filtered through a nylon screen (pore size, 100 μm), and mature fat cells and fat droplets were allowed to float for 30 min. The infranatant was filtered through a nylon screen (pore size, 40 μm), and the filtrate was centrifuged for 10 min at 700 × *g* at room temperature. The pellet (stromal-vascular fraction) was recovered in 5 ml culture medium (DMEM supplemented with 10% FBS, 33 μM D-biotin, 17 μM pantothenate, 100 μM ascorbate, 3 nM insulin, 3 nM H_2SeO_3, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 25 U/ml nystatin) and centrifuged for 10 min at 700 × *g* at room temperature. The pellet was resuspended in 5 ml culture medium and filtered through a nylon screen (pore size, 40 μm), and the filtrate-containing precursor cells were inoculated into 6-cm dishes or six-well plates (PRIMARIA, Becton Dickinson and Co., Lincoln Park, NJ) at a density of 2500–5000 cells/cm^2. Culture medium was changed on day 1 and every second day thereafter. Precursor cells proliferated actively under these conditions, reached confluence on day 6 or 7, and were fully differentiated into mature brown adipocytes by day 10. The culture medium with thyroid hormone-stripped FBS (16) was used for 24–72 h before harvest. Studies were performed during the differentiation period (days 8–9).

Cell culture of FRTL-5 rat thyroid cells

FRTL-5 rat thyroid cells (American Type Culture Collection, Interthyr Research Foundation, Baltimore, MD) were grown in Coon's modified F-12 medium supplemented with 5% calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, and a mixture of six hormones (6H): bovine TSH (10 mU/ml), insulin (10 μg/ml), hydrocortisone (1 nM), human transferrin (5 μg/ml), somatostatin (10 ng/ml), and glycyl-L-hystidyl-L-lysine acetate (10 ng/ml). Three days before RNA isolation, cells were grown in a medium with five hormones (5H) lacking bovine TSH.

RNA isolation and Northern analysis

Total RNA was isolated from interscapular BAT, cultured brown adipocytes, or FRTL-5 cells by a modified acid guanidinium thiocyanate-phenol-chloroform method according to Chomczynski and Sacchi (17). Polyadenylated [poly(A)$^+$] RNA was isolated from total RNA using Dynabeads oligo(deoxythymidine)$_{25}$ (DynAl, Oslo, Norway) according to the manufacturer's instructions. Northern analysis was performed as previously described (18). Rat TSH receptor complementary DNA (cDNA), provided by Dr. L. D. Kohn (19), was digested with *Hin*dIII and *Xho*I and subcloned into pGEM-7Zf. Plasmid rDII 5–1/pBluescript SK, which contains rat DII cDNA, was provided by Dr. D. L. St. Germain (12). UCP-1 cDNA fragment (527 bp) (20) was amplified from total RNA of rat BAT with RT-PCR (18) using the sense primer TGAGAGTTCG-GTACCCACATC and the antisense primer GTGCAGATGGCTTTGT-GCT and was subcloned into pCRII (Invitrogen, San Diego, CA). Briefly, complementary RNA (cRNA) probes for TSH receptor, DII, UCP-1, and β-actin were synthesized with T7 RNA polymerase (Nippon Gene, Tokyo, Japan) and [α-^{32}P]UTP. Twenty micrograms of total RNA or 1 μg poly(A)$^+$ RNA were electrophoresed on 1.4% agarose gel containing 2 M formaldehyde and transferred overnight in 20 × SSC (1 × SSC = 150 mM sodium chloride and 15 mM trisodium citrate) to a nylon membrane (Biodyne, Pall BioSupport Corp., East Hills, NY). RNA was cross-linked to the nylon membrane with a UV Stratalinker (Stratagene, La Jolla, CA). The membrane was prehybridized with hybridization buffer (50% formamide, 0.2% SDS, 5% dextran sulfate, 50 mM HEPES, 5 × SSC, 5 × Denhardt's solution, and 250 μg/ml denatured salmon sperm DNA) at 68 C for 2 h. Subsequently, the membrane was hybridized at 68 C overnight with the hybridization buffer containing cRNA probe. The membrane was washed twice in 2 × SSC-0.1% SDS at 25 C for 10 min and three times in 0.1 × SSC-0.1% SDS at 68 C for 1 h. Autoradiography was established by exposing the filters to x-ray film (Kodak XAR-2, Eastman Kodak Co., Rochester, NY) at −70 C. The probe was stripped off, and blots were rehybridized with another cRNA probe. The mRNA level was quantitated by densitometry using NIH Image version 1.61, and was corrected for β-actin.

Quantitative RT-PCR of DII

Total RNA was isolated from cultured rat brown adipocytes as described above. RT-PCR was performed as previously described (18) with minor modifications. Briefly, single strand cDNA synthesis was performed on 1 μg total RNA using random hexamers and murine leukemia virus reverse transcriptase (GeneAmp RNA PCR kit, Roche, Branchburg, NJ) in 20 μl. Subsequently, quantitative RT-PCR was performed according to the method described by Zhao (21) and Tu (22) with modifications. For the competitive PCR, rDII 5–1/pBluescript SK was used to prepare a mutant DII plasmid. To construct a mutant rDII plasmid, a 156-bp fragment between two *Bsa*AI restriction sites (nucleotides 688–843) within the coding region was deleted, and the plasmid was religated. The sense primer for the PCR (DII-S) was nucleotides 545 GAGTGCACAGGAGACTGACTG 645, and the antisense primer (DII-A) was nucleotides 1350 CTTCTCCAGCCAACTTCGGAC 1330 from the *Eco*RI 5′-cloning site.

For the amplification of DII in a 50-μl PCR reaction, 10 μl RT products were mixed with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM DII-S and DII-A, 200 μM deoxynucleotide triphosphates, 2 fg mutant DII-containing plasmid (mDII), and 1.25 U AmpliTaq Gold polymerase (Perkin-Elmer Corp., Branchburg, NJ). Subsequently, 60 cycles of PCR amplification were carried out with denaturation at 94 C for 0.5 min and annealing/extension at 60 C for 1.5 min after 9-min preincubation at 95 C. For the calibration, a standard curve was plotted by performing PCR with serial dilutions (16–0.25 fg/50 μl) of the wild-type DII (wDII) at the same time as the experimental samples. Subsequently, 10 μl of PCR products were electrophoresed, the gel was stained with SYBR Green (Molecular Probes, Inc., Eugene, OR), and products were visualized using a UV transilluminator. The PCR products were quantitated by densitometry using NIH Image version 1.61. The ratio of wDII to mDII band was then determined for the standards and unknowns. For quantitation, the relative DII density ratio was plotted against the serially diluted concentrations of the standard DII plasmids. The logarithm of the wDII to mDII ratio plotted against the logarithm of the initial amount of wDII plasmid added yielded a linear relationship.

Measurement of DII activity

DII activity was measured as previously described (23) with minor modifications (24). Briefly, BAT or cultured brown adipocytes were homogenized or sonicated in homogenizing buffer (100 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 20 mM dithiothreitol). Homogenates of BAT were centrifuged for 15 min at 1500 × *g* at 4 C, and resultant infranatants were used for DII activity measurement. Homogenates or sonicates were incubated in a total volume of 50 μl containing 2 nM [^{125}I]T_4, which was purified using LH-20 (Pharmacia Biotech, Uppsala, Sweden) column chromatography on the day of experiment, 1 mM EDTA, 1 mM PTU, and 20 mM dithiothreitol, pH 7.0, for 1 h at 37 C. The reaction was terminated by the addition of 100 μl 2% BSA and 800 μl 10% trichloroacetic acid. Separated ^{125}I was counted with a

γ-counter. Nonenzymatic deiodination was corrected by subtracting I^- released in tissue-free tubes. The protein concentration was determined by Bradford's method using BSA as a standard (25). Deiodinating activity was linear within the range of the protein concentration used and was expressed as femtomoles of I^- released per mg protein/h after multiplication by a factor of 2 to correct random labeling at the equivalent 3′ and 5′ positions.

Measurement of cAMP concentration

Cells in six-well culture plates were washed twice with Hanks' buffer, pH 7.4, and incubated in low salt isotonic solution [NaCl-free Hanks' buffer supplemented with 220 mM sucrose, 0.5 mM 3-isobutyl-1-methylxanthine, 1.5% (wt/vol) BSA, and 20 mM HEPES, pH 7.4] with or without 0.01–10 mU/ml bovine TSH (Sigma) for 30 min. At the end of incubation, the incubation buffer was removed, and the cAMP concentration was measured by RIA.

Statistics

Statistical differences were calculated using Student's *t* test or Duncan's multiple range test.

Results

Expression of TSH receptor mRNA in rat BAT

The results of Northern analysis of TSH receptor mRNA in rat BAT and cultured FRTL-5 rat thyroid cells are shown in Fig. 1. Hybridization signals with 5.6 and 3.3 kb were identified in FRTL-5 cells, in agreement with the previous observation (19). Hybridization signals of identical size were also demonstrated in rat BAT, indicating the presence of TSH receptor mRNA in rat BAT.

Effects of cold exposure on DII mRNA and activity, UCP-1 mRNA, and TSH receptor mRNA in rat BAT

In the next experiment, the effects of cold exposure on DII mRNA, DII activity, UCP-1 mRNA, and TSH receptor mRNA in BAT were studied. The rats were killed after cold exposure (4 C) for 1, 6, and 24 h. The hybridization signals for DII mRNA and UCP-1 mRNA were significantly increased by cold exposure, as shown in Fig. 2A. As shown in Fig. 2B, DII mRNA (●) and UCP-1 mRNA (○) were increased as early as 1 h after the beginning of cold exposure. DII activity was significantly increased by 6 h after the beginning of cold exposure, as shown in Fig. 2C. These results were in agreement with previous studies that demonstrated cold exposure induction of DII and UCP-1 expression in BAT (7, 8, 10). In contrast to the results for DII and UCP-1, hybridization signals with 5.6 and 3.3 kb for TSH receptor mRNA were significantly decreased by cold exposure, as shown in Fig. 3A. As shown in Fig. 3B, the sum of hybridization signals

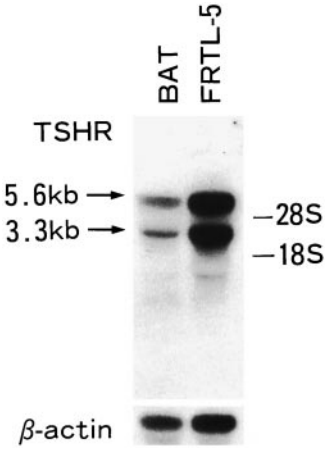

FIG. 1. Expression of TSH receptor (TSHR) mRNA in rat BAT. Northern analysis of total RNA (20 μg/lane) isolated from FRTL-5 rat thyroid cells and rat BAT was performed using rat TSHR and rat β-actin cRNA probes.

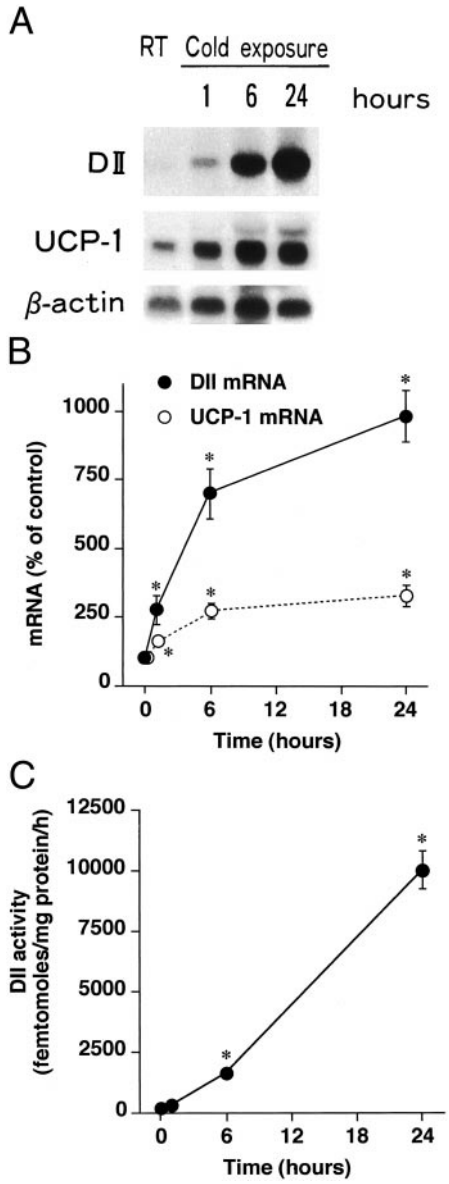

FIG. 2. Effect of cold exposure on DII mRNA, DII activity, and UCP-1 mRNA in rat BAT. A, Representative results of Northern analysis of total RNA (20 μg/lane) isolated from BAT of rats at room temperature (25 C; RT) and rats that were exposed to cold (4 C for 1, 6, and 24 h) using rat DII, rat UCP-1, and rat β-actin cRNA probes. B, DII mRNA (●) and UCP-1 mRNA (○) in BAT of rats that were exposed to cold. The OD of the DII or UCP-1 band was corrected for β-actin, and the results were expressed as a percentage of the value obtained for control rats at RT. Each *point* shown represents the mean ± SE of six animals. C, DII activity in BAT of rats that were exposed to cold. The DII activity shown represents the mean ± SE of six animals. *, $P < 0.01$ compared with control rats at RT.

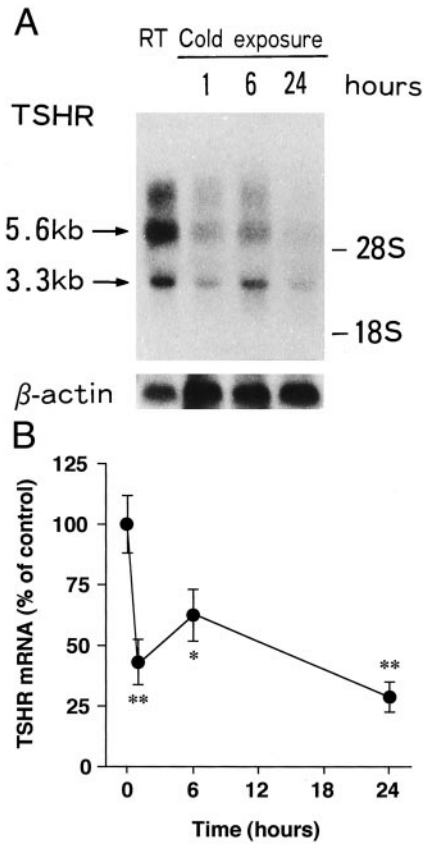

FIG. 3. Effect of cold exposure on TSH receptor (TSHR) mRNA in rat BAT. A, Representative results of Northern analysis of total RNA (20 μg/lane) isolated from BAT of rats at room temperature (RT) and rats that were exposed to cold (4 C for 1, 6, and 24 h) using rat TSHR and rat β-actin cRNA probes. B, TSHR mRNA in BAT of rats that were exposed to cold. The sum of the OD for the 5.6- and 3.3-kb mRNA was corrected for β-actin. The results were expressed as a percentage of the value obtained for control rats at RT and represent the mean ± SE of six animals. *, $P < 0.05$; **, $P < 0.01$ (compared with control rats at RT).

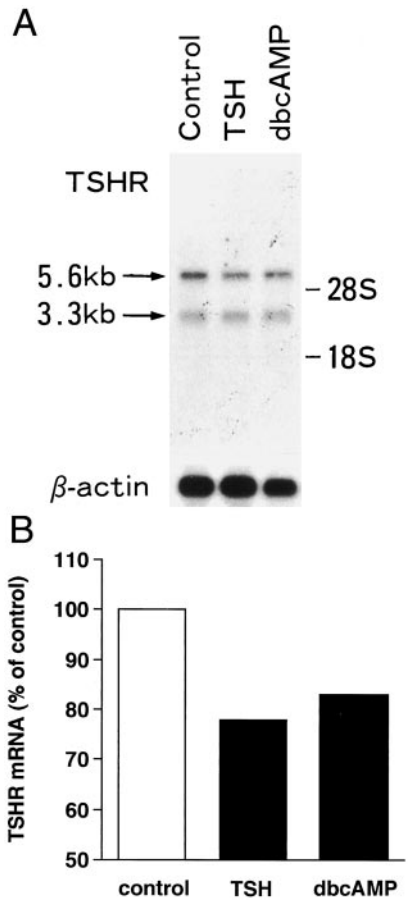

FIG. 4. Effects of TSH and $(Bu)_2$cAMP on TSH receptor (TSHR) mRNA in cultured rat brown adipocytes. A, Northern analysis of poly(A)$^+$ RNA (1 μg/lane) isolated from cultured rat brown adipocytes was performed using rat TSHR and rat β-actin cRNA probes. B, TSHR mRNA was corrected for β-actin. The results were expressed as a percentage of the control value. Brown adipocytes were incubated with 1 mU/ml TSH or 1 mM $(Bu)_2$cAMP for 2 h.

of 5.6 and 3.3 kb for TSH receptor mRNA in BAT was significantly decreased within 1 h after the beginning of cold exposure. These results suggest that TSH receptor mRNA in BAT is down-regulated by cold exposure.

Effects of TSH and $(Bu)_2$cAMP on TSH receptor mRNA expression in cultured rat brown adipocytes

To study the physiological function of TSH receptors in BAT *in vitro*, we performed primary culture of rat brown adipocytes. Figure 4A shows the results of Northern analyses of rat TSH receptor mRNA in poly(A)$^+$ RNA isolated from cultured rat brown adipocytes. Hybridization signals with 5.6 and 3.3 kb of TSH receptor mRNA were clearly demonstrated in cultured rat brown adipocytes, and the signals of TSH receptor mRNA were decreased by incubation with TSH or $(Bu)_2$cAMP as shown in Fig. 4B.

Stimulation of cAMP accumulation by TSH in cultured rat brown adipocytes

In the next experiment, the effects of various concentrations of TSH on cAMP accumulation in cultured rat brown adipocytes were studied. As shown in Fig. 5, cAMP accumulation in brown adipocytes was stimulated by TSH in a dose-dependent manner. These results suggest the expression of functional TSH receptors coupled to G protein that activates adenylate cyclase system in rat BAT.

Stimulation of DII expression by TSH in cultured rat brown adipocytes

To study the role of TSH receptor in BAT-specific protein expression, we analyzed DII mRNA and DII activity in cultured rat brown adipocytes. Characterization of iodothyronine deiodinase activity in cultured rat brown adipocytes revealed that the K_m for T_4 was 6.86 nM, and iodothyronine deiodinase activity was inhibited by 1 mM iopanoic acid, but was not affected by 1 mM PTU. These results indicate that iodothyronine deiodinase activity in cultured rat brown adipocytes fulfills the characteristics of DII. Because the preliminary experiments showed that DII mRNA in cultured rat brown adipocytes was not always enough to quantitate by Northern analysis, we performed quantitative RT-PCR of DII (22). The standard curve was obtained using sequential dilutions of wDII and a constant amount of mDII. An inverse

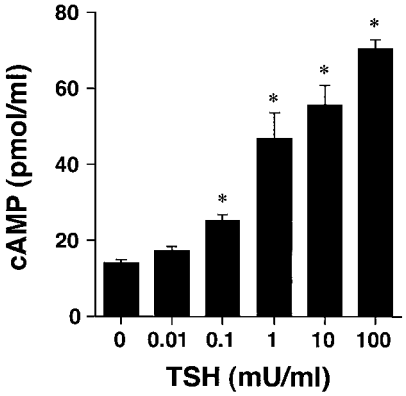

FIG. 5. Stimulation of cAMP accumulation by TSH in cultured rat brown adipocytes. Cells were incubated in low salt isotonic solution (NaCl-free Hanks' solution supplemented with 220 mM sucrose, 0.5 mM 3-isobutyl-1-methylxanthine, 1.5% BSA, and 20 mM HEPES, pH 7.4) with the indicated concentration of TSH for 30 min. Each *bar* represents the mean ± SE of three wells. *, $P < 0.01$ compared with control (no TSH).

relationship between wDII and mDII band intensities was observed, and the logarithm of the ratio of wDII to mDII plotted against the logarithm of the initial amount of wDII cDNA added yielded a linear relationship. Using the standard curve, we measured the DII mRNA level in cultured brown adipocytes incubated with various concentrations of TSH. As shown in Fig. 6A, the ratio of wDII to mDII was increased in TSH-treated brown adipocytes. The calculated DII mRNA level was increased in brown adipocytes incubated with TSH in a dose-dependent manner as shown in Fig. 6B. DII activity was also increased by incubation with TSH in a dose-dependent manner as shown in Fig. 6B. NE and $(Bu)_2$cAMP also increased DII mRNA and DII activity in cultured rat brown adipocytes (data not shown).

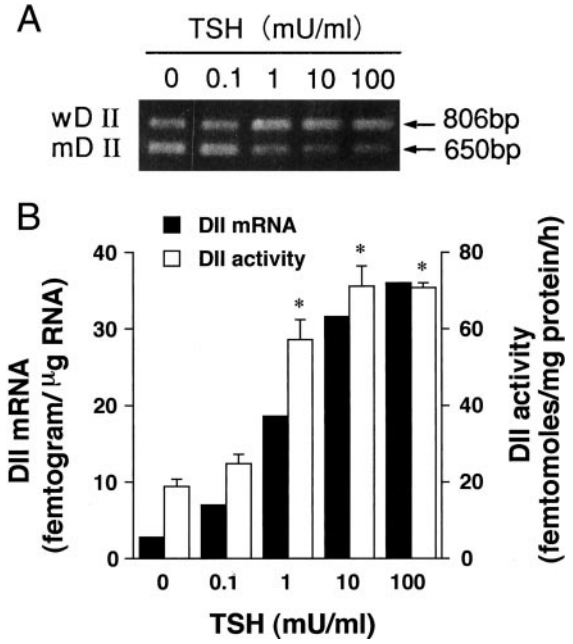

FIG. 6. Stimulation of DII expression by TSH in cultured rat brown adipocytes. A, Quantitative PCR of DII using cDNA templates reverse transcribed from 0.5 μg total RNA isolated from cultured brown adipocytes incubated with the indicated concentrations of TSH. wDII and mDII corresponding to 806 and 650 bp, respectively, are indicated. Standard samples from 1:2 serial dilution of rat DII cDNA (wDII; 16–0.5 fg) and reverse transcribed samples were amplified with a constant 2 fg mDII (competitor) by PCR. A competitive PCR standard curve was obtained by the logarithm of wDII/mDII ratio plotted against the logarithm of the amounts of added wDII template. B, DII mRNA (■) and DII activity (□) in cultured brown adipocytes. Cells were incubated with the indicated concentration of TSH for 2 h for RNA isolation and for 8 h for the measurement of DII activity. The DII activity shown represents the mean ± SE of three wells. *, $P < 0.01$ compared with control (no TSH).

Stimulation of UCP-1 mRNA expression by TSH in cultured rat brown adipocytes

Fig. 7A shows the results of Northern analysis of UCP-1 mRNA in cultured rat brown adipocytes. The hybridization signal of UCP-1 mRNA in cultured rat brown adipocytes was increased by incubation with TSH. As shown in Fig. 7B, UCP-1 mRNA, which was corrected for β-actin, was increased by TSH in a dose-dependent manner.

Discussion

The present results clearly demonstrate the expression of TSH receptor mRNA in rat BAT and cultured rat brown adipocytes for the first time. TSH receptor mRNA in rat BAT was significantly down-regulated by cold exposure of the rats. In cultured rat brown adipocytes, TSH receptor mRNA was down-regulated by incubation with TSH or $(Bu)_2$cAMP. TSH increased the intracellular cAMP concentration in cultured rat brown adipocytes in a dose-dependent manner, indicating the expression of TSH receptors coupled to G protein that activates adenylate cyclase system in rat BAT. We described the expression of DII mRNA in cultured rat brown adipocytes for the first time in the present study. TSH stimulated DII mRNA, DII activity, and UCP-1 mRNA in rat brown adipocytes in a dose-dependent manner. These results indicate that functional TSH receptors are expressed in rat BAT and that TSH stimulates the expression of BAT-specific proteins (6, 7).

In FRTL-5 rat thyroid cells, TSH and the subsequent cAMP production cause a time-dependent positive and then negative regulation of TSH receptor gene expression (19, 26). A *cis* DNA element similar to the cAMP response element (CRE) has been identified in the minimal promoter of the rat TSH receptor gene, and it has been shown that this element functions as a constitutive enhancer of TSH receptor promoter activity (27). Recently, it has been shown that TSH drives the induction of the inducible cAMP early repressor, isoform of the CRE modulator gene in rat thyroid gland and FRTL-5 cells, and that inducible cAMP early repressor binds to a CRE-like sequence in the TSH receptor promoter and represses its expression (28). It is well known that cold exposure-induced thermogenesis in BAT is controlled mainly by sympathetic nervous system accompanied by an increase in intracellular cAMP. It is also established that plasma TSH is markedly elevated by cold exposure in the rat (29). In the present study TSH or cAMP induced down-regulation of TSH receptor mRNA in cultured rat brown adipocytes, suggesting the role of TSH and/or cAMP in down-regulation of expression of TSH receptors in rat BAT. Down-regulation of

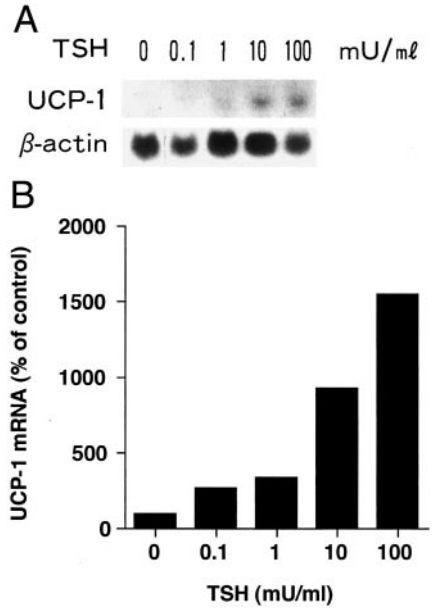

FIG. 7. Stimulation of UCP-1 mRNA by TSH in cultured rat brown adipocytes. A, Northern analysis of total RNA (20 μg/lane) isolated from cultured rat brown adipocytes was performed using rat UCP-1 and rat β-actin cRNA probes. B, UCP-1 mRNA in cultured rat brown adipocytes. The OD of the UCP-1 band was corrected for β-actin, and the results were expressed as a percentage of the value for control (no TSH). Brown adipocytes were incubated with the indicated concentration of TSH for 2 h.

TSH receptors in rat BAT and cultured rat brown adipocytes, which is also observed for TSH receptors in thyroid, further suggests the expression of functional TSH receptors in rat BAT.

In the present study TSH stimulated not only intracellular cAMP in cultured rat brown adipocytes, but also BAT-specific proteins, namely DII and UCP-1, which are known to play important roles in thermogenesis. Because DII and UCP-1 are not expressed in rat WAT, these results strongly suggest that functional TSH receptors are expressed in rat brown adipocytes *per se*. Although as little as 10 μU/ml TSH regularly caused a significant stimulation of cAMP production in rat FRTL-5 cells, 100 μU/ml TSH was required to stimulate cAMP production in cultured rat brown adipocytes. Therefore, the sensitivity of TSH receptor to TSH in rat brown adipocytes is lower than that in thyroid follicular cells, which may be due to the lower expression of TSH receptors in BAT compared with rat FRTL-5 cells, as demonstrated in the Northern analysis. Although it has been shown that a high concentration of hCG is able to stimulate TSH receptor, more than 10^4 mIU/ml hCG is required to stimulate TSH receptor (30). Thus, it is unlikely that possible contaminations, such as LH or FSH in the bovine TSH preparation, stimulate TSH receptor in cultured rat brown adipocytes.

DII has been reported to be expressed in BAT, central nervous system, pituitary, pineal gland, and Harderian gland in the rat (9). DII mRNA and DII activity are regulated by a β-adrenergic mechanism in rat pineal gland and Harderian gland (24, 31). Moreover, DII mRNA and DII activity are increased by incubation with cAMP-elevating agents in cultured rat astrocytes and rat pineal gland (24, 32). In the present study TSH stimulated intracellular cAMP accumulation, DII mRNA, and DII activity, and $(Bu)_2$cAMP increased DII mRNA and DII activity in cultured rat brown adipocytes. These results suggest that TSH stimulates cAMP production, resulting in activation of functional DII expression in rat BAT. Although new protein synthesis is not required for cAMP-mediated stimulation of DII mRNA (24), it remains to be elucidated whether the changes in DII mRNA induced by TSH are due to an increased transcription or a decreased degradation.

UCP-1 in BAT plays important roles in cold exposure-induced nonshivering thermogenesis or diet-induced thermogenesis (6, 7). It has been reported that NE induces UCP-1 expression through a cAMP-mediated β-adrenergic mechanism at the transcriptional level (7). T_3 has been reported to be required for optimal UCP-1 gene expression in response to the noradrenergic stimulus *in vivo* and in brown adipocytes *in vitro* (8). T_3 induces the transcription of the UCP-1 gene and also stabilizes its mRNA in fetal brown adipocyte primary culture (33). Taken together, TSH stimulation of UCP-1 mRNA expression observed in the present study may be caused by elevated cAMP concentration produced by TSH and/or increased intracellular T_3 production from T_4 by DII, which is stimulated by TSH. Although it is not known whether TSH causes an increased transcription or a decreased degradation of UCP-1, the presence of CRE and thyroid hormone response elements in the promoter of the rat UCP-1 gene (8) suggests the transcriptional regulation of UCP-1 expression by TSH.

BAT plays important roles in nonshivering thermogenesis and in diet-induced thermogenesis in rodents. The serum TSH level is known to be elevated in the neonatal period (34), in a cold environment (29), and in the hypothyroid state (35) in the rat. Although the physiological roles of BAT in human adults are not well understood, BAT is responsible for nonshivering thermogenesis, and sufficient BAT is present to account for all of the thermogenesis in newborns in humans (36). It is well known that the circulating TSH level is markedly elevated in human newborns (37, 38). As the sensitivity of TSH receptor to TSH in cultured rat brown adipocytes is relatively low, the possible physiological roles of elevated TSH level in BAT thermogenesis in those conditions require further studies.

In conclusion, we report that functional TSH receptors are expressed in rat BAT, and TSH increases the expression of DII and UCP-1 in rat brown adipocytes. These results suggest the previously unrecognized roles of TSH and its receptors in functions of BAT.

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