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Expression of Type 2 Iodothyronine Deiodinase in Human Osteoblast Is Stimulated by Thyrotropin

Tadashi Morimura, Katsuhiko Tsunekawa, Takayuki Kasahara, Koji Seki, Takayuki Ogiwara, Masatomo Mori, and Masami Murakami

Department of Medicine and Molecular Sciences and Department of Clinical Laboratory Medicine, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

Thyroid hormones play important roles in bone growth, development, and turnover. To exert its biological activity, T_4 needs to be converted to T_3 by iodothyronine deiodinase. In human thyroid gland as well as rat brown adipose tissue, type 2 iodothyronine deiodinase (D2) expression is regulated by a TSH receptor-cAMP-mediated mechanism. TSH receptor knockout mice demonstrated the direct effects of TSH on bone via TSH receptors found on osteoblast and osteoclast precursors. In the present study we investigated the possible expression and function of iodothyronine deiodinase and TSH receptors in human osteoblast-like osteosarcoma (SaOS-2) cells and normal human osteoblast (NHOst) cells. Iodothyronine deiodinase activity was detected in SaOS-2 cells and NHOst cells, and all of the characteristics of deiodinating activity were compatible with those of D2. Northern analysis demonstrated D2 mRNA expression in SaOS-2 cells and NHOst cells. D2 mRNA levels as well as D2 activities were rapidly increased by dibutyryl cAMP or forskolin in SaOS-2 cells and NHOst cells. TSH receptor mRNA was demonstrated in SaOS-2 cells and NHOst cells, and D2 mRNA and D2 activity were stimulated by TSH in both cells. In addition, all T_3 receptor isoforms were detected by RT-PCR in SaOS-2 cells and NHOst cells. The present results indicate the expression of functional TSH receptors and D2 in human osteoblasts and suggest previously unrecognized roles of TSH receptors and local T_3 production by D2 in the pathophysiology of human osteoblasts. (*Endocrinology* 146: 2077–2084, 2005)

THYROID HORMONES PLAY important roles in bone growth, development, and turnover by influencing the rate of bone resorption and formation. Children with juvenile hypothyroidism or subjects with syndromes of resistance to thyroid hormone, in which mutations of the T_3 receptor-β (TRβ) gene exist, have growth arrest, delayed skeletal maturation, and epiphyseal dysgenesis (1). In contrast, it has been demonstrated that excessive thyroid hormone in the body is associated with bone loss (2). Bone mass is reduced in patients with hyperthyroidism or in those receiving long-term thyroid hormone suppression therapy, and patients with a history of thyrotoxicosis have an increased risk of bone fracture (2). Despite the clinical importance of the regulatory action of T_3 on bone, little is known about the mechanisms by which this occurs. Recent studies have identified the presence of thyroid hormone receptors in osteoblast-like cells and immortalized osteosarcoma cells (3, 4). Direct responsiveness of osteoclasts to T_3 has been disputed, and the majority of T_3 actions in bone are believed to be mediated by osteoblasts (5, 6).

To exert its biological activity, T_4, which is a major secretory product of the thyroid gland, needs to be converted to T_3 by iodothyronine deiodinase (7). Type 1 iodothyronine deiodinase (D1) and type 2 iodothyronine deiodinase (D2) catalyze the conversion of T_4 to T_3. D1 is present in thyroid gland, liver, kidney, and many other tissues, whereas D2 is present in a limited number of tissues, including central nervous system, anterior pituitary, and brown fat in the rat (7). The K_m of D2 is approximately 1–5 nM for T_4 , which is 100 times lower than that of D1. D1, but not D2, is highly sensitive to inhibition by the antithyroid drug 6-propyl-2-thiouracil (PTU). D1 activity is known to decrease in the hypothyroid state and is believed to have a primary role in maintaining circulating T_3 levels. D2 activity, in contrast, increases in the hypothyroid state and is considered to play a critical role in providing local T_3 to regulate the intracellular T_3 concentration (7). Although the source of T_3 mainly depends on circulating T_3 in most tissues, local intracellular conversion of T_4 to T_3 is an important source of T_3 in certain tissues where D2 exists (7).

A cDNA encoding D2 was cloned from *Rana catesbeiana* tissues (8), and its mammalian counterpart was subsequently isolated from rat brown fat (9). In humans, D2 mRNA was unexpectedly detected in thyroid gland and other tissues, suggesting previously unrecognized roles of D2 in those tissues (10–13).

TSH, which is secreted from adenohypophyseal thyrotrophs, binds TSH receptors on the plasma membrane of thyroid follicular cells to regulate thyroid functions (14). D2 is regulated by a TSH receptor-cAMP-mediated mechanism in human thyroid gland (15). We have demonstrated that functional TSH receptors are also present in rat brown adipose tissue, and D2 in rat brown adipocytes is also regulated by a TSH receptor-cAMP-mediated mechanism (16). Recently, a study using TSH receptor knockout mice has shown the direct effects of TSH on bone components of skeletal

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Abbreviations: $(Bu)_2$cAMP, Dibutyryl cAMP; D1, type 1 iodothyronine deiodinase; D2, type 2 iodothyronine deiodinase; DTT, dithiothreitol; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IOP, iopanoic acid; NHOst, normal human osteoblast; PTU, 6-propyl-2-thiouracil; TR, thyroid hormone receptor; V_{max}, maximum velocity.

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remodeling, osteoblastic bone formation, and osteoclastic bone resorption mediated via TSH receptors found on osteoblast and osteoclast precursors (17, 18), suggesting physiological roles of TSH receptors in bone metabolism. These results prompted us to study the possible expression and interaction of functional TSH receptors and D2 in human osteoblasts.

In the present study we studied the possible expression of functional TSH receptors and D2 in human osteoblasts, namely human osteoblast-like osteosarcoma (SaOS-2) cells and normal human osteoblast (NHOst) cells.

Materials and Methods

Materials

[^{125}I]T_4 , [^{125}I]rT_3, and [α-^{32}P]UTP were purchased from NEN Life Science Products Corp. (Boston, MA). AG 50W-X2 resin and protein assay kit were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA). All other chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO) or Wako Pure Chemical Industries Ltd. (Osaka, Japan) unless otherwise indicated.

Cell culture

SaOS-2 cells were obtained from American Type Culture Collection (Manassas, VA) and NHOst cells were obtained from Cambrex (San Diego, CA). NHOst cells used in the present study were established from 1-yr-old female and met all specifications for normal morphology, including positive staining for alkaline phosphatase and bone mineralization (von Kossa stain) (19–21). SaOS-2 cells were cultured in RPMI 1640, which was supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 25 IU/ml nystatin. NHOst cells were cultured in osteoblast growth medium (BioWhittaker, San Diego, CA) containing 10% fetal calf serum and 100 μg/ml ascorbic acid and were used by six population doublings according to the manufacturer's instructions. Cells were maintained at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. For the experiments these cells were seeded in six-well plastic culture plates for measurement of deiodinase activity or in 60-mm plastic culture dishes for Northern analysis and RT-PCR analysis, and cells that reached confluence were used in all experiments. After the cells became confluent, the medium was replaced with thyroid hormone-stripped medium (22) for 12 h. The cells were then incubated in the medium containing compounds to be tested for the indicated hours.

Measurement of iodothyronine deiodinase activity

Iodothyronine deiodinase activity was measured as previously described (23) with minor modifications (24, 25). Briefly, cells were scraped off, and transferred into 1.5 ml ice-cold buffer [PBS containing 20 mM dithiothreitol (DTT)]. After centrifugation at 3000 rpm for 10 min at 4 C, the supernatant was discarded. Pellets were sonicated in 220 μl assay buffer (100 mM potassium phosphate, pH 7.0, containing 1 mM EDTA and 20 mM DTT)/tube and were incubated in a total volume of 100 μl with the indicated amount of [^{125}I]T_4 or [^{125}I]rT_3 , which was purified on the day of experiment, in the presence or absence of 1 mM PTU or 1 mM iopanoic acid (IOP). The reaction was terminated by the addition of 100 μl 2% BSA and 800 μl 10% trichloroacetic acid. The released ^{125}I was separated by column chromatography using AG 50W-X2 resin, as previously described (24), and counted. The protein concentration was determined by Bradford's method using BSA as a standard (26). The deiodinating activity was calculated as femtomoles of I^- released per milligram of protein per hour. After the characterization of deiodinase activity, the deiodinating activity was measured in duplicate by I^- release from 2 nM [^{125}I]T_4 in the presence of 1 mM PTU at 37 C for 2 h. In some experiments, reaction products were analyzed by HPLC (Hitachi, Tokyo, Japan) (27). Briefly, the incubation mixtures were extracted with 2 vol absolute ethanol, evaporated, dissolved in acetonitrile/water (32:68), applied to C_{18} column (Shimazu Co., Kyoto, Japan), and eluted with acetonitrile/water/phosphoric acid (32:68:0.1). The flow rate was 1 ml/min, and each 0.5-min fraction was collected and counted for radioactivity.

RNA preparation and Northern analysis

Total RNA was isolated from each dish, and Northern analysis was performed as previously described (12). Fifteen micrograms of total RNA per lane were electrophoresed on a 1.4% agarose gel containing 0.66 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 membrane with a UV Stratalinker (Stratagene, San Diego, CA). The membrane was prehybridized with the hybridization buffer (50% formamide, 0.2% sodium dodecyl sulfate, 5% dextran sulfate, 50 mM HEPES, 5× SSC, 5× Denhart's solution, and 100 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 a human D2 cRNA probe (12). The membrane was washed twice in 2× SSC-0.1% sodium dodecyl sulfate at 25 C for 15 min and twice in 0.1× SSC-0.1% SDS at 68 C for 1 h. Autoradiography was established by exposing the filters for 3–7 d to x-ray film (XAR-2, Eastman Kodak Co., Rochester, NY) at −70 C. After detection of D2 mRNA, the probe was stripped off, and blots were rehybridized with human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cRNA probe. Hybridization and washing were performed as described above, and the membrane was exposed for 30 min. Exposure time was adjusted to obtain a linear intensity of hybridization signals. mRNA levels were quantitated by densitometry using NIH Image version 1.61, and the OD of the D2 band was corrected for G3PDH. RNA samples for comparison were analyzed on the same blot, and each experiment was performed at least twice with basically identical results.

RT-PCR of TSH receptors and thyroid hormone receptor (TR) isoforms

Total RNA was isolated from SaOS-2 cells, NHOst cells, and human thyroid tissue obtained from a patient with Graves' disease. After treatment with deoxyribonuclease I (Invitrogen Life Technologies, Inc., Gaithersburg, MD), single-strand cDNA synthesis was performed on 1 μg total RNA using a GeneAmp RNA PCR Kit (Roche, Branchburg, NJ) as previously described (28) with minor modifications. For PCR, the synthesized sense and antisense primers for human TSH receptor were AGAAATAGCCCCGAGTCCCGTGGA (nucleotides −26 to −3) and GGTTTCATTGCATAGTCCCT (nucleotides 518–537) (29, 30); for human TR isoforms (31), the primers were: TRα1, AAGATGCCCT-CAACTCACC (nucleotides 1659–1677) and TCTTCATTTGTCTTCTC-CCC (nucleotides 1851–1870); TRα2, ATCCCGTAAGACCTCCTTCC (nucleotides 1653–1672) and AAACAGACTCATGCCCGAC (nucleotides 1901–1919); TRβ1, GAGGAGAAGAAATGTAAAGG (nucleotides 550–569) and GGAATAGGATGGATGGAG (nucleotides 700–717); and TRβ2, GGGCTGGAGAATGCATGCGTAGACT (nucleotides 41–65) and ATTCACTGCCCAGGCCTGTTCCATA (nucleotides 255–279). Ten microliters of the RT reaction product were amplified in 50 μl PCR buffer containing 50 pmol of each oligonucleotide primer. One unit of AmpliTaq Gold (Roche) was added before the first denaturation (9 min at 95 C). Samples were then subjected to 60 cycles consisting of 30 sec at 94 C and 90 sec at 60 C. The last extension was carried out for 10 min. The reaction products were analyzed by 1% agarose gel electrophoresis, and the resulting bands were visualized by ethidium bromide staining.

Measurement of cAMP production

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 0.1–10 mU/ml bovine TSH for 30 min. At the end of incubation, the incubation buffer was removed, and the cAMP concentration was measured by RIA using a cAMP kit (Yamasa Co., Chosi, Japan) as described previously (32, 33).

Statistics

All values are expressed as the mean ± SE. Statistical differences were evaluated by *t* test.

Results

Characteristics and cAMP stimulation of iodothyronine deiodinase expression in SaOS-2 and NHOst cells

In the preliminary experiments the basal iodothyronine-deiodinating activity was found to be low in SaOS-2 cells. Because D2 activity was stimulated through a cAMP-mediated pathway in human tissues (10, 12, 13, 15), and the presence of an adenylate cyclase system was described in human bone (34), the effects of dibutyryl cAMP [$(Bu)_2$cAMP] and forskolin on the deiodinating activity in SaOS-2 cells were studied. SaOS-2 cells were incubated with $(Bu)_2$cAMP or forskolin for 6 h, and deiodinating activity was measured by the release of I^- from 2 nM [^{125}I]T_4 or [^{125}I]rT_3 in the presence of 20 mM . The deiodinating activity in SaOS-2 cells was significantly increased by $(Bu)_2$cAMP (10^{-3} M) and forskolin (10^{-5} M), as shown in Fig. 1A. The deiodination in SaOS-2 cells was dependent on the protein concentration and the incubation period for up to 2 h (data not shown). Incubation at 4 C or preheating the cell sonicate at 56 C for 30 min abolished the deiodination (data not shown). Neither T_4 nor rT_3 deiodinating activity was influenced by the addition of 1 mM PTU, but both were completely inhibited by the addition of 1 mM IOP (data not shown). From the double-reciprocal plot shown in Fig. 1B, kinetic constants of T_4 deiodination were calculated to be: K_m = 4.96 nM and maximum velocity (V_{max}) = 370.37 fmol I^- released/mg protein·h in $(Bu)_2$cAMP-stimulated SaOS-2 cells. When [^{125}I]rT_3 was used as the substrate, kinetic constants of rT_3 deiodination were calculated to be: K_m = 6.41 nM and V_{max} = 312.50 fmol I^- released/mg protein·h in $(Bu)_2$cAMP-stimulated SaOS-2 cells. When sonicates of SaOS-2 cells were incubated with 2 nM [^{125}I]T_4 in the presence of 20 mM DTT and 1 mM PTU and subsequently analyzed by HPLC, there were only three definable peaks corresponding to I^-, T_4, and T_3 , and radioactivity in the I^- peak was comparable to that in the T_3 peak (data not shown). These results indicate that the characteristics of the deiodinating activity in SaOS-2 cells are compatible with those of D2, and its activity is stimulated through a cAMP-mediated pathway.

To investigate whether D2 activity is also present in human normal osteoblasts, we examined the expression of D2 in cultured NHOst cells. The deiodinating activity was clearly detected and increased by forskolin (10^{-5} M) in NHOst cells, as shown in Fig. 1A. Incubation at 4 C or preheating the cell sonicate at 56 C for 30 min abolished deiodination (data not shown). Neither T_4 nor rT_3 deiodinating activity was influenced by the addition of 1 mM PTU, but both were completely inhibited by the addition of 1 mM IOP (data not shown). From the double-reciprocal plot, kinetic constants of T_4 deiodination were calculated to be: K_m = 1.60 nM and V_{max} = 666.67 fmol I^- released/mg protein·h in forskolin-stimulated NHOst cells, as shown in Fig. 1B. When [^{125}I]rT_3 was used as the substrate, kinetic constants of rT_3 deiodination were calculated to be: K_m = 3.63 nM and V_{max} = 526.31 fmol I^- released/mg protein·h in forskolin-stimulated NHOst cells. These results indicate that D2 activity is also present and stimulated through a cAMP-mediated pathway in NHOst cells. D2 activity was higher in NHOst cells than in SaOS-2 cells at the basal level and the forskolin-simulated level, and D2 activity in these human osteoblast cells was compatible with that in human skeletal muscle cells (12) and human vascular smooth muscle cells (13).

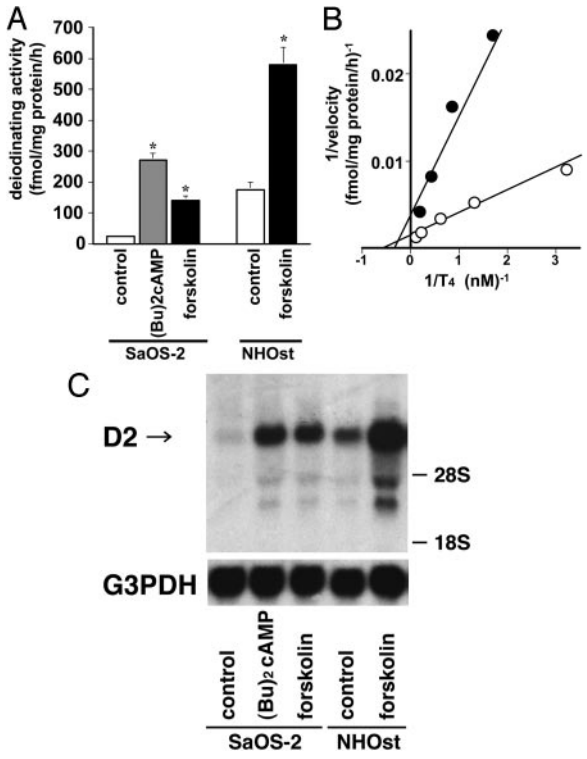

FIG. 1. D2 activity and D2 mRNA in SaOS-2 cells and NHOst cells. A, $(Bu)_2$cAMP and forskolin stimulation of deiodinating activity in SaOS-2 cells and forskolin stimulation of deiodinating activity in NHOst cells. Cells were incubated with medium only (control) or with medium containing $(Bu)_2$cAMP (10^{-3} M) or forskolin (10^{-5} M) for 6 h. Deiodinating activity was measured as described in *Materials and Methods*. The deiodinating activity shown represents the mean ± SE of three wells. *, $P < 0.01$ compared with control SaOS-2 and NHOst cells, respectively. B, Double-reciprocal plot of T_4 deiodination by $(Bu)_2$cAMP-stimulated SaOS-2 cells (●) and by forskolin-stimulated NHOst cells (○). Incubations were performed for 1 h at 37 C with various concentrations of [^{125}I]T_4. Kinetic constants were calculated to be: K_m = 4.96 nM, V_{max} = 370.37 fmol I^- released/mg protein·h in SaOS-2 cells; and K_m = 1.60 nM, V_{max} = 666.67 fmol I^- released/mg protein·h in NHOst cells. C, Northern analysis of D2 mRNA using human D2 and G3PDH cRNA probes in SaOS-2 and NHOst cells incubated with medium only (control) or with medium containing $(Bu)_2$cAMP (10^{-3} M) or forskolin (10^{-5} M) for 4 h. Each lane represents 15 μg total RNA obtained from cells in an individual dish.

Northern analysis of total RNA extracted from SaOS-2 cells and NHOst cells using human D2 cRNA probe was performed. As shown in Fig. 1C, hybridization signals of D2 mRNA, approximately 7.5 kb in size, were clearly demonstrated in SaOS-2 cells and NHOst cells, as reported in other cells and tissues (10, 12, 13, 15), and D2 mRNA was increased by treatment with $(Bu)_2$cAMP or forskolin. These results indicate that D2 mRNA is expressed and stimulated through a cAMP-mediated pathway in SaOS-2 cells and NHOst cells.

D2 mRNA was higher in NHOst cells than in SaOS-2 cells at the basal level and the forskolin-stimulated level, in agreement with the difference in D2 activity in those cells.

In the time-course study of SaOS-2 cells shown in Fig. 2, both D2 mRNA and D2 activity were increased by $(Bu)_2$cAMP (10^{-3} M) and forskolin (10^{-5} M) within 3 h and reached peak levels at 6 h. The rapid increase in D2 mRNA and D2 activity by the cAMP-elevating agent is in agreement with the results obtained using cultured rat astrocytes (35) and cultured rat pineal gland (36). These results indicate that D2 expression is pretranslationally stimulated through a cAMP-mediated pathway.

Regulation of D2 activity in SaOS-2 cells by thyroid hormones through a ubiquitin-proteasome pathway

One of the important characteristics of D2 is the negative regulation of its activity by thyroid hormones (7). To study the effects of physiological concentrations of thyroid hormones on deiodinating activity in SaOS-2 cells, 10^{-9}–10^{-7} M thyroid hormones were added to the culture medium for 6 h before harvesting the cells. As shown in Fig. 3A, deiodinating activity was decreased by thyroid hormones, and the potency of the inhibitory effect was $T_4 > rT_3 > T_3$ in forskolin-stimulated SaOS-2 cells, in agreement with previous observations for D2 in other tissues and cells (12, 13, 25).

Endogenous D2 activity is posttranslationally regulated by its substrate (37, 38), which accelerates its degradation through the ubiquitin-proteasome pathway (39). It was demonstrated that treatment with MG132, a specific proteasome inhibitor, blocked rT_3-induced down-regulation of D2 activity in rat pituitary tumor (GH_4C_1) cells (39). To study the effects of rT_3 and/or MG132 on D2 activity in forskolin-stimulated SaOS-2 cells, 10^{-6} M MG132 (Peptide Institute, Inc., Osaka, Japan) was added 10 min before the addition of 10^{-7} M rT_3. As shown in Fig. 3B, although rT_3 decreased D2 activity, MG132 increased D2 activity 2-fold over that in forskolin-stimulated SaOS-2 cells and blocked rT_3-induced down regulation of D2 activity. These results demonstrated that thyroid hormone inhibited D2 activity in SaOS-2 cells

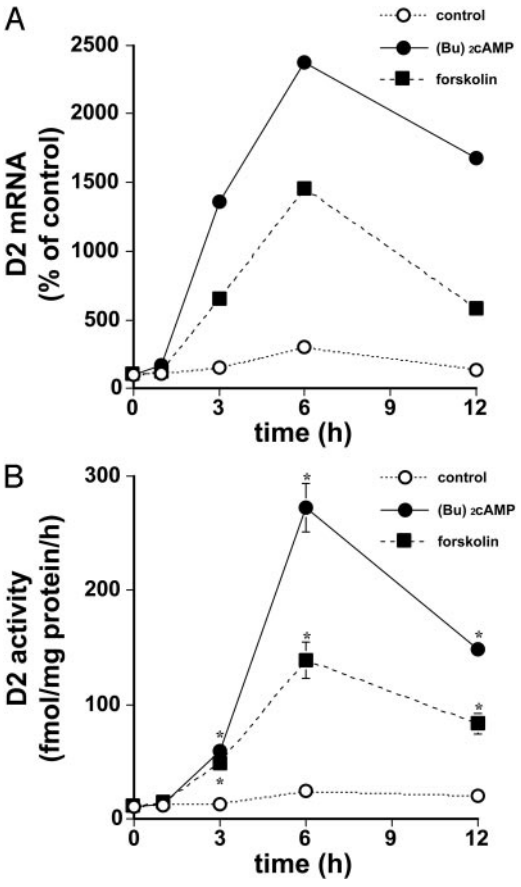

FIG. 2. Time course of forskolin and $(Bu)_2$cAMP stimulation of D2 mRNA and D2 activity in SaOS-2 cells. A, D2 mRNA (D2 mRNA/G3PDH mRNA ratio) in SaOS-2 cells incubated with medium only (control) or with medium containing $(Bu)_2$cAMP or forskolin for various hours. The OD of the D2 band was corrected for G3PDH, and the results were expressed as a percentage of the control value (0 h). B, D2 activity in SaOS-2 cells incubated with medium only (control), or with medium containing $(Bu)_2$cAMP (10^{-3} M) or forskolin (10^{-5} M) for various time periods. The D2 activity shown represents the mean ± SE of three wells. *, $P < 0.01$ compared with control (0 h).

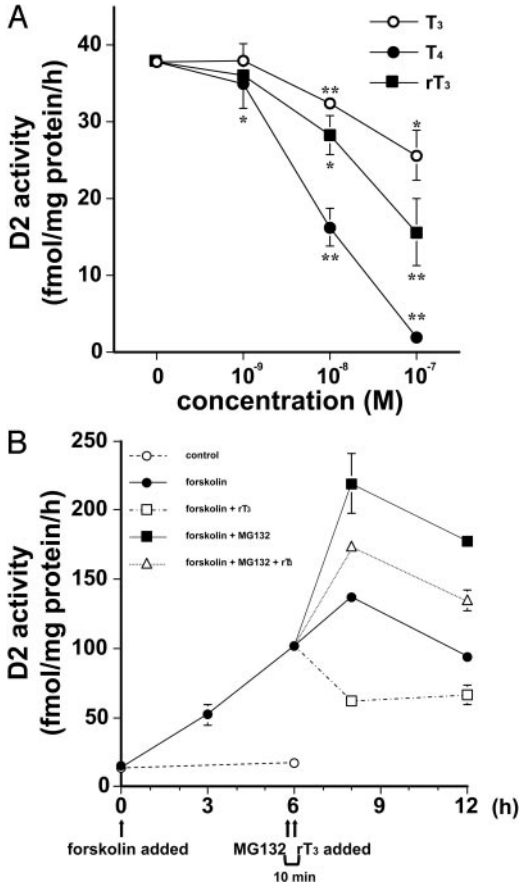

FIG. 3. Regulation of D2 activity in SaOS-2 cells by thyroid hormones. A, D2 activity in SaOS-2 cells incubated with forskolin (10^{-5} M; control) or forskolin (10^{-5} M) and various concentrations of T_3, T_4, or rT_3 for 6 h. D2 activity shown represent the mean ± SE of three wells. *, $P < 0.05$; **, $P < 0.01$ (compared with control). B, D2 activity in SaOS-2 cells incubated with medium only (control) or medium with forskolin (10^{-5} M) only, forskolin with rT_3 (10^{-7} M), forskolin with MG132 (10^{-6} M), or forskolin with MG132 and rT_3. Cells were incubated with forskolin for 6 h, and MG132 was added 10 min before the addition of rT_3. The D2 activity shown represents the mean ± SE of three wells.

through a ubiquitin-proteasome pathway, in agreement with the results obtained using other cells and tissues (39, 40).

Identification of TSH receptors and stimulation of D2 expression by TSH in SaOS-2 and NHOst cells

To examine the possible expression of TSH receptors in SaOS-2 cells, the effect of TSH on cAMP accumulation in SaOS-2 cells was studied. SaOS-2 cells were incubated with various concentrations of TSH for 6 h. As shown in Fig. 4A, cAMP accumulation in SaOS-2 cells was increased by TSH in a dose-dependent manner. These results suggest that the expression of TSH receptors coupled to G protein activates adenylate cyclase system in SaOS-2 cells.

RT-PCR of TSH receptor mRNA in SaOS-2 and NHOst cells was performed as shown in Fig. 4B. RT-PCR of TSH receptor mRNA in human thyroid tissue demonstrated a single amplified DNA fragment of 563 bp, which corresponds to the expected size of DNA. The same DNA fragment was amplified in RT-PCR of RNA from SaOS-2 and NHOst cells, although the amount of the amplified products was less than that in the thyroid. Total RNA that was treated in an identical manner, except the substitution of water for reverse transcriptase showed no band. These results demonstrated the expression of TSH receptor mRNA in SaOS-2 and NHOst cells.

Because D2 expression has been demonstrated to be regulated by TSH in human thyroid gland and rat brown adipose tissue (15, 16), we studied the effects of TSH on D2 mRNA and D2 activity in SaOS-2 and NHOst cells. D2 mRNA and D2 activities in SaOS-2 cells (Fig. 5, A and B) and NHOst cells (Fig. 5, C and D) were significantly increased by TSH in a dose-dependent manner. These results indicate that D2 expression is pretranslationally regulated by a TSH receptor-cAMP-mediated mechanism in SaOS-2 and NHOst cells.

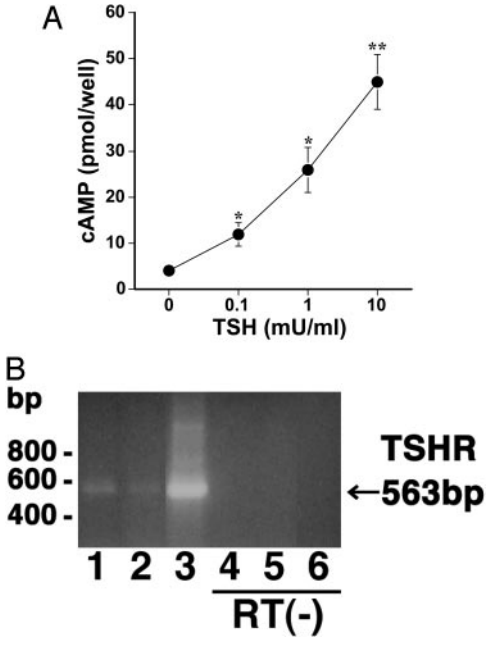

FIG. 4. Stimulation of cAMP accumulation by TSH in SaOS-2 cells and expression of TSH receptors in SaOS-2 cells and NHOst cells. A, Stimulation of cAMP accumulation by TSH in SaOS-2 cells. Cells were incubated with the indicated concentration of TSH for 30 min. Each *bar* represents the mean ± SE of three wells. *, $P < 0.05$; **, $P < 0.01$ [compared with control (0 mU/ml TSH)]. B, RT-PCR of TSH receptor mRNA in SaOS-2 cells, NHOst cells, and human thyroid tissue. RT-PCR was performed on 1 μg total RNA, and the reaction products were analyzed by 1% agarose gel electrophoresis as described in *Materials and Methods*. Lane 1, SaOS-2 cells; lane 2, NHOst cells; lane 3, human thyroid tissue; lane 4, SaOS-2 cells (no RT); lane 5, NHOst cells (no RT); lane 6, human thyroid tissue (no RT).

Identification of TR isoforms by RT-PCR in SaOS-2 and NHOst cells

To investigate whether TR isoforms are expressed in SaOS-2 and NHOst cells, we performed RT-PCR analysis of TR isoforms in those cells. All TR isoforms were clearly

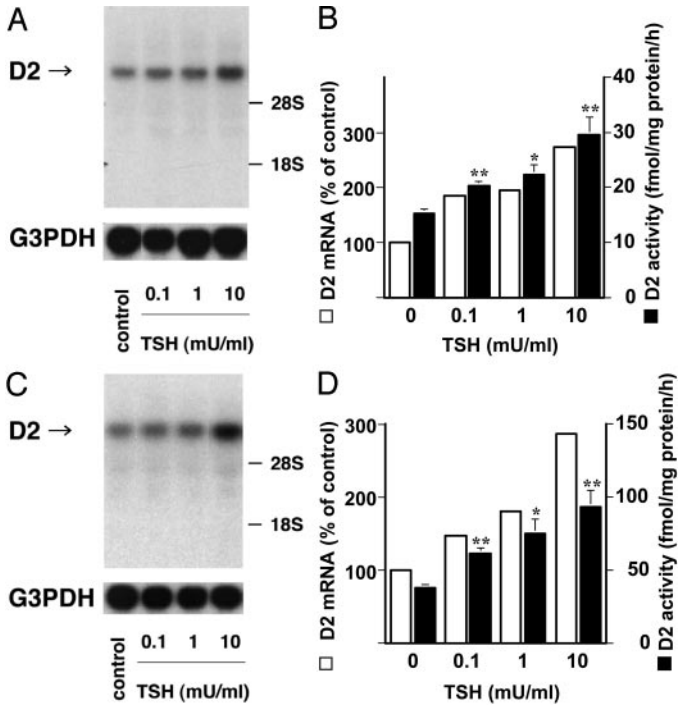

FIG. 5. Stimulation of D2 expression by TSH in SaOS-2 cells and NHOst cells. A, Northern analysis of D2 mRNA using human D2 and G3PDH cRNA probes in SaOS-2 cells incubated with the indicated concentration of TSH for 4 h. Each lane represents 15 μg total RNA obtained from cells in an individual dish. B, D2 mRNA (D2 mRNA/G3PDH mRNA ratio) and D2 activity in SaOS-2 cells stimulated by TSH. Cells were incubated with the indicated concentration of TSH for 4 h for RNA isolation and for 6 h for the measurement of D2 activity. The D2 activity shown represents the mean ± SE of three wells. *, $P < 0.05$; **, $P < 0.01$ [compared with control (0 mU/ml TSH)]. The OD of the D2 band was corrected for G3PDH, and the results were expressed as a percentage of the control value (0 mU/ml TSH). C, Northern analysis of D2 mRNA using human D2 and G3PDH cRNA probe in NHOst cells incubated with the indicated concentration of TSH for 4 h. Each lane represents 15 μg total RNA obtained from cells in an individual dish. D, D2 mRNA (D2 mRNA/G3PDH mRNA ratio) and D2 activity in NHOst cells. Cells were incubated with the indicated concentration of TSH for 4 h for RNA isolation and for 6 h for the measurement of D2 activity. The D2 activity shown represents the mean ± SE of three wells. *, $P < 0.05$; **, $P < 0.01$ [compared with control (0 mU/ml TSH)]. The OD of the D2 band was corrected for G3PDH, and the results were expressed as a percentage of the control value (0 mU/ml TSH).

demonstrated by RT-PCR in both SaOS-2 and NHOst cells. Total RNA, treated in an identical manner except for the substitution of water for reverse transcriptase, showed no band in RT-PCR for any TR isoform (data not shown).

Discussion

The present results have clearly demonstrated that iodothyronine-deiodinating activities are present in SaOS-2 and NHOst cells. The deiodinating activities in SaOS-2 and NHOst cells were dependent on the protein concentrations, incubation period, temperature, and substrate concentrations. These characteristics clearly indicate the enzymatic nature of those cells. The deiodinating activities in SaOS-2 and NHOst cells were not inhibited by 1 mM PTU and were demonstrated to have low K_m for T_4. Iodothyronine deiodinating activities in SaOS-2 and NHOst cells, therefore, have characteristics compatible with D2 (7). Northern analysis using a human D2 cRNA probe clearly demonstrated hybridization signals of approximately 7.5 kb in size in SaOS-2 and NHOst cells. These results indicate that D2 is expressed in SaOS-2 and NHOst cells.

In the present study, D2 activity and D2 mRNA were higher in NHOst cells than in SaOS-2 cells at the basal level and after forskolin stimulation. Lower D2 expression in a tumor cell line derived from osteosarcoma compared with that in normal osteoblast cells suggests that D2 expression may increase in differentiated osteoblast cells, because D2 expression in papillary thyroid carcinoma was lower than that in normal thyroid tissue (15). In earlier studies, 5′-deiodinase activity was not detected in UMR-106 rat osteoblastic osteosarcoma cells (41). D2 expression may be different among rat tissues and human tissues, because D2 is highly expressed in human thyroid gland, but is absent in rat thyroid gland (10, 15).

D2 activity is negatively controlled by thyroid hormones (7). The potency of the inhibitory effect of thyroid hormones was $T_4 > rT_3 > T_3$, as demonstrated in SaOS-2 cells in the present study. Both T_4 and rT_3 are more potent than T_3 , suggesting that this effect does not require TRs. T_4 and rT_3 suppress D2 activity mainly at the posttranslational level through acceleration of the degradation rate of D2 protein (39). Recently, a ubiquitin-proteasome pathway has been demonstrated to be involved in the posttranslational regulation of D2 activity by thyroid hormones (39). In the present study, MG132, a specific proteasome inhibitor, blocked rT_3 suppression of D2 activity, indicating that proteasomal degradation is involved in the regulation of D2 activity by thyroid hormones in SaOS-2 cells. Deiodination in SaOS-2 cells is, therefore, negatively regulated by thyroid hormones, mainly through posttranslational mechanisms involving a ubiquitin-proteasome pathway, which also supports the idea that authentic D2 is expressed in SaOS-2 cells. Because D2 expression in osteoblasts is suggested to be increased in the hypothyroid state, D2 in human osteoblasts may play a role in the protection of human bone from local T_3 deficiency in hypothyroidism.

In the present study, both D2 mRNA levels and D2 activities were rapidly stimulated by forskolin or $(Bu)_2$cAMP in SaOS-2 and NHOst cells, suggesting the pretranslational regulation of D2 expression thorough a cAMP-dependent mechanism. The rapid stimulation of D2 mRNA and D2 activity was also observed in human thyroid follicular cells (15), human skeletal muscle cells (12), human vascular smooth muscle cells (13), rat brown adipocytes (16), rat pineal gland (36), and mouse pituitary tumor cells (25). Recently, a functional cAMP response element was demonstrated to be present in the promoter region of the human D2 gene (15, 42). Taken together, these findings suggest that D2 expression in SaOS-2 and NHOst cells is regulated by a cAMP-dependent mechanism at the transcriptional level. Because PTH is known to regulate the function of osteoblasts through a cAMP-mediated pathway (43), it is of considerable interest to study the potential stimulatory effect of PTH on D2 expression in human osteoblasts.

The physiological importance of intracellular thyroid hormone activation by D2 has been demonstrated in certain tissues. Adenohypophyseal T_3 production by D2 plays a significant role in feedback regulation of TSH secretion by thyroid hormones (44). In rat brown adipose tissue, the expression of uncoupling protein is regulated by locally generated T_3, which is provided by D2 (45). In the present study, TRs were demonstrated in SaOS-2 and NHOst cells by RT-PCR, suggesting that T_3 locally produced by D2 might play a role through TRs in osteoblasts. It is of interest to study the possible role of T_3 locally produced by D2 in the regulation of osteoblast-specific gene expression, which may be associated with bone maturation and skeletal remodeling.

We previously reported that D2 mRNA and D2 activity were detected in cultured human thyroid cells and rat brown adipocytes and were stimulated by TSH in dose-dependent manners in those cells (15, 16). These results suggest that D2 expression in human thyroid cells and rat brown adipocytes is regulated by a TSH receptor-mediated mechanism. The present results have clearly demonstrated the expression of TSH receptors in SaOS-2 and NHOst cells. TSH increased D2 mRNA and D2 activity in SaOS-2 and NHOst cells, suggesting that D2 expression was regulated by a TSH receptor-cAMP mediated pathway, as demonstrated for human thyroid follicular cells and rat brown adipocytes (15, 16). These results suggest that D2 in human osteoblasts is increased in suppressed thyroid hormone levels and is further increased by TSH, which is elevated in primary hypothyroidism. TSH receptors may, therefore, be involved in the homeostasis of T_3 by regulating D2 expression in human osteoblasts.

Increased bone turnover is observed in patients with Graves' disease, and serum alkaline phosphatase concentrations correlate with thyroid-stimulating antibody in patients with Graves' disease (46). These observations suggest that bone metabolism in Graves' disease is related to thyroid-stimulating antibody as well as circulating thyroid hormone levels. Local T_3 production from T_4 in osteoblasts by D2, which is regulated by a TSH receptor-mediated mechanism, may be involved in the reduction of bone mass in patients with Graves' disease.

Recently, a study using TSH receptor knockout mice has shown the direct effects of TSH on bone components of skeletal remodeling, osteoblastic bone formation, and osteoclastic bone resorption mediated via TSH receptors found on osteoblast and osteoclast precursors (17, 18). TSH receptor

knockout mice demonstrated osteoporosis together with focal osteosclerosis even with normal circulating thyroid hormone levels (17). TSH was shown to inhibit osteoblast differentiation and osteoclast formation via TSH receptors independently of serum thyroid hormone levels that is controlled by thyroid function under the regulation of TSH (17). It is, therefore, tempting to speculate that the osteoporosis observed in human hyperthyroidism may be related to suppressed circulating TSH levels, but not to increased circulating thyroid hormone levels (17, 18). Based on the present observations, TSH positively regulates, and thyroid hormones negatively regulates intracellular T_3 production by controlling D2 in human osteoblasts, suggesting that intracellular T_3 provided by D2 may modulate the function of osteoblasts regulated by TSH via TSH receptors expressed in osteoblasts. Additional studies are required to elucidate the pathophysiological roles of D2 in human osteoblasts that are regulated by TSH and thyroid hormones in the regulation of bone maturation and skeletal remodeling.

In summary, the present results demonstrate the expression of functional TSH receptors and D2 in human osteoblasts, presenting novel perspectives on the roles of TSH receptors and thyroid hormone metabolism in the pathophysiology of human bone.

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Address all correspondence and requests for reprints to: Dr. Masami Murakami, Department of Clinical Laboratory Medicine, Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan. E-mail: mmurakam@showa.gunma-u.ac.jp.

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