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Presence of thyrotropin receptor in hepatocytes: not a case of illegitimate transcription

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Abstract

The function of thyrotropin (TSH) in the thyroid gland is mediated by thyrotropin receptor (TSHR). In addition to the thyroid, TSHR expression has been described in some non-thyroidal tissues, although it is uncertain whether TSHR is present in hepatocytes. One study has reported hepatic expression of TSHR mRNA, but this was considered to be because of illegitimate transcription, and there has not been a study investigating its protein expression and function in hepatocytes. Here, we examined the expression of TSHR in human and rat liver tissues, as well as human normal hepatocyte cell line L-02. Our results demonstrated that hepatic TSHR mRNA could be detected and had the same sequence as that of thyroid-derived mRNA. TSHR protein was also expressed and mainly located in the hepatocyte cell membrane. Moreover, bovine TSH and immunoglobulin from sera of patients with Graves' disease stimulated cAMP production in these cells. Taken together, these data show that TSHR is present and functional in hepatocytes, and this expression is not a case of illegitimate transcription. Given the pivotal role of the liver in body metabolism and many human diseases, our findings provide important implications for a potentially novel physiopathological role of TSH *via* acting on the TSHR in hepatocytes besides its classical role in regulating the thyroid function.

Keywords: thyrotropin • thyrotropin receptor • hepatocyte • cAMP

Introduction

Thyrotropin (TSH) mediates its function through highly specific interactions with the thyrotropin receptor (TSHR) [1]. In many autoimmune thyroid disorders, such as Graves' disease (GD), TSHR is a target of autoimmune antibodies, leading to dysfunction of the thyroid gland [2]. Several studies have demonstrated that TSHR expression is not confined to thyrocyte but can be detected in a number of non-thyroidal cells, such as lymphocytes [3], adipocytes [4], retroocular fibroblasts [5], erythrocytes [6], osteocytes [7], neuronal cells and astrocytes [8]. Although the role of TSHR in these non-thyroid tissues is unclear, its expression and activity in these non-thyroid tissues may have physiopathological relevance [9].

Recently, two separate studies reported that the level of hepatic production of C-reactive protein directly correlated with TSH levels in subclinical hypothyroidism patients [10, 11], which indicated a potential association between TSH and hepatocytes. To date, the existence of TSHR in hepatocytes is a contentious issue. Previous studies have selected liver tissues as negative controls when detecting TSHR expression in chicken and rats [4, 12]. Agretti *et al.* [13] successfully detected TSHR mRNA in liver tissues of patients with non-thyroidal diseases. However, because of the very low levels of TSHR mRNA transcript, the authors speculated that this likely represented illegitimate transcription, which was defined as low transcription of a tissue-specific gene in non-specific cells [14]. In the present study, we extensively investigated the mRNA and protein expression and function of TSHR in human and rate hepatocytes. Our results have important implications for a novel physiopathological role of TSH *via* the TSHR in liver.

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Table 1 Sequence information on the primers used for PCR

Genes	Sequences	Product size (bp)	Annealing temperature (°C)	Gene Bank
Human	5′-CCTCTCATCACTGTTAGCAA-3′	324	55	NM_000369
TSHR-1	5′-TACTCTTCTGAGATTTGGCC-3′			
Human	5′-TTTGACAGCCATTATGACTACACC-3′	839	59	NM_000369
TSHR-2	5′-TTGGAGTTGCTAACAGTGATGAGA-3′			
Rat	5′-AAGCTGGATGCTGTTTACCT-3′	169	56.9	NM_012888
TSHR	5′-GTTCTTCGCGATCAGCTCTT-3′			

Materials and methods

Cell culture and sample collection

The normal human hepatocyte cell line L-02 was purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. L-02 cells were cultured as described previously [15].

Human samples of normal tissues adjacent to the surgical excision of liver ($n = 5$) or thyroid ($n = 3$) were obtained from patients undergoing elective surgery in Shandong Provincial Hospital and were confirmed by pathologists. Rat liver samples ($n = 6$) were collected from male Wistar rats or Sprague Dawley rats (weighing 200–220 g), obtained from Shandong University. The rat thyroid cell line FRTL-5 was cultured as we previously described [16]. Normal human thyroid tissues and FRTL-5 cells were used as positive controls.

Immunoglobulin (IgG) was isolated from the sera of healthy individuals ($n = 4$) and from primary untreated patients with GD ($n = 4$). The diagnosis of GD was made on the basis of authoritative criteria. In this study, the characteristics of all patients with GD included diffuse thyroid goiter (stage III), ultrasonographic hypoechoic pattern, elevated serum thyroid hormone levels, low serum levels of TSH and the presence of serum TSHR autoantibodies (TRAb, 64.5 ± 3.6 U/l). These patients had no other detectable autoimmune diseases and acute or chronic liver diseases.

All tissue and blood samples were obtained with the informed consent of the patients. The study conformed to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Shandong Provincial Hospital. All animals received humane care in compliance with the guidelines from the Animal Care and Use Committee of Shandong University.

RT-PCR and sequencing

Total RNA was isolated using Trizol buffer (Invitrogen Life Technologies, Carlsbad, CA, USA). To eliminate genomic DNA contamination, isolated RNA was first treated by RNase-free DNase I (New England Biolabs, Ipswich, MA, USA). cDNA was synthesized from 5 μg of the isolated RNA performed with a commercial RT kit (MBI Fermentas, Vilnius, Lithuania). Equal amounts of cDNA were subjected to PCR (TaKaRa Biotechnology, Dalian, China) with specific primers for human TSHR or for rat TSHR. Primer sequences and corresponding annealing temperatures used in the PCR are provided in Table 1. The PCR conditions were as follows: 1 cycle at 95°C for 5 min.; 35 cycles at 94°C for 30 sec.; annealing for 30 sec., 72°C for 1 min. and 1 cycle at 72°C for 7 min. Positive controls were derived from human thyroid tissues and FRTL-5 cells; for the negative control, double distilled water was substituted for cDNA.

After the reaction, 10 ml of the PCR products were subjected to electrophoresis on 0.8% (for human TSHR-2) or 2% (for human TSHR-1 and rat TSHR) agarose gels. For confirmation of the sequenced product, the left PCR fragments were purified using a DNA extraction Kit (MBI Fermentas) and sequenced in both directions on ABI PRISM® 3100—Avant Genetic Analyzer with a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA), as previously described [17]. Three independent experiments were performed.

Immunoprecipitation

Total lysates of all samples were prepared using RIPA lysis buffer (Shenneng Bo Cai Co. Ltd, Shanghai, China). Protein concentrations were determined using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). To initiate immunoprecipitation, 2 μg (10 μl) of monoclonal anti-TSHR antibodies (sc-32263, Santa Cruz Biotechnology, CA, USA) were added into each Eppendorf tube containing 500 μl whole lysate (total 500 μg protein). Tubes were incubated at 4°C overnight, with continuous gentle shaking.

Following an incubation with 20 μl of Protein G agarose beads (Upstate, Lake Placid, USA) at 4°C for 2 hr, samples were washed three times with cold-PBS buffer, resuspended and boiled in 60 μl double-concentrated electrophoresis sample buffer. Human TSHR was detected by Western blotting performed with mouse monoclonal anti-TSHR antibodies (ab6047, Abcam, Cambridge, UK), and rat TSHR was detected using goat polyclonal anti-TSHR antibodies (sc-7816, Santa Cruz). The immunocomplexes were detected using the chemiluminescence method (ECL-plus kit, Amersham Biosciences UK Limited, UK). Three independent experiments were performed for both human and rat samples.

Immunofluorescent microscopy

The cellular distribution of TSHR was examined in L-02 cells by immunofluorescent microscopy. Briefly, cells were seeded on polyornithine-coated glass cover slips and cultured for 48 hrs, after which they were washed with cold PBS and fixed with 4% paraformaldehyde for 30 min. on ice. The cells were washed twice with cold PBS containing 0.1% Tween-20 for 5 min., blocked with 10% goat serum/PBS for 30 min. and incubated with

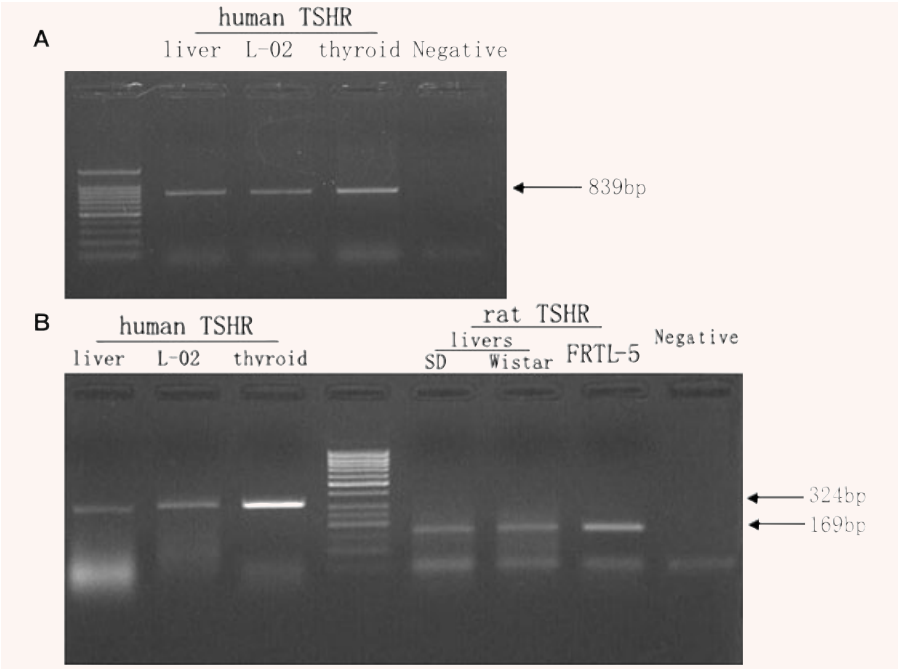

Fig. 1 TSHR mRNA is expressed in liver tissue and hepatocyte cell lines. RT-PCR for human TSHR and rat TSHR using various pairs of primers was performed as described in the Materials and Methods. For the negative control, double distilled water was substituted for cDNA. These data are representative of three separate experiments. (**A**) The PCR products were separated on a 0.8% agarose gel with a 100 bp ladder as size marker. (**B**) The PCR products were separated on a 2% agarose gel with a 50 bp ladder as size marker.

mouse monoclonal anti-human TSHR antibodies (Santa Cruz Biotechnology) in 5% goat serum/PBS at 4°C overnight. After further washing, the cells were incubated with secondary FITC-conjugated AffiniPure goat antimouse IgG (Zhongshan Goldenbridge Biotechnology Co. Ltd, Beijing, China). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), and the resultant immunofluorescence was viewed under a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). All images were acquired using the same intensity and photodetector gain to allow for quantitative comparisons between sections of relative levels of immunoreactivity. As a negative control, the identical procedure was performed in the absence of the primary antibody. Four independent experiments were performed.

cAMP assay

L-02 cells were incubated in serum-free media without antibiotics, containing 0.5 mM 3-isobutyl-1-methylxanthie (IBMX; Biomol, Plymouth Meeting, PA, USA) as a cAMP phosphodiesterase inhibitor alone or together with bTSH (Sigma-Aldrich, MO, USA), 100 μg/ml GD IgGs or 100 μg/ml normal IgG for 1 hr. Cell lysates were collected to detect cAMP contents performed with the cAMP Direct Immunoassay Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. At least four independent experiments were performed in all groups, and results were expressed as picomoles cAMP per milligram protein.

Statistical analysis

All values are given as mean ± standard deviation (SD). Statistical analyses were performed with a one-way ANOVA followed by LSD test, with a significance level set at 0.05.

Results

Presence of TSHR mRNA in hepatocytes

After PCR amplification, distinct bands of 839 and 324 bp were all observed in human liver tissue and L-02 cells. Importantly, the size of the bands obtained from human samples corresponded with those obtained from human thyroid tissue (Fig.1 A and B). Similarly, a band of 169 bp was clearly detectable in the liver tissues of two rat species, consistent with that from the positive control, FRTL-5 cells (Fig.1 B).

Specificity confirmed by sequence

To validate the specificity of the amplified fragment, PCR products of TSHR from L-02 cells, human liver and thyroid samples were purified and sequenced in both directions. The results demonstrated that the TSHR sequences of L-02 cells and human liver tissue were identical (Fig.2 A and B). In addition, both of these sequences were identical to the published mRNA sequence of human TSHR derived from thyroid tissue.

Presence of TSHR protein in hepatocytes

Immunoprecipitation of TSHR was performed to assess TSHR protein expression in hepatocytes. As seen in Figure 3, a protein band of approximately 62 kD was detected in the positive controls.

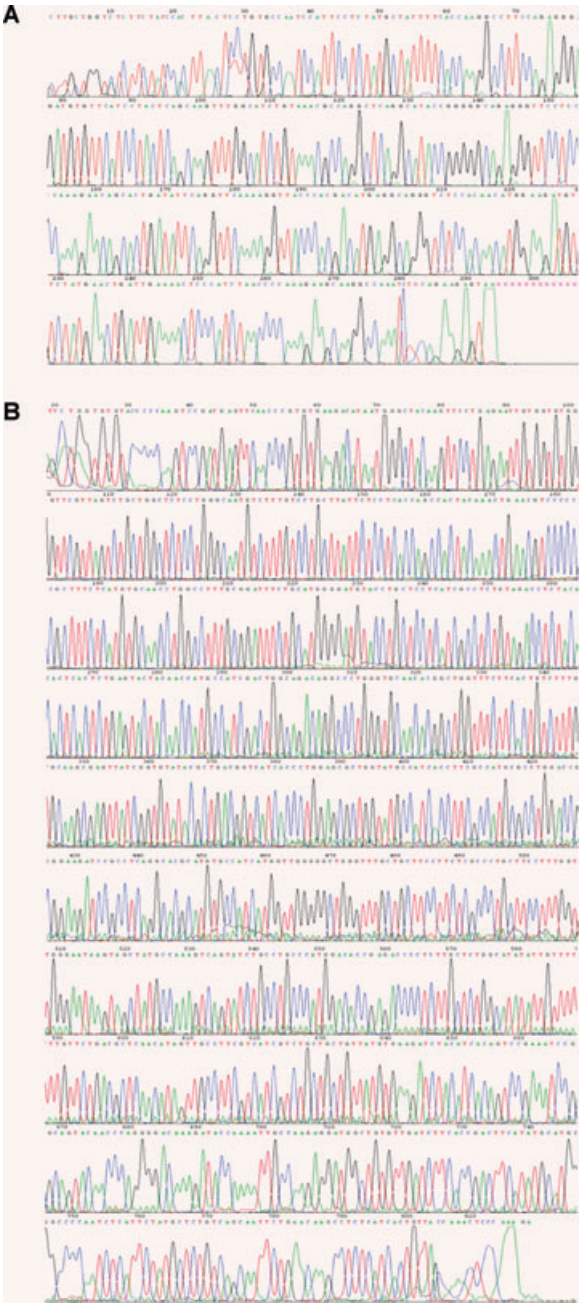

Fig. 2 Confirmation of the sequence of human TSHR PCR products. Human TSHR PCR fragments were purified and sequenced as described in the Materials and Methods. The results demonstrated that the sequences of both L-02 cells and human liver tissue were identical and were consistent with the published mRNA sequence of human TSHR derived from thyroid tissue. (**A**) Representative of the sequence of human TSHR using the TSHR-1 pair of primers (Table 1). (**B**) Representative of the sequence of TSHR using the TSHR-2 pair of primers (Table 1).

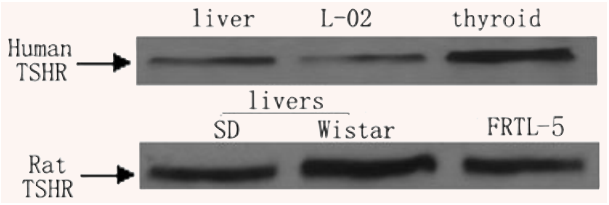

Fig. 3 TSHR protein is present in human or rat samples. The presence of TSHR protein was detected by immunoprecipitation using anti-human TSHR or anti-rat TSHR antibodies as described in the Materials and Methods. Human thyroid tissue and rat thyroid FRTL-5 cell line were positive controls. Data are representative of three separate experiments.

Importantly, bands of the same size were detected in human liver tissues, L-02 cells and rat liver tissues.

To examine the cellular distribution of TSHR protein in hepatocytes, immunofluorescent microscopy was performed on L-02 cells. As shown in Figure 4A1, immunoreactivity for the TSHR protein was clearly observed (green). As expected, the green fluorescence was present in the cell membrane. In contrast, the negative control (represented by the L-02 cells processed in the absence of the primary antibodies) showed no staining (Fig. 4B1).

Both bTSH and GD IgGs stimulated cAMP production in L-02 cells

To determine whether the TSHR protein observed in hepatocytes was functional, we investigated the effect of bTSH stimulation on cAMP production. Compared with the control, bTSH significantly increased the cAMP levels by 114% at 2 μM bTSH ($P < 0.05$) and by 179% at 4 μM bTSH ($P < 0.01$). In addition, high (4 μM) concentrations of bTSH stimulated a two-fold increase in cAMP production relative to low (0.2 μM) concentrations ($P < 0.01$), indicating that bTSH increases cAMP production in hepatocytes in a dose-dependent manner (Fig. 5A).

The production of cAMP in L-02 cells was also assayed after incubation with GD IgGs or normal IgGs. As shown in Figure 5B, GD IgGs (100 μg/ml) significantly increased cAMP production compared with control or cells treated with normal IgGs (both $P < 0.05$). We did not find any difference in cAMP production between the control and normal IgGs-treated cells.

Discussion

The evidence presented in this paper demonstrates that both TSHR mRNA and protein were present in human and rat liver tissues, as well as in the cultured normal human hepatocyte cell line L-02. Furthermore, the TSHR in hepatocytes is functional, as

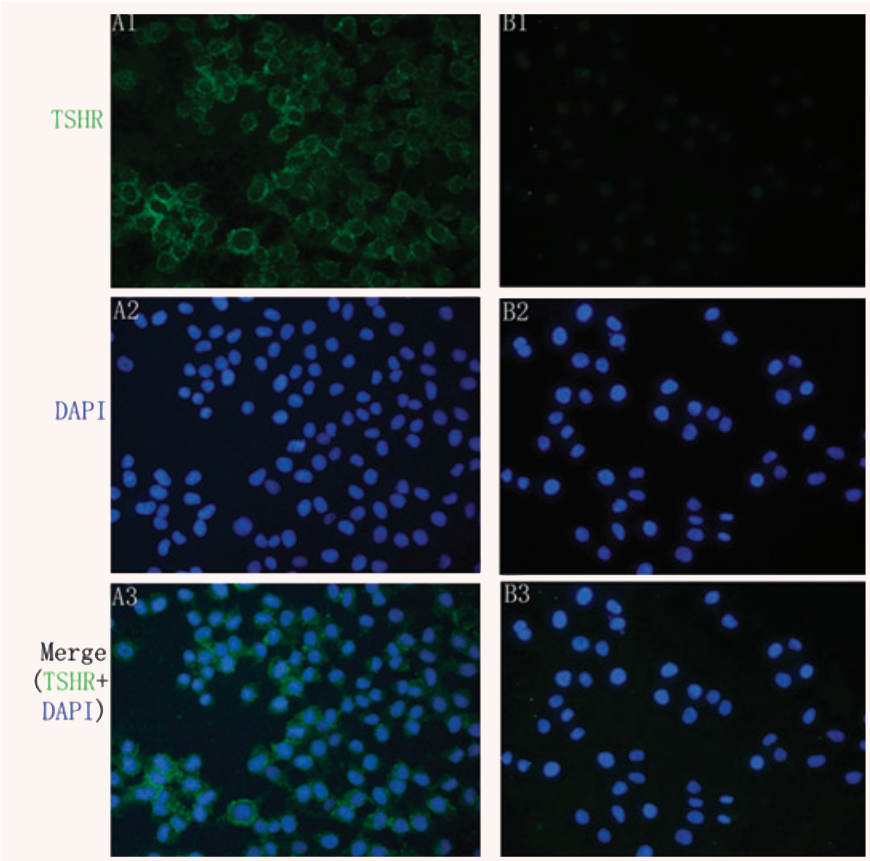

Fig. 4 TSHR is localized to the membranes of L-02 cells. L-02 cells were seeded on polyornithine-coated glass cover slips and cultured for 48 hrs. TSHR was detected using monoclonal anti-TSHR antibodies. The green colour represents TSHR protein (**A1**, FITC-conjugated), whereas the blue colour depicts the DAPI-stained nucleus (**A2** and **B2**). The merged images of TSHR and DAPI were obtained after superposition of the green and blue channels (**A3** and **B3**). The negative control was performed, as described, without the primary antibody (**B1**). Representative images from four experiments performed on different days are shown. Magnification ×400.

indicated by the ability of bTSH and GD IgGs to stimulate cAMP production in L-02 cells.

Identification of TSHR in non-thyroid tissues such as brain, bone, heart, adipose, kidney, lymphocytes, pituitary, thymus and fibroblast has been reported [18]. However, whether TSHR is expressed in hepatocytes has been uncertain. Evidence for TSHR mRNA expression in human liver tissues was first described by PCR [13], but owing to a very low level of TSHR mRNA transcript, the authors speculated that the gene was transcribed illegitimately. It is therefore thought that it is unlikely that these rare transcripts have an important biological function [14]. In the present study, we demonstrated the presence of TSHR protein with immunological activity in the liver tissue of both human beings and rats. Moreover, the translated TSHR protein was mainly localized to the hepatocyte membrane, reminiscent of its distribution on the thyrocyte membrane.

To further determine whether the TSHR protein observed in hepatocytes is functional, we measured the bTSH- and GD IgGs-stimulated cAMP responses. A classical functional assay for TSHR in thyroid cells is the testing of its signaling pathway leading to intracellular cAMP production [19]. Measurement of cAMP concentrations after TSH treatment is also commonly used to determine functional TSHR in non-thyroid cells, such as fibroblasts and adipocytes [20, 21]. In this study, we clearly demonstrated that bTSH-stimulated cAMP production in a dose-dependent fashion, indicating that the TSHR protein expressed in hepatocytes is functional. To our knowledge, this is the first evidence that hepatocytes possess a functional TSHR. It should be noted that the bTSH concentrations (0.2–4 μM) used in the present study were higher than that in normal people or patients with hypothyroidism, similar to the concentrations used for thyrocytes in culture [22] or for non-thyrocytes in culture, such as 3T3-L1 preadipocytes [23] and fibroblasts [20]. The reason for the need for a lower concentration of TSH in human body is possibly the synergistic action of co-existing growth factors/cytokines such as IGF-1 to augment TSH signaling *in vivo* [22]. Of greater interest is the result that GD IgGs from TRAb positive sera also stimulated cAMP production in hepatocytes, whereas normal IgGs did not show the same effect, similar to that reported in fibroblasts [20].

Albeit as yet unknown, given the pivotal role of the liver in metabolism and numerous disease processes, the functional expression of TSHR in hepatocyte has important physiopathological implications. The TSH-stimulated cAMP accumulation in hepatocyte demonstrates a novel physiological link between the liver function and the endocrine system through the TSH and TSHR. Given the presence of this functional TSHR in hepatocytes,

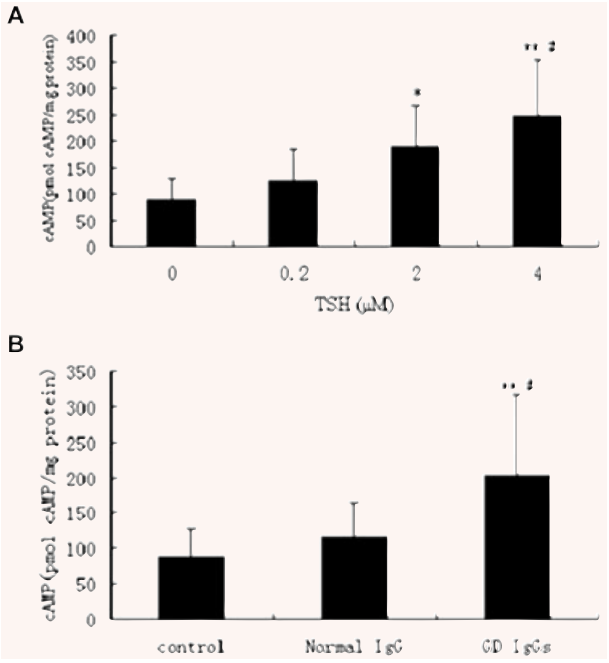

Fig. 5 Both bTSH and GD IgG stimulate intracellular cAMP production in L-02 cells. (**A**) Cells were incubated with bTSH at the indicated concentrations for 1 hr. Intracellular cAMP contents were measured as described in the Materials and Methods. Results are presented as the mean ± SD from 4 to 7 independent samples for each group. $*P < 0.05$, $**P < 0.01$ *versus* control, $\#P < 0.01$ *versus* cells treated with 0.2 μM bTSH. (**B**) L-02 cells were treated for 1 hr with 100 μg/ml GD IgG or 100 μg/ml normal IgG. Results are presented as the mean ± SD from 7 to 10 independent samples for each group. $**P < 0.01$ *versus* control, $\#P < 0.05$ *versus* cells treated with normal IgG.

a possible TSHR-antigenic link may also exist between the thyroid and the liver. This link could explain a pathogenic relationship between TRAb and human diseases, which has been known for decades [2]. Although there are many clinical and laboratory associations between thyroid and liver diseases [24], previous studies have only focused on the relationship between thyroid hormones and the liver [24]. There are many unexplained metabolic derangements in endocrine conditions associated with abnormal TSH. For example, Christ-Crain *et al.* [10] and Tuzcu *et al.* [11] independently reported the elevation of hepatic C-reactive protein [25] in patients with subclinical hypothyrodism, which is characterized by an increase in serum TSH levels but normal ranges of thyroid hormone levels. It would be interesting to see if these hepatic derangements were caused by abnormal TSH *via* acting on the TSHR in hepatocytes.

Taken together, our results demonstrate the presence of both TSHR mRNA and protein in hepatocytes of both human beings and rats. This hepatic TSHR was able to respond specifically to bTSH and GD IgG stimulation, suggesting that this expression of TSHR is not a case of illegitimate transcription. Further studies are required to examine the potential pathophysiological roles (if any) that TSH and TSHR may have in influencing hepatocyte function.

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