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Tachykinin Family Genes and their Receptors are Differentially Expressed in the Hypothyroid Ovary and Pituitary

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Key Words
Tachykinin • Pituitary • Ovary • Granulosa cell • Hypothyroid • Thyroid hormone • Reproductive disorder

Abstract
Plasma tachykinin levels are known to be altered with sexual acyclicity and loss of reproductive function. Ovulatory dysfunction, as seen in postmenopausal women, is also often encountered in hypothyroid patients. To know the involvement of different tachykinin genes in hypothyroidism-associated reproductive disorders, we performed DD-PCR with the pituitary RNA of control and hypothyroid rats to see the differentially expressed gene profile. Subsequently, we selected a few clones, tachykinin being one of them. Since its expression was up regulated in hypothyroidism as it does in the sexually acyclic females, we wanted to correlate these two phenomena with hypothyroidism associated reproductive disorders. We observed differential expression of tac2 along with other tk genes and their receptors in rat pituitary and ovary, which suggests that hypothyroidism affects the expression of these genes in these tissues. The experiments were repeated in ovarian tissue obtained at surgery from hypothyroid human patients, which showed similar expression pattern of TAC3 (equivalent to rat tac2) and their receptors as in rat ovary. Significant reduction of tac2 expression in reproductively less active rat ovary suggests the association of tac2 with reproductive senescence. Our results suggest that decline in reproductive function in hypothyroidism is associated with altered expression level of tac2 and its receptors. Further investigation in this area could elucidate the possible mechanism of tachykinins' involvement in loss of sexual cyclicity and other reproductive disorders associated with hypothyroidism.

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Introduction

The tachykinins (tks) are bioactive peptides and are widely distributed within the mammalian peripheral and central nervous system and play a well-recognized role as excitatory neurotransmitter. The mammalian tks are encoded by three different genes, termed as pre-

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protachykinin TAC1, TAC3 and TAC4 according to the Human Genome Organization (HUGO). This nomenclature replaces the previously used terms PPT-A/PPT-1 (TAC1), PPT-B/PPT-II (TAC3) and PPT-C (TAC4) [1]. Tac1, the first gene that was cloned from bovine brain was initially believed to have two splice variants, one containing substance P (SP /tac1) and the second containing Neurokinin A (NKA/tac2) [2]. Later, it was realized that a discrete genomic segment encoded NKA by alternative RNA splicing of the same gene to yield α-tac1 and β-tac1 [3]. TAC1 expression has been found in all human tissues, particularly the whole brain, heart, colon, spleen, fetal brain and mammary gland except for the placenta, a tissue not innervated by the peripheral nervous system [4, 5]. The classification of the mammalian tks has been mentioned in Table 1 [2, 4, 6-13].

Gene	Peptide	Reference
TAC1 /tac1	SP/NKA NPK/NP γ	2, 6-11
TAC3 / tac2	NKB	12, 13
TAC4	HK-1,EKA,EKB, EKC, EKD	4

Table 1. Classification of the mammalian tachykinins

Tk exerts its effects on target cells by at least three specific guanine nucleotide coupled receptors, the tachykinin receptors [NK-1R, -2R and -3R encoded by Tacr1, -r2 and -r3] [14]. Each tk appears to preferentially activate a distinct receptor, although at low ligand concentration. Tacr1 is preferentially activated by SP, tacr2 by NKA, and tacr3 by NKB [15]. The conserved carboxyl terminal domain of tks is essential for receptor activation, whereas their amino terminal dictates receptor specificity. However, tks are not highly selective to bind and activate their receptors at high ligand concentration.

Tks are also found in endocrine glands and have been shown to exert paracrine actions on the secretion of hormones with potent stimulatory activity on the contraction of smooth muscle [16]. The possible presence of tk in the oocytes and the surrounding cells may be a contributing factor to stimulate the contraction of smooth muscle after the oocyte is shed and picked up by the fallopian tube; it will help transport the cell inside the tubes [17]. In mammals, the most abundant tks are SP, NKA, NPK, NPγ and NKB, which act on the hypothalamo-pituitary-gonadal axis to regulate reproductive functions [18-20]. It is widely known that chronic estradiol-induced loss of LH-surge causes loss of sexual cyclicity [21]. Other reports suggest that estradiol increases hypothalamic levels of SP and NKA [22] and also substance P receptor gene expression in pancreatic acinar cells [23]. The increased levels of hypothalamic NKA, SP and NKB in aged female rats and decreased level of pineal NKA concentrations suggest that such up- and down regulations may have some relationship with the loss of sexual cyclicity due to aging. It seems evident, that aging alters the regulation of the tk content in CNS, but this influence was specific for each tissue [24, 25]. In ovary, both nervous tissue and the granulosa, luteal and interstitial cells express tk-encoding genes [26]. Relatively few studies have been the focus of the possible direct intra-ovarian role of tk [27].

To study the differentially expressed gene profile in hypothyroidism, we performed DD-PCR with two major centers of reproductive axis i.e pituitary and ovary. We performed DD-PCR with pituitary and ovarian RNA and subsequently selected a few clones, tachykinin1 (tac1) being one of them, obtained from pituitary RNA. Hypothyroidism is associated with different types of reproductive dysfunction, including ovarian atrophy [28], disturbed folliculogenesis, anovulation [29], infertility and increased frequency of recurrent unexplained foetal loss [30]. Since tks are intraovarian modulators as well as they likely exert a paracrine modulatory activity on the secretion of hormonal steroids [31], their altered expression in both pituitary and in ovary could influence the steroid hormone release via autocrine and paracrine functions. In our earlier report we showed the involvement of various genes, including lysyl hydroxylase, matrix metalloproteases, collagens in hypothyroid associated reproductive dysfunction [32, 33]. In the present study we wanted to investigate whether tks are also involved in hypothyroid associated ovarian dysfunction or not and this would be another aspect to study its regulatory mechanism. We showed the status of tac1, tac2 and their receptors in hypothyroid rat ovary and pituitary. We also showed that tac2 expression was significantly reduced in reproductively less active rat ovary, suggesting its association with reproductive senescence. We did not obtain tac1 expression in the ovary of 4-weeks old rat before 6m of age; hence performed the detail and subsequent studies with tac2, which was expressed in both pituitary and ovary at all ages under study. We were able to show the expression of TAC3 (equivalent to rat tac2) gene and its receptors in ovaries of human hypothyroid patients to satisfy its clinical value. Cumulatively, our results indicate that altered expression of tac2 attribute to the reproductive senescence due to hypothyroidism.

Materials and Methods

Animals and treatment

As described previously, newborn pups were rendered hypothyroid (hypothyroid rats) through their mother's milk because the mothers take 0.02 % 6-N-propyl-2-thiouracil (PTU; Sigma, St. Louis, MO) with the drinking water [32, 34]. The hypothyroid pups were given intra-peritoneal T_3 injection at a dose of 15µg/ 100 gm body weight [35]. For T_3 and TSH measurement, serum from 10 individual rats was collected and the mean value of 10 samples is presented. We strictly followed all details of the animal protocol approved by Institutional Animal Ethics Committee during the experiments. The rats mentioned as +PTU+T_3 were i.p injected daily with T_3 (15 µg/ 100g body wt) in hypothyroid rats. Here the rats were injected from d15 of their birth for 15 consecutive days. After that blood was collected from each set and serum samples were prepared from control, hypothyroid and experimental rats followed by RIA and ELISA for T_3 and TSH assay respectively (Table 2). For RNA work with ovary and pituitary, 26d old rats were treated with pregnant mare's serum gonadotropin (PMSG) [32]. After 48hrs of this treatment, the rats were anaesthetized, dissected and respective tissues were collected and washed in PBS and RNA isolation was followed.

Human sample collection

The patients gave written informed consent for the studies, as required by the Ethics committee of the Institute of Post Graduate Medical Education and Research, Kolkata. The study group consisted of 6 hypothyroid and 6 non-hypothyroid (control) subjects. Their age was 20-50yrs. All subjects were apparently healthy and did not take any regular medication. Blood samples were collected from each group and isolated serum samples were subjected to T_3 and TSH assay by RIA and ELISA respectively (Table 2). Ovary samples were collected from control and hypothyroid human patients undergoing oophorectomy with hysterectomy for control of menorrhagia and the tissue bits were collected in TRI-reagent (RNA isolation solution), for further analysis. Venous blood samples were obtained for the measurement of plasma T_3 and TSH.

Differential Display PCR (DD-PCR)

Differences in patterns of gene expression were determined using DD-PCR kit provided by Clonetech (Palo Alto, CA, USA). Briefly, cDNA was synthesized from total RNA (2µg) isolated from rat pituitary using cDNA synthesis primer provided by the vendor. The resulting cDNAs were amplified by PCR with a 25-nt oligomer of random sequence and with oligo dT primers. A total reaction of 20 µl contained 1X PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl), 3mM $MgCl_2$, 200 µM dNTPs (along with [α-P^{32}]deoxy-ATP, and 1 unit of Taq DNA Polymerase (Clonetech). Amplification was performed using the following protocol- 1 cycle at 94°C for 5min, 40°C for 5 min and 68°C for 5min; then 2 cycles at 94°C for 2min, 40°C for 5min and 68°C for 5min; and then 23 cycles at 94°C for 20sec, 60°C for 30sec and 68°C for 2min. Amplified products were resolved on a 5% denaturing polyacrylamide gel. Auto-radiographs of dried gels were prepared by overnight exposure to BioMAX MR film (Kodak, Rochester, NY). Bands corresponding to differentially expressed mRNAs were excised from the acrylamide gel, reamplified using PCR and then cloned into the T/A cloning vector using the InsT/A cloning kit (MBI Fermentas, Hanover, MD).

	T_3 (ng/ml)	TSH (µIU/ml)
Con	2.4 ± 0.02	0.30 ± 0.03
Con+PTU-T_3	0.48 ± 0.02	3.78 ± 0.025
Con+PTU+T_3	2.28 ± 0.01	0.24 ± 0.018

	T_3 (ng/ml)	TSH (µIU/ml)
Normal patients	1.9 ± 0.02	1.8 ± 0.021
Hypothyroid patients	0.8 ± 0.01	9.23 ± 0.87

Table 2. Serum samples were collected from control, hypothyroid and T_3-treated hypothyroid rats and T_3, TSH were assayed as mentioned in the methods section and are represented in the table. Similarly serum T_3 and TSH levels were assayed from normal and hypothyroid human patients and the mean values are represented in the table.

Radio Immuno Assay (RIA)

In rats and human patients, plasma T_3 was determined by RIA using T_3 RIA kit as previously described (Riak-4 radiopharmaceuticals operations, BRIT, BARC, India) [32, 36] The sensitivity of T_3 was 0.24 ng/ml of the sample based on 90% B/B_0 intercept.

ELISA

In rats and human patients, ELISA was performed for serum TSH using Pathozyme TSH kit (Omega Diagnostics Ltd, UK) as described previously [32]. The minimum detectable concentration of TSH by Pathozyme TSH kit was estimated to be 0.2 µIU/ml

RNA isolation

Total RNA was isolated from rat ovary and pituitary (from control, hypothyroid group and T_3-injected hypothyroid rats) and also from human ovarian tissue using RNA isolation reagent (TRI-Reagent, Sigma) following to the manufacturers protocol [32].

Northern Blot analysis

Northern blotting and hybridization was performed following the methods described earlier [37]. Briefly, 10µg of total RNA, isolated from pituitary, was loaded in each lane of 1% formaldehyde-agarose gel, electrophoresed, and transferred

Gene Product	Gene Acc. no.	Forward Primer	Reverse Primer	Size of the amplicon
GAPDH (rat)	BC059110	5'GCCATCAACGACCCCTTC3'	5'AGCCCCAGCCTTCTCCA3'	237 bp (nt 937-1173)
Tac1 (rat)	NM_012666	5'GGTGGCGGTCTTTTTTCTC 3'	5'GATAGTGCGTTACAGGGTTT 3'	394 bp (nt 117-510)
Tac2 (rat)	NM_019162	5'GGGGATCCAGGAGCGCCATGCTGTT 3'	5'GGGAATTCCTATTTGAGGA CGCCAA3'	348 bp (nt 118-465)
Tac4 (rat)	AY471575	5'CTTGCCCTGTTTCTCCTGAT3'	5'TCCGCTGACTGGATGGGAT 3'	227 bp (nt 13-239)
GAPDH (human)	NM_002046	5'TGGCGTCTTCACCACCAT 3'	5'TGAGTCCTTCCACGATACCAA 3'	227 bp (nt 372-598)
TACR3 (human)	NM_001059	5'TATGGTGTGGTGGT GGCAGT3'	5'TCTGGAAGCGGCAGTAGTTG3'	211bp (nt 417-627)
TAC3 (human)	NM_013251	5'TGACTTCTTTGTGGGACTTATGG3'	5'TATTCTGCTCTCGGGGGATACT3'	117bp (nt 409-525)
Tacr3 (rat)	NM_017053	5'ATTTGGATTTTGGCATTTCTAC3'	5'ATCATCTTTACCACCTTTCGTT3'	284bp (nt 806-1089)

Table 3. The oligonucleotide primers and the respective amplicon sizes of different genes used in the semi quantitative RT-PCR has been listed in the table.

to a nylon membrane (Millipore, USA) by capillary transfer method. Pre-hybridization was allowed for 2hours in the buffer containing 6 X SSC with 50% formamide, 1 X Denhardt's solution, 100 µg/ml salmon sperm DNA and 1% SDS at 42°C. Hybridization was carried out in the same condition with the [$^{\alpha\text{-}32}$P] dATP-labelled tachykinin gene fragment for 18h.Then the membrane was washed for 90min at 65°C in 2 X SSC containing 0.1% SDS with subsequent three changes of buffer. The hybridized membrane was exposed to Kodak X-ray film and kept at -80°C as per required exposure time. RNA molecular size markers (0.2kb-6 kb) used in the test was purchased from MBI Fermentas.

Reverse Transcription-Polymerase Chain Reaction

First strand cDNA synthesis was carried out with 2 µg total RNA isolated from rat pituitary and ovary as well as from human ovaries using Revert Aid M-Mulv Reverse Transcriptase (MBI Fermentas), following the manufacturer's instruction. Into the tube, oligo $(dT)_{18}$ primer, RT reaction buffer, RNase inhibitor, dNTPs were mixed (final volume 20µl) and incubated at 42°C for 1h for the first strand cDNA synthesis. 2µl of the synthesized cDNA was used as template for semi-quantitative PCR with gene specific primers and relative expression was observed with GAPDH primer (38). A 50µl PCR volume was made by adding 2.5U Taq DNA polymerase (Invitrogen, USA), to a PCR mixture containing 1X reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% Triton-X-100 and 2.5 mM $MgCl_2$], 200 µM of each dNTPs (Sigma, USA), 0.4 pmol of each primer (sense and antisense). The PCR was performed with initial denaturation at 94°C for 5min, and then 35 cycles of denaturation at 94°C for 30sec, annealing at specific temperature for each set of primers for 30sec, extension at 72°C for 35sec, final extension at 72°C for 10min in the last cycle (Perkin-Elmer 9700). The RT-PCR products were cloned and sequenced. The primers used for RT-PCR and the amplicon sizes are listed in Table-3. The expression profile of different tachykinin genes and their receptors was also verified by real time quantitative RT-PCR (Q-PCR) method and the similar kind of expression profile was observed here. We have not incorporated the Q-PCR data in this report to avoid redundancy.

Sequencing and analysis

Sequencing of the plasmid DNA and the PCR products was done by ABI Prism Automatic DNA Sequencer (Perkin Elmer). Sequence alignment and data analysis was done through BLAST search from NCBI GenBank. [39].

Statistical analysis

All data are expressed as the mean ± SD, statistical analysis, which was performed by Student's t-test. $P<0.05$ was considered to be significant. The experiments were repeated at least three times in duplicate unless otherwise stated.

Results

Identification of Tachykinin clone by DD-PCR

We performed DD-PCR with RNA isolated from the pituitaries of control and hypothyroid rats. We identified a number of up- and down regulated cDNA clones in hypothyroid condition (data not shown). The differentially expressed cDNAs thus obtained were cloned and then sequenced. One of such clones happened to be tachykinin (tac1).

Differential expression of tac1 in pituitary of hypothyroid and T_3-supplemented hypothyroid rats

To reconfirm the authenticity of the DD-PCR result, we performed Northern hybridization with control and hypothyroid pituitary RNA using tac1 cDNA as a probe (Fig. 1a). Like DD-PCR results, the expression level of tac1 in hypothyroid pituitary was markedly higher compared to control. In Fig. 1 (a), the rRNA bands of control and hypothyroid pituitary indicate equal loading in both lanes.

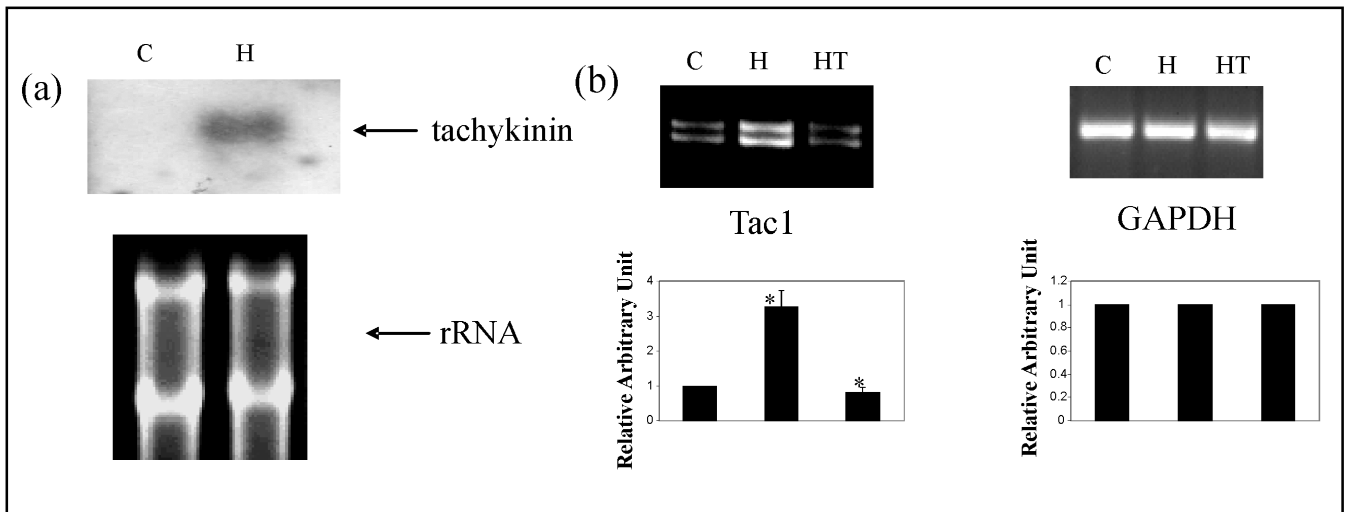

Fig. 1. Differential expression of tac1 in pituitary of hypothyroid and T_3 supplemented hypothyroid rats. RNA was isolated from control (C) and hypothyroid (H) rat pituitary and the isolated RNAs were electrophoresed on agarose gel and Northern hybridized with radio-labeled tac1 cDNA fragment as probe. The hybridized band and the ethidium bromide stained rRNA bands (as loading control) have been shown (Fig. 1a). RT-PCR was performed with pituitary RNA isolated from control (C), hypothyroid (H), and T_3-injected hypothyroid (HT) rats using gene specific primers for tac1 and GAPDH (Fig 1b). In the lower panel of the RT-PCR data, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. In tac1 RT-PCR result, average pixel value of both the bands has been represented. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. *P < 0.05.

RT-PCR was performed with pituitary RNA isolated from control (C), hypothyroid (H) and T_3-injected hypothyroid rats (HT) using gene specific primers for tac1 and GAPDH. As shown in Fig. 1(b), the expression levels of tac1 was significantly higher in hypothyroid condition compared to control. Upon T_3 supplementation to the hypothyroid rats, the expression levels of tac1 reverted back to the normal level. The double band obtained in this RT-PCR data suggests the amplification of tac1 splice variants in this tissue. GAPDH expression level was unaltered under the above-mentioned conditions.

Differential expression of tac2 and its receptor in pituitary of hypothyroid and T_3-supplemented hypothyroid rats

RT-PCR was performed with pituitary RNA from control (C), hypothyroid (H) and T_3-injected hypothyroid (HT) rats using gene specific primers of tac2 and also with tacr3 and GAPDH. In Fig. 2, the result shows that tac2 was significantly down regulated in hypothyroid condition. The expression level of tac2 did not revert back to its normal level in T_3-injected hypothyroid pituitary. The expression level of tacr3 was markedly induced under hypothyroid condition. Upon T_3-supplementation to the hypothyroid rats, the expression of this receptor had a tendency to revert back to the control level. GAPDH expression level was unaltered under the above-mentioned conditions.

Differential expression of tac2 and its receptor in ovary of hypothyroid and T_3-supplemented hypothyroid rats

RT-PCR was performed with ovarian RNA from control (C), hypothyroid (H) and T_3-injected hypothyroid (HT) rats using gene specific primers of tac2 and also with tacr3 and GAPDH. In Fig. 3, the result indicates, as in pituitary, tac2 is significantly down regulated in hypothyroid ovary and its decreased expression level did not revert back to its normal value even after T_3 supplementation. The expression level of tacr3 was down regulated in hypothyroid ovary and upon T_3 supplementation to the hypothyroid rats, the expression of tacr3 reverted back to its normal value. GAPDH expression level was unaltered under the above-mentioned conditions.

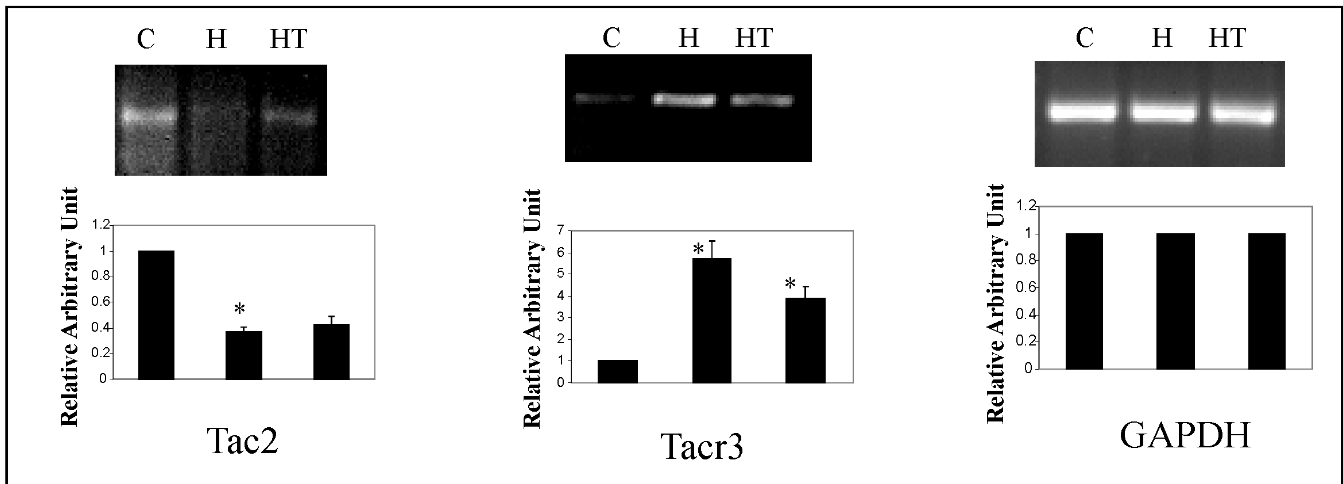

Fig. 2. Differential expression of tac2 and its receptor in pituitary of hypothyroid and T_3-supplemented hypothyroid rats. RT-PCR was performed with pituitary RNA isolated from control (C), hypothyroid (H), and T_3- supplemented hypothyroid (HT) rats using gene specific primers for tac2, tacr3 and GAPDH. In the lower panel of the RT-PCR data, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. *P < 0.05.

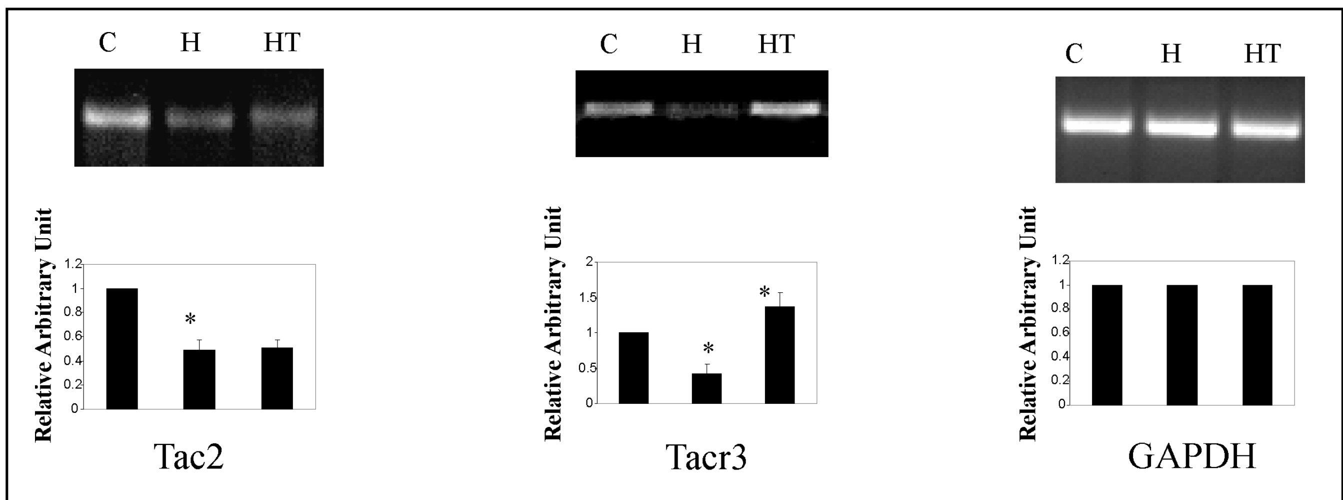

Fig. 3. Differential expression of tac2 and its receptor in rat ovary of hypothyroid and T_3-supplemented hypothyroid rats. RT-PCR was performed with ovarian RNA isolated from control (C), hypothyroid (H) and T_3-supplemented hypothyroid (HT) rats using gene specific primers for tac2, tacr3 and GAPDH. Lower panel of each RT-PCR data represents scanning data of the respective band intensities, where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. *P < 0.05.

The expression levels TAC3 (equivalent to rat tac2) and its receptor in hypothyroid human ovary

RT-PCR was performed with ovarian RNA isolated from normal (C) and from hypothyroid (H) human patients undergoing oophorectomy, using gene specific primers of TAC3 (equivalent to rat tac2), TACR3 and GAPDH. As shown in Fig. 4, TAC3 expression level was down regulated in hypothyroid patients, although not very significantly. The expression level of TACR3 was induced in hypothyroid human ovary.

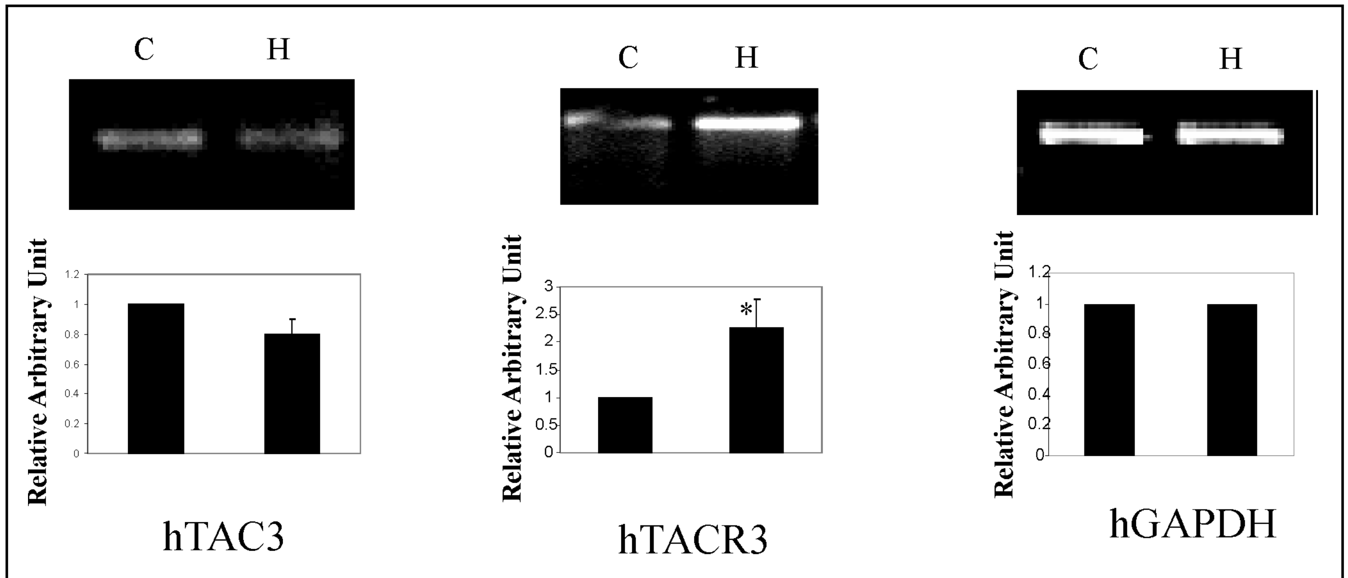

Fig. 4. Differential expression of TAC3 (equivalent to tac2 in rat) and its receptor in the ovary of hypothyroid patient. RT-PCR was performed with ovarian RNA isolated from control (C) and hypothyroid (H) human subjects (undergoing oophorectomy) using gene specific primers for TAC3, its receptor TACR3 and GAPDH. In the lower panel of the RT-PCR data, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. $*P < 0.05$.

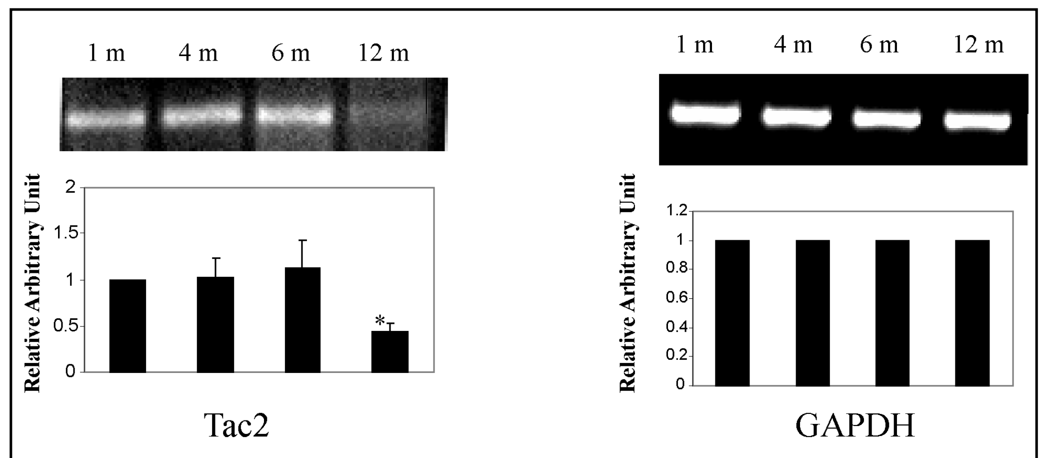

Fig. 5. The differential expression of tac2 in the rat ovaries of different age-groups. RT-PCR was performed with ovarian RNA isolated from different age group of rats i.e, 1m, 4 m, 6 m and 12 m using gene specific primers for tac2 and GAPDH. Lower panel of each RT-PCR data, scanning data of the respective band intensities have been shown where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. $*P < 0.05$.

Differential expression of tac2 in the rat ovaries of different age-groups

RT-PCR (Fig. 5) was performed with ovarian RNA isolated from 1-, 4-, 6- and 12months old rats and the result shows that the tac2 expression level was increased gradually from 1 m of age onwards and the value was maximum at 6 m. But at 12m, its expression became significantly less. However, GAPDH expression was unaltered under the above-mentioned conditions.

Differential expression of tac4 in pituitary and ovary of hypothyroid and T_3-supplemented hypothyroid rats

We wanted to check the expression level of other tk gene in rat pituitary and ovary. RT-PCR was performed with RNA isolated from control (C), hypothyroid (H) and T_3-supplemented hypothyroid (HT) rats using gene specific primers of tac4 and GAPDH (Fig. 6). The RNAs were isolated from rat pituitary and ovary. Tac4 gene

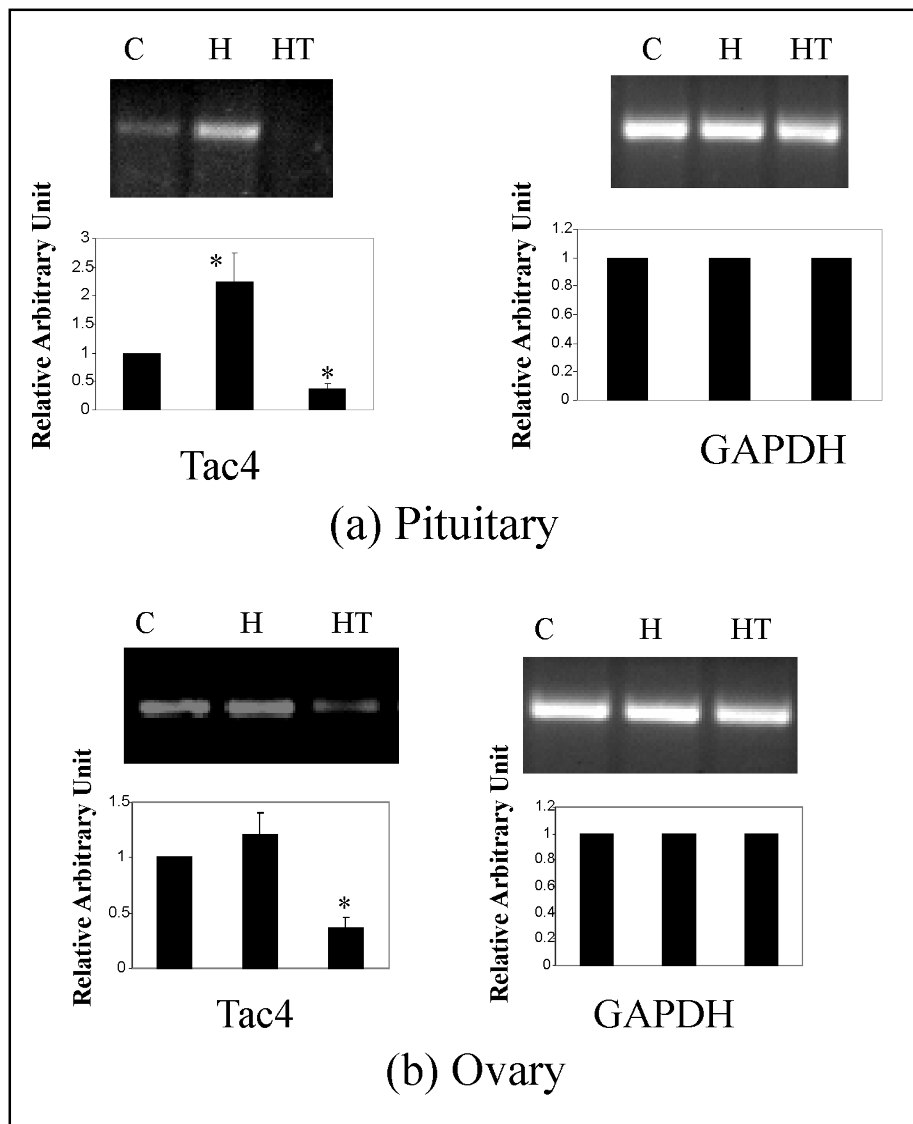

Fig. 6. The differential expression level of tac4 gene in pituitary and ovary of hypothyroid and T_3-supplemented hypothyroid rats. In Fig (a) and (b), RT-PCR was performed using gene specific primers for tac4 and GAPDH with pituitary and ovarian RNA respectively isolated from control (C), hypothyroid (H) and T_3-injected hypothyroid (HT) rats. In the lower panel of each RT-PCR data, scanning data of the respective band intensities have been shown, where pixel value of each band was calculated by the ImageJ software. The above experiments were performed three times in duplicate and the scanning data is the mean ± SD. *P < 0.05.

expression, however, was significantly increased in hypothyroid condition in both pituitary (a) and ovary (b). In both the tissues, expression levels of these genes were significantly reduced upon T_3-supplementation to hypothyroid rats, whereas GAPDH expression was unaltered under these conditions.

Discussion

The presence of tachykinins have been demonstrated in endocrine glands, where they likely exert a paracrine modulatory effect on hormonal secretions and the conceived dogma that the tks are exclusively restricted to nervous tissue thus has been challenged [26]. Tks of both neuronal and non-neuronal (like ovarian granulosa and luteal cells) origin are differentially expressed in various types of reproductive cells and may play role in female reproductive function [17]. Tks have a direct contractile effect in uterine smooth muscle [40] and have been implicated in menstrual and pregnancy related disorders [41-46].

Various types of reproductive disorders are associated with hypothyroidism. Differential expression of different tk genes has relationship with the loss of sexual cyclicity [25]. As similar reproductive failure is prevalent in hypothyroidism, we wanted to correlate the differential expression of tk with reproductive malfunction in hypothyroidism and identified the up regulation of tac1 gene in the pituitary of hypothyroid rat. Previous report suggests that hypothyroidism due to thyroidectomy produced large increases in the preprotachykinin-A

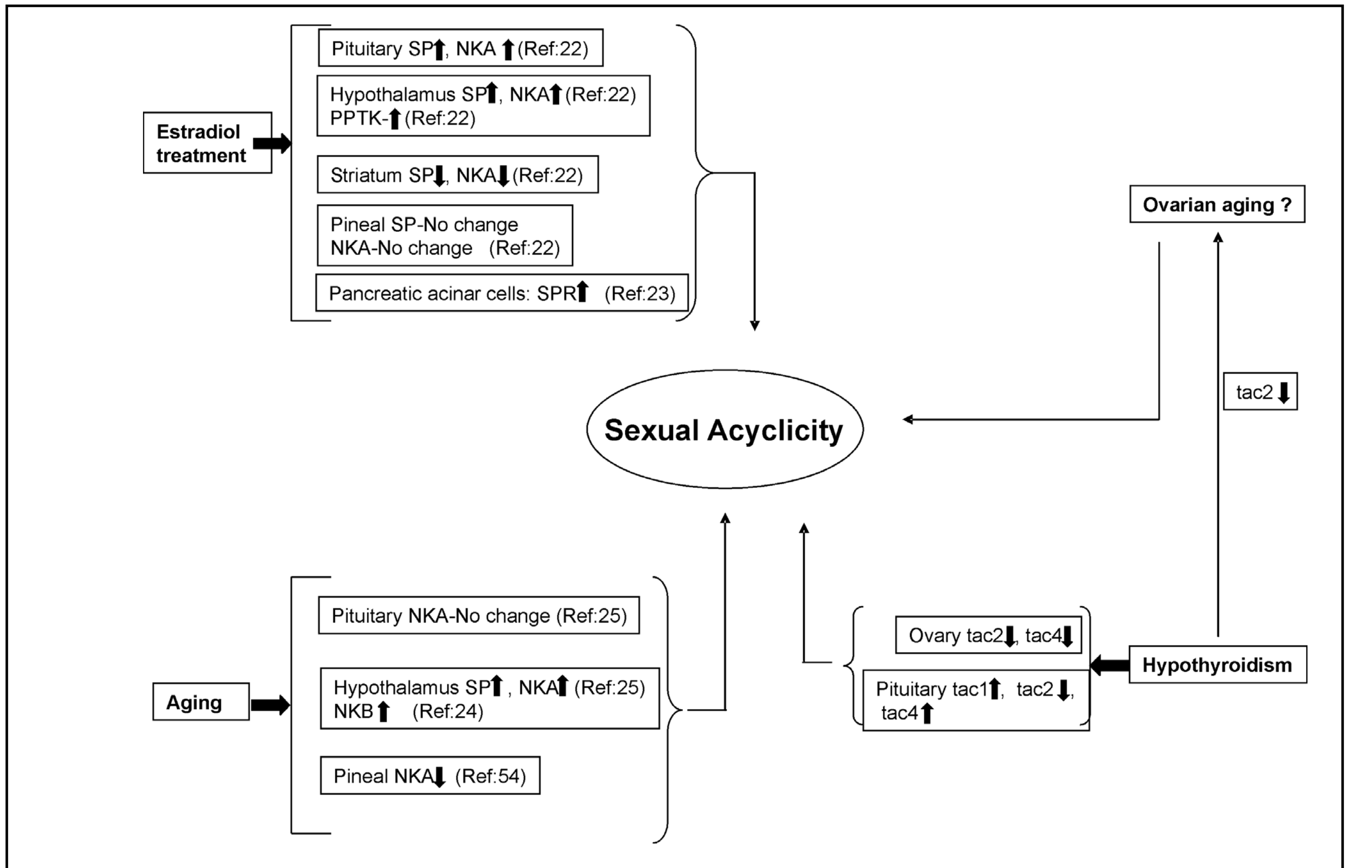

Fig. 7. The scheme shows that the expression of different tachykinin genes is affected in sexual acyclicity caused by different factors including hormones, like estradiol, aging and hypothyroidism. The upward arrows indicate up regulation and the downward arrows indicate down regulation of a particular tachykinin gene.

mRNA level in pituitary, whereas with T_4 replacement the mRNA level was much decreased [47]. Hence our model (hypothyroidism caused by PTU treatment) is supported by similar results obtained in hypothyroidism made by thyroidectomy. In addition to this, existing report suggests that rats infused with SP show an increased serum level of TSH [48]; whereas we observed that in hypothyroid rats (where TSH was increased), SP (tac1) level was also increased in pituitary. Therefore, these two observations suggest that tac1 and TSH are inducible to each other. In ovary, tac1 expression was not detected before 6m of age, whereas tac2 expression was detected in both the tissues at the age of 4-weeks. Therefore, to get a complete and useful data on comparative expression profiles in two major centers of reproductive axis, i.e. pituitary and ovary and subsequently to have a clear idea about the inter-relationship between tk and hypothyroidism-associated reproductive disorders; we focused our study mostly on tac2.

In this report we show that tac2 expression is significantly down regulated in hypothyroid condition both in pituitary and in ovary, which did not revert back upon T_3-supplementation. This data reinforces a recently published result, which suggests that neonatal treatment with capsaicin (induces release and depletion of SP in female rats and mice), resulted in decreased reproductive success compared to control [17, 49-52]. According to the order of potency of the tk receptors, NKB activates tacr3 most compared to tacr1 and -r2 [26]. The expression level of tacr3 remained significantly low in hypothyroid rat ovary and was recovered after T_3-supplementation. As tac2 expression did not recover, it is expected that even after T_3-supplementation the tac2 or more precisely NKB mediated pathway in ovary and in pituitary remains affected which might exert the decline in reproductive success. Similarly, in hypothyroid human patients also TAC3 (equivalent to tac2 in rat) gene was down regulated, whereas TACR3 was up regulated. Tacr1

and -r2 expression level varies in rat ovary and pituitary and also in human subjects, which suggests that expression of other tk receptors also affected due to hypothyroidism (data not shown). The differential expression status of various tk genes in PTU-induced hypothyroidism or in T_3-supplemented hypothyroid ovaries and pituitaries could be addressed in analogy with the effect of PTU on parathyroid. Previous reports suggest that prolonged PTU treatment i.e., prolonged TSH stimulation could lead to hyperparathyroidism and that is not reversed even with supplementation with thyroxin [53]. Again the specific effect of PTU could be endorsed considering another aspect where it is reported that neuropeptides containing nerve fibers are localized to parathyroid gland. The report suggests a novel neuroendocrine pathway whereby SP regulates PTH secretion through NK-1 receptors [54]. Although we did not check tac1 expression level in parathyroid gland, it is obvious that tac1 is upstream of PTH secretion. Since our observation suggests that tac1 expression level is increased in hypothyroid rat pituitary, it may induce PTH secretion.

Aging of CNS was associated with widespread changes in TK expression [55]. Here we investigate the association of altered expression of tac2 with reproductive senescence. The first overt sign of reproductive aging in female rats occurs around 8-12 months of age, when the preovulatory LH surge becomes attenuated and delayed; it probably results from a decrease in response of the hypothalamic-pituitary axis to the positive feedback action of estradiol. Around 9-11m of age female rats cease cycling altogether [21]. Our data shows that at 12m, when the reproductive efficiency falls, tac2 expression was reduced significantly in rat ovary. The alteration in tac2 may have implications in the pathophysiology of the elderly, as the increased NKB gene expression in the hypothalamus of postmenopausal women is secondary to estrogen withdrawal [55]. Again, pineal NKA concentrations markedly decreased when the female rats reached acyclicity which may be due to the senescence of the neuroendocrine-reproductive axis [56]. Hence, decline in reproductive function in acyclic aged female rats was associated with both increase and decrease of tks in hypothalamus and pineal gland respectively [24, 56]. Therefore, as we have shown, the decreased tac2 expression associated with sexual acyclicity due to hypothyroidism is supported by the fact that tac2 is similarly down regulated in aging-induced sexual acyclicty. Since tac4 is associated with pregnancy as well as normal and premature labor [40], its altered expression could affect these physiological phenomena. The tac4 expression profile in pituitary and ovary shows that in these tissues its expression was induced in hypothyroid condition, suggesting its involvement in hypothyroid associated reproductive disorders.

We demonstrate here that expressions of tac2 along with other tk genes and their receptors are affected due to hypothyroidism. We have also shown the decreased tac2 expression with the loss of reproductive efficiency. The steroid hormone-induced sexual senescence has been shown to play a major role in controlling the expression of different tk genes in different tissues. The expression of different tk genes is affected in sexual acyclicity caused by different factors including hormones, like estradiol, aging and hypothyroidism and that has been represented as a schematic diagram (Fig. 7). It is yet to be ascertained, whether altered expression of tks and their receptors are the primary cause of reproductive senescence. If so, in future, the modulation of expression of the important tks at the transcriptional and translational level may be performed with specific and selective agents to combat different reproductive disorders associated with hypothyroidism and aging. Further extensive study is needed to unravel the specific role of tachykinin family genes in reproductive senescence caused by hypothyroidism and aging.

Abbreviations

PTU (Propyl Thio Uracil); T_3 (Triiodothyronine); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); Tk (Tachykinin); NK (Neurokinin); SP (Substance P); NP (Neuropeptide); TRI reagent (Tripure RNA Isolation reagent); DD PCR (Differential Display PCR); RT PCR (Reverse Transcriptase Polymerase Chain Reaction); TSH (Thyroid Stimulating Hormone); SSC (Standard Saline Citrate); SDS (Sodium Dodecyl Sulphate).

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