CLINICAL STUDY

Expression of cAMP-responsive element binding protein and inducible cAMP early repressor in hyperfunctioning thyroid adenomas

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Abstract

Objective: The pathogenesis of thyroid hyperfunctioning adenomas is still only partially understood and controversy exists about the frequency of gain-of-function mutations of the TSH receptor or Gsα gene, which activate the cAMP pathway. The nuclear transcription factors cAMP-responsive element binding protein (CREB) and inducible cAMP early repressor (ICER) are among the final targets of this signalling cascade.

Design: In our study we focused on the expression of CREB and ICER genes in the nodular as well as in the extranodular tissue of hyperfunctioning tumours of the thyroid.

Methods: RT-PCR and Western blot analysis were performed in a series of 14 patients. The presence of an activating mutation of the TSH receptor or of the Gsα gene was ascertained by direct sequencing.

Results: The levels of CREB transcripts did not significantly differ in the adenomas and in the normal tissues (CREB/GAPDH, mean optical density ± S.E.: 0.98±0.18 vs 0.88±0.27 respectively, P = not significant N.S.), although case-to-case variability was observed. The absence of a significant difference between the adenoma and the surrounding normal tissue was maintained after dividing the patients into two groups, according to TSH receptor status. Accordingly, no significant difference in the levels of CREB protein (total and Ser133-phosphorylated) was observed between the nodular and the extranodular tissue. In addition, no difference was found in the levels of ICER transcripts (ICER/GAPDH, mean optical density ± S.E.: 0.52±0.11, nodule vs 0.36±0.11, normal thyroid, P = N.S.), independently of the TSH receptor gene status (i.e. wild-type or mutated).

Conclusions: Our results support the recent hypothesis that the activation of the cAMP pathway in hyperfunctioning adenomas of the thyroid might be counteracted by opposite events and suggest that complex molecular mechanisms might take part in the pathogenesis of hyperfunctioning tumours.

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Introduction

Hyperfunctioning autonomous thyroid adenoma, a cause of non-autoimmune hyperthyroidism mostly in iodine-deficient countries, is a benign neoplasm presenting as a single nodule in an otherwise normal thyroid gland (1, 2). The constitutive activation of the cyclic adenosine 3′, 5′-monophosphate (cAMP) pathway, due to the presence of somatic-activating mutations of the gene encoding the stimulatory α-subunit of the G protein, namely Gsα (3) or, with a greater frequency, of gain-of-function mutations of the thyrotrophin (TSH) receptor gene (4), has been related to the pathogenesis of hyperfunctioning adenomas of the thyroid, as recently reviewed by Duprez et al. (5). However, controversy still exists on the frequency of TSH receptor gene mutations, their detection rate ranging from 3% (6) to about 80% (7–10) in different series. In particular, a lower prevalence was observed in some countries such as Japan and the United States, and in some Italian series (11). In addition, in vitro evidence showed complex and non-homogeneous functional/morphological responses of the thyroid cells to the activation of the cAMP cascade, as reviewed by Derwahl et al. (12). It is known that, besides protein kinase A (PKA), both the protein kinase C (PKC) and the tyrosine-kinase cascade may control human thyroid growth and differentiation (13). Therefore, it is likely that different molecular mechanisms may take part in the pathogenesis of hyperfunctioning adenomas of the thyroid.
A final step in the TSH-stimulated cAMP cascade is the activation, upon PKA-mediated phosphorylation, of the nuclear transcription factors cAMP-responsive element binding protein (CREB) and cAMP-responsive element modulator (CREM) (14). These factors bind to specific palindromic DNA sequences, namely cAMP response elements (CREs), thus modulating gene expression. The peculiar aspect of CREB and CREM genes is that they can encode different isoforms by mechanisms of alternative exon splicing, alternative promoter usage and autoregulation of promoters (14). Some CREB and CREM isoforms, once phosphorylated, stimulate gene expression, whereas others act as repressors of gene expression (14). While the promoter of CREB gene is autoregulated by cAMP signalling, the promoter of CREM gene is not (14). Instead, an alternative internal promoter of the CREM gene directs the expression of repressor isoforms, indicated as inducible cAMP early repressors (ICER I, ICER II and ICER III), in response to cAMP signalling (14). Previous in vitro experiments have demonstrated that the cAMP mitogenic effects require the activation of CREB and CREM in FRTL5 thyroid cells (15) and in dog thyroid cells in primary culture (16) respectively. The expression of ICER was also enhanced by acute, but not chronic, TSH stimulation in dog thyroid cells (16). In addition, it has been shown that the long-term unresponsiveness of ICER to TSH may be due to ICER-mediated downregulation of the TSH receptor gene expression (17). Therefore, the CREB/CREM system may participate in the molecular events originating thyroid adenomas. In order to determine the involvement of cAMP-dependent nuclear transcription factors in solitary hyperfunctioning thyroid adenomas, in the present study we investigated the expression of CREB (total and Ser133-phosphorylated) and ICER in the adenoma as well as in the surrounding normal tissue obtained at surgery. In a preliminary analysis of the TSH receptor gene, hyperfunctioning thyroid adenomas were subdivided into two groups: those harbouring the wild-type TSH receptor (wtTSHr) gene and those harbouring an activating mutation of this gene (mutTSHr). The relationship between the TSH receptor gene status (wild-type or mutated) and the levels of expression of CREB and ICER was also analysed.

Materials and methods

Patients

Fourteen patients (12 females and 2 males, age 35–76 years), affected by a solitary hyperfunctioning thyroid adenoma and undergoing surgery, were included in the study, after informed consent had been obtained. At the time of surgery, subnormal TSH levels and normal free tri-iodothyronine and free thyroxine levels were detected. The size of the nodules (maximum diameter) ranged from 19 to 39 mm. Thyroid lobectomy was performed and samples from the adenoma and from the surrounding normal gland were obtained, frozen and kept at −80°C.

Analysis of TSH receptor and Gsα mutations

Genomic DNA was extracted from hyperfunctioning nodules and the surrounding normal tissue. Direct sequencing of exon 9 and 10 of the TSH receptor gene and of codon 201 and 227 of the Gsα gene was performed as described previously (8). At least two different PCR amplifications from genomic DNA were sequenced on double strands with sense and antisense primers. To confirm the presence of a TSH receptor mutation, all mutations were subcloned in a plasmid, and sequences were repeated on individual clones. Contamination problems were ruled out by including PCR control samples with no DNA as template. Extraction of DNA and pre-PCR reactions were performed in different rooms from the post-PCR reactions.

RT-PCR

RT-PCR was performed on total RNAs (0.5 μg for each reaction) extracted from the nodular and normal tissue, according to the method described by Chomczynski & Sacchi (18). A commercially available kit (SuperScript One Step RT-PCR System; Stratagene, La Jolla, CA, USA) was used to prepare the mixture for RT-PCR. Three different experiments were performed for each RNA sample. For the detection of CREB transcripts, a pair of previously described specific primers (19) spanning sequences at the 5’- and 3’-end of CREB mRNA were used. The sequence of the sense primer was: 5’-ATGACCATGGAATCTGGAGC-3’. The sequence of the antisense primer was: 5’-TTAATCTGATTTGTGGCAGT-3’. For the analysis of ICER transcripts, a pair of primers was designed by using the computer program Oligo 4.0 (Primer Analysis Software, National Biosciences Inc., Plymouth, MN, USA). The sequence of the sense primer (ICER-L), which spanned sequences of the internal promoter and exon γ of the CREM gene, was: 5’-CTGATGAGGAAAATGCAATGCAG-3’. The sequence of the antisense primer (ICER-R), spanning sequences of exon lb of the CREM gene, was: 5’-TCGGCTCTCCAGA-CATTTTAC-3’. The primers were synthesized by Roche Diagnostics (Monza, Italy). RT-PCR was performed by using the Programmable Thermal Controller PTC-100 (MJ Research, Inc., Watertown, MA, USA). Reverse transcription was performed at 50°C for 30 min. For cDNA amplification the following conditions were established: 1 min at 94°C (denaturation); 1 min at 60°C (for CREB) or at 56°C (for ICER) (annealing); 1 min at 70°C (extension). The quality of RNAs was assessed by performing additional RT-PCR using primers specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, as described previously (20). Preliminary experiments were performed to determine
the PCR cycles corresponding to the exponential phase of amplification. Thereafter, the PCR reactions were always stopped in this phase. Finally, in each RT-PCR experiment, a ‘no RNA’ reaction was added, as a negative control.

Detection of RT-PCR products

The detection of RT-PCR products was performed as described previously (21). Briefly, cDNAs were subjected to agarose gel electrophoresis and subsequent Southern blot on nylon membranes (Roche Diagnostics). Immobilised cDNAs were hybridised to CREB and ICER specific oligonucleotide probes, synthesized by Roche Diagnostics. The sequence of the probes was completely different from the sequence of the primers. The sequences of CREB probe (CREB-P), ICER probe (ICER-P) and GAPDH probe were respectively: 5′-GTACAGGGCCTTGCAAAACATTA-3′, 5′-GGAGTGGTGATGGCTGCATCG-3′ and 5′-CTAAGCAGGTGTGGTGCGA-3′. The hybridisation temperatures were respectively: 57°C, 63°C and 57°C. The hybridised cDNAs were detected by using an immunoocheluminiscent method (Roche Diagnostics), as described previously (21).

The levels of CREB and ICER II transcripts were evaluated by calculating the ratio between the optical densities (expressed in densitometric units) of the signals representing CREB or ICER II RT-PCR products and those corresponding to GAPDH, as described for comparative PCR (22–25). This PCR application is widely used to determine the levels of a specific transcript, provided that it is correctly performed. In our hands, semi-quantitative RT-PCR was validated by adding the same amount of RNA to each reaction, by stopping the amplification in the exponential phase and by using the same time of exposure of the films. In addition, the reproducibility of the results was confirmed in different experiments. It should also be mentioned that quantitative RT-PCR applications, such as competitive PCR, are problematic in the case of genes such as CREB and ICER, because the coexistence of various isoforms makes it virtually impossible to construct specific competitors.

Sequence analysis of ICER RT-PCR products

RT-PCR for the determination of ICER transcripts generated signals of different length and their specificity was validated by additional sequence analysis, as described previously (25). Briefly, after gel electrophoresis, the RT-PCR products were excised from the agarose and purified by means of a commercially available kit (Agarose Gel DNA Extraction Kit; Roche Diagnostics). Sequence analysis was performed by using the AmpliCycle Sequencing Kit (Perkin Elmer, Branchburg, NY, USA), according to the manufacturer’s instructions. For sequencing, ICER-L and ICER-R primers were radiolabelled with [32P]γATP.

The 9600 Perkin Elmer thermal cycler (Perkin Elmer) was used for direct sequencing, using the following conditions: 30 sec at 94°C (denaturation) and 30 sec at 70°C (annealing and extension), for a total of 30 cycles. Amplified products were electrophoresed on acrylamide gel (6%) in the presence of 7 mol/l urea. The gels were then blotted on a Whatman 3M filter (Whatman International Ltd, Maidstone, Kent, UK). After drying, the filters were transferred to an X-ray cassette and exposed to X-ray films for 12 h.

Protein extraction

Frozen tissues were ground and kept in lysis buffer (20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.2 mmol/l EDTA, 1% Triton X 100, 1 mmol/l Na3VO4, 1 mmol/l phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, 1 x phosphatase inhibitor cocktail 1 and 1 x phosphatase inhibitor cocktail 2 (Sigma, St Louis, MO, USA)), for 2 h at 4°C. Thereafter, tissues were homogenised with an Ika Werk RN18 potter (Janke & Kunkel, Staufen, Germany) and protein concentration was determined.

Western blot analysis

Proteins (30 µg) were diluted in equal volumes of reducing 2 × Laemlli’s sample buffer (62.5 mM Tris, pH 6.8, containing 10% glycerol, 2% SDS, 2.5% pyronin and 200 mM dithiothreitol), incubated at 95°C for 5 min and loaded onto 10% polyacrylamide-bisacrylamide gel. After separation in SDS-PAGE, proteins were transferred onto nitrocellulose (Sigma) and stained with Ponceau to verify equal protein loading. The nitrocellulose was blocked in 5% skim-milk powder for 1 h in Tris-buffered saline containing 0.1% Tween 20 (pH 7.4), washed and then immunostained with a rabbit polyclonal anti-CREB antibody (1:1000) or with a rabbit polyclonal anti-phospho-CREB (Ser133) antibody (1:1000), specific for the 43 kDa phosphorylated CREB protein (New England Biolabs, Beverly, MA, USA) followed by a peroxidase-conjugated secondary anti-rabbit IgG antibody (1:2000) (New England Biolabs). Antigen–antibody complexes were detected by incubation with LumiGLO chemiluminescent reagent and peroxide (New England Biolabs) and exposure to autoradiography film. Quantification was obtained by densitometric scanning.

Statistical analysis

Differences between CREB mRNA, ICER II mRNA or phosphorylated CREB protein levels in the adenoma and in the surrounding normal tissue were analysed by Student’s t-test.
Results

Analysis of TSH receptor gene mutations

Direct sequencing of exon 9 and 10 of the TSH receptor gene, which contain all the activating mutations described so far, revealed the presence of a mutation in 8 out of 14 hyperfunctioning nodules. Most mutations were located in the VI transmembrane domain (patients 1, F631L; 4, T630L; 5, T632I; 9, P639S; 10, T632I). In one case, the TSH receptor gene mutation was localised in the third intracellular loop (patient 14, A623V). In another case (patient 2), the mutation was located in the second transmembrane domain (M453T). Patient 12 harboured a mutation in the extracellular domain of the TSH receptor codified by exon 9 (S281N). No TSH receptor mutation was identified in the extranodular tissue. Only wild-type sequences of the Gsα gene were identified in both adenomas and extranodular tissues.

All the mutations identified had been already described in different laboratories (5, 8) and had been found to constitutively activate the cAMP pathway after transient expression in COS 7 cells. The P639S mutation has previously been shown to also activate the phospholipase C–diacylglycerol cascade (26).

Detection of CREB mRNA

Because in several cases the amount of tumoral tissue was too limited to successfully perform Northern blot analysis or RNase protection assay, the presence of CREB and ICER transcripts in hyperfunctioning thyroid nodules and in the normal extranodular tissue was determined by RT-PCR. In each experiment, RNA contamination was excluded by adding a ‘no RNA’ control reaction (not shown). Three different experiments were performed for each RNA sample, both for CREB and ICER mRNA determination, in order to assess the reproducibility of the results. No evident difference was observed among different experiments.

When RT-PCR was performed using primers specific for the CREB gene, a major signal of 1026 bp corresponding to the full-length transcript of CREB was detected, after agarose gel electrophoresis, Southern blotting and hybridisation to a CREB-specific probe, in both the nodular and the normal tissue of 10 out of 14 thyroids (nos 1, 2, 3, 4, 5, 6, 7, 8, 10, 13). The CREB signal was found only in the adenoma of one thyroid (no. 14) and only in the normal tissue of one thyroid (no. 9). In two cases, no CREB transcript was detected either in the nodular or in the extranodular thyroid tissue (nos 11 and 12). In Fig. 1A the results of a typical experiment are reported; the results were virtually identical in all the experiments which were performed. Transcripts for GAPDH were found by RT-PCR in all RNA samples (Fig. 1B), indicating that the failure to detect CREB signals in some cases was not due to RNA degradation.

The levels of CREB transcripts in RNAs from the nodular and extranodular tissue were determined by densitometric analysis as the CREB/GAPDH ratio, as previously described in different cell systems (25, 27–29). Densitometric analysis was performed on CREB...
(Fig. 1A) and GAPDH (not shown) chemilumigrams. The levels of CREB transcript in the nodular tissue were not significantly different from the extranodular tissue, either considering the patients as a whole (CREB/GAPDH ratio: 0.98±0.18 in the nodular vs 0.88±0.27 in the extranodular tissue, mean optical density±S.E., P = not significant (N.S.)) or dividing them into two groups according to the TSH receptor status (mutTSHr: 0.83±0.24, nodular vs 0.60±0.29, extranodular tissue, P = N.S., n = 8; wtTSHr: 1.17±0.27, nodular vs 1.25±0.49, extranodular tissue, P = N.S., n = 6). However, individual variations were observed, indicating a complex pattern of expression of CREB in hyperfunctioning adenomas.

**Detection of total and phosphorylated CREB protein**

Western blot analysis was performed in order to detect, in addition to CREB transcripts, the presence of CREB protein in thyroid tissues from patients with thyroid adenomas, harbouring either a wtTSHr or a mutTSHr gene. Western blot was carried out in those cases in which there was enough material left after RNA extraction (n = 7). CREB protein was detected using either a polyclonal anti-CREB antibody specific for the total CREB protein, or a polyclonal anti-CREB-P Ser133 antibody, which recognizes the 43 kDa phosphorylated CREB protein. The anti-CREB antibody revealed comparable levels of CREB protein in extracts of both nodular and extranodular tissues, as assessed by densitometric analysis (nodular: 55.7±11.5 vs extranodular: 56.4±11.7, mean optical density±S.E., P = N.S.) (Fig. 2A). Noticeably, in each of the cases, the levels of CREB protein in the adenoma versus the corresponding normal tissue paralleled the data regarding CREB mRNA. Therefore, data regarding the levels of CREB protein confirmed the results of the analysis of CREB transcript. Analogously, the anti-CREB-P Ser133 antibody revealed the presence of similar amounts of phosphorylated, hence activated, CREB protein in the nodular and extranodular thyroid tissue (nodular: 46.58±7.5 vs extranodular: 60.1±11.37, mean optical density±S.E., P = N.S.) (Fig. 2B). No correlation was observed between the levels of CREB protein (total and phosphorylated) and the TSH receptor status.

**Detection of ICER mRNA**

Similarly to CREB transcripts, ICER transcripts were detected by hybridisation of RT-PCR products to a specific probe. Specific signals, which corresponded exactly to the signals detected previously in pituitary adenomas (25), were found in both the nodular and the normal tissue of 8 out of 14 thyroids (nos 1, 2, 3, 4, 7, 10, 13, 14), and only in the adenoma of 5 thyroids (nos 5, 6, 8, 9, and 12), as shown in Fig. 3, in which the results of a typical experiment are reported. No signal was repeatedly detected in the thyroid specimens of patient no 11.

The expected 657 bp and 257 bp signals were subjected to sequence analysis and were found to correspond to ICER I and II respectively (not shown), as described previously in pituitary adenomas (25). Additional sequencing of the two middle RT-PCR products, which were detected in some cases (Fig. 3), was performed. The results confirmed the specificity of these amplified fragments, which contained partial sequences of the 657 bp product (ICER I). These additional amplified signals might be the result of alternative splicing. The levels of the RT-PCR signal corresponding to ICER II were analysed by densitometry. ICER II mRNA levels in the nodular and in the extranodular tissue did not significantly differ (ICER/GAPDH ratio: 0.52±0.11, nodular versus 0.36±0.11, extranodular tissue, mean optical density±S.E.,

![Figure 2](https://via-free-access.bioscientifica.com/146.png)

**Figure 2** Western blot analysis of protein extracts from extranodular (E) and nodular (N) tissue, using (A) a polyclonal anti-CREB antibody specific for total CREB protein or (B) a polyclonal anti-phospho-CREB antibody. The 43 kDa product represents full-length CREB protein. The numbers on (A) indicate the patients.
was also confirmed when the patients were considered separately, on the basis of the presence or absence of a TSH receptor mutation.

The levels of CREB protein were determined by Western blot analysis. According to mRNA data, no significant difference was observed between the nodular and the extranodular tissue of hyperfunctioning adenomas, independently of the TSH receptor status. The observed parallelism between CREB mRNA and protein levels, in those cases in which both analyses could be performed, confirmed the validity of the RT-PCR results. Because Ser\textsuperscript{133}-phosphorylation is a requirement for CREB to act as a transcriptional activator, the levels of phosphorylated CREB were further determined. Again, as observed with total CREB, no significant difference was found between the nodular and the extranodular thyroid tissue.

Our data might, in principle, suggest that the constitutive activation of the cAMP-dependent cascade, as occurs in thyroid adenomas bearing activating mutations of TSH receptor gene, is not associated with increased CREB expression or activation. This issue has been recently investigated in a study by Brunetti et al. (31), in which the levels of Ser\textsuperscript{133}-phosphorylated CREB appeared to be reduced in hyperfunctioning adenomas compared with the corresponding normal thyroid tissue, independently of the presence of TSH receptor or G\textsubscript{\alpha} gene mutations. In addition, no correlation between phosphorylated CREB and either PKA activity or protein phosphatase expression was found. The authors concluded that the PKA/CREB system does not seem to play a role in thyroid cell proliferation. Another recent report showed the absence of difference in the cAMP content in hyperfunctioning adenomas of the thyroid with or without activating mutations of the TSH receptor or of the G\textsubscript{\alpha} gene, as compared with the extranodular tissue (32). This finding was related to the presence of an elevated cAMP-degrading activity by phosphodiesterases (PDEs), and primarily by the cAMP-specific PDE4, selectively in adenomas bearing a mutated TSH receptor. Similar findings have been obtained in growth hormone-secreting pituitary adenomas, in which the similar levels of phosphylated-CREB observed in tumours harbouring or not harbouring gain-of-function mutations of the G\textsubscript{\alpha} gene (33) were...
related to the higher PDE4 activity found in the former group (33, 34). It is noteworthy that cAMP-specific PDEs are activated by PKA-mediated phosphorylation and that the expression of the corresponding genes is modulated by a CRE contained in the promoter region (35). Therefore, in this scenario, our results suggest, at variance with the conclusions of Brunetti et al. (31), that the PKA/CREB system might participate in the pathogenesis of hyperfunctioning adenomas of the thyroid, but its role is likely to be confined to the early molecular events, before the onset of counter-acting mechanisms takes place. Nevertheless, the certain degree of variability in the levels of expression and activation of CREB in the nodular and extranodular thyroid tissue that we observed in individual cases highlights the possibility that different molecular events may be involved in the pathogenesis of thyroid adenomas. There is evidence, for instance, that PKA activation is required to trigger DNA synthesis in thyroid cells, but may not be sufficient (36). It is also known that cAMP, in addition to the PKA/CREB system, may mediate the expression/activation of other mitogenic factors such as c-jun, c-fos (37), the Ras-like GTPase Rap 1 and the guanine-nucleotide exchange factor Epac (for exchange protein directly activated by cAMP) (38). Furthermore, besides PKA, both the PKC and the tyrosine-kinase cascade have been related to the control of thyroid cell growth (13). Recent evidence has also demonstrated the involvement of JAK/STAT in the TSH-signalling pathway (39). These data might, at least in part, explain the case-to-case variability that we observed and also the unexpected absence of CREB expression in a few cases.

A unique feature of our study was the analysis of the levels of the CREM isoforms ICER. ICER, which essentially consists of only the DNA-binding domain of CREM (14), escapes from PKA-dependent phosphorylation and, therefore, in contrast to other CRE-binding factors, the main determinant of its activity resides in its intracellular concentration and not in the degree of phosphorylation. The analysis of ICER levels in our series of hyperfunctioning adenomas of the thyroid revealed the absence of a significant difference between the nodular and the normal tissue. According to CREB data, these results also suggest the presence of counter-acting mechanisms blunting the activation of the cAMP pathway. In addition, our experimental results are in agreement with the observation that an early, yet only transitory, increase in the levels of expression of ICER takes place following the activation of the cAMP pathway by TSH in thyroid cells in primary culture (16). Finally, the case-to-case differences and the absence of ICER expression that we observed in some cases, similarly to CREB, further suggest that different events are likely to be related to thyroid cell proliferation and function in hyperfunctioning adenomas. In this regard, it is also possible that the heterogeneity among the different samples (i.e. size of the adenoma, duration of the disease at the time of surgery) contributed, at least in part, to the differences that we observed in individual cases.

In conclusion, our data are in agreement with the hypothesis that the activation of the cAMP-dependent pathway in hyperfunctioning adenomas of the thyroid harbouring gain-of-function mutations of the TSH receptor or of the Gαs gene appears to be counteracted by opposite events (32) and suggest that a unique mechanism may not unequivocally explain the pathogenesis of this disease. These results prompt further investigation in order to unmask other factors and pathways involved in the proliferation and function of thyroid cells in hyperfunctioning tumours.

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