Thyrotropin modifies activation of nuclear factor κ B by tumour necrosis factor α in rat thyroid cell line

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We have recently demonstrated that nuclear factor κ B (NF- κ B) mediates the tumour necrosis factor α (TNF- α)-dependent expression of the gene encoding interleukin 6 (IL-6) in rat thyroid FRTL-5 cells cultured in the presence of thyrotropin (TSH). In the present study we investigated how TSH is involved in the activation of NF- κ B by TNF- α in the cells. Electrophoretic mobility-shift assay revealed that, in the absence of TSH, TNF- α activated a single protein–DNA complex containing the p50 subunit but not other NF- κ B subunits such as p65. In contrast, two distinct protein–DNA complexes were activated in the presence of TSH: the faster-migrating complex contained only p50 subunit; the slower-migrating complex consisted of p65–p50 heterodimer. This TSH effect was mimicked by forskolin and thyroid-stimulating antibodies obtained from patients with Graves's disease, suggesting that an increase in intracellular cAMP is responsible for the induction of different NF- κ Bs by

TNF- α . A transient transfection study with a luciferase reporter gene driven by multimerized NF- κ B sites demonstrated that TNF- α increased the luciferase activities only in the presence of TSH, and that this increase was inhibited by the co-transfection of mutant p65, which prevented the function of wild-type p65 in a dominant-negative manner. Accordingly, TNF- α activated the expression of the IL-6 gene in the presence of TSH but not in its absence. Although the expression of the p105 gene, another known target for NF- κ B, was increased by TNF- α in the absence of TSH, the presence of TSH further increased the mRNA level. Taken together, these observations indicate that the presence of TSH is crucial for the NF- κ B-mediated actions of TNF- α on thyroid follicular cells.

Key words: autoimmune thyroid disease, cAMP, forskolin, interleukin 6, thyroid-stimulating antibody.

INTRODUCTION

Infiltration of lymphocytes into the thyroid gland is a common histological feature of autoimmune thyroid diseases (ATD). Such lymphocytes obtained from patients with Hashimoto's thyroiditis [1,2] and with Graves's disease [2,3] have been shown to produce tumour necrosis factor α (TNF- α). The existence of TNF- α receptors has been demonstrated in human thyroid follicular cells [4], human thyroid carcinoma cells [5,6] and rat thyroid FRTL-5 cells [5], suggesting that TNF- α exerts its effects on thyroid follicular cells. Indeed, several studies have shown the pleiotropic effects of TNF- α on the cells. TNF- α has an inhibitory effect on proliferation [7,8], iodide uptake [9] and the expression of iodothyronine type I 5'-deiodinase [10,11]. Of particular importance in the context of ATD, TNF- α increases the expression of MHC class I [12] and intercellular adhesion molecule-1 [13–15] and also the aberrant expression of MHC class II induced by interferon- γ [16,17]. These molecules are required for the presentation of both exogenous and intrinsic antigens and for T-cell adhesion and activation. It is therefore postulated that TNF- α is important in the autoantigen presentation of the thyroid follicular cells in ATD. Furthermore, it has been demonstrated that TNF- α increases the production of various inflammatory cytokines such as interleukin 6 (IL-6) [18–20]. IL-6 promotes B-cell differentiation and T-cell activation [21] and is suggested to recruit the lymphocytes into the thyroid gland. It is therefore considered that thyroid follicular cells are directly involved in the intrathyroidal autoimmune responses in ATD by presenting autoantigens and by producing cytokines.

It has been shown in various cell types that the transcription of the genes of inflammatory molecules such as cytokines, MHC class I and II and cell adhesion molecules are under the control of a transcription factor, nuclear factor κ B (NF- κ B) (reviewed in [22–24]). The prototypic NF- κ B is a dimer consisting of the p65 (RelA) and p50 subunits. At least three subunits, p52, c-Rel and RelB, have been identified in addition to p50 and p65. In the inactive state, the NF- κ B dimer is sequestered in the cytoplasm bound to an inhibitory protein, I κ B. Several stimuli, including TNF- α , induce the activation of NF- κ B by promoting the degradation of I κ B and nuclear translocation of the NF- κ B dimer released from the I κ B. The activated NF- κ B binds to the regulatory NF- κ B elements in the target genes and controls their transcription. We have recently shown that the TNF- α -dependent induction of IL-6 expression is mediated by the activation of NF- κ B in rat thyroid FRTL-5 cells [25].

Thyrotropin (TSH) is a prime regulator for the function and proliferation of thyroid follicular cells. Although the role of TSH in ATD remains unclear, Pujol-Borrell et al. [26] have proposed that the increased serum TSH levels frequently observed in patients with Hashimoto's thyroiditis and the thyroid-stimulating antibodies (TSAb) present in Graves's disease might contribute to the pathogenesis of ATD. Their hypothesis was supported by the following findings. TSH alone or in combination with interferon γ induced MHC class II expression in cultured human thyrocytes [27]. A similar effect of dibutyl cAMP was observed, indicating that TSAb might induce class II expression [26]. Furthermore, Chiovato et al. reported that TSH enhances the expression of the microsomal antigen on the surface of FRTL-5

Abbreviations used: ATD, autoimmune thyroid diseases; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; NF- κ B, nuclear factor κ B; PKA, cAMP-dependent protein kinase; TNF, tumour necrosis factor; TSAb, thyroid-stimulating antibodies; TSH, thyrotropin.

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cells [28] and that the administration of L-thyroxine for replacement or suppressive therapy decreases the circulating anti-microsomal antibody titres in hypothyroid but not euthyroid patients with Hashimoto's thyroiditis [29]. In accordance with these findings, Reinhardt et al. [30] demonstrated that the incidence of spontaneous lymphocytic thyroiditis and levels of anti-thyroglobulin antibody in BB/Wor rats were decreased by the suppression of TSH levels with L-thyroxine. It is therefore likely that TSH and TSAb alter the intrathyroidal autoimmune responses in ATD, probably by modulating the responsiveness of the thyroid follicular cells to cytokines.

In the present study we therefore investigated the effects of TSH on the activation of NF- κ B and on the expression of IL-6 by TNF- α in rat thyroid FRTL-5 cells, which express the differentiated phenotypes of thyroid follicular cells [31]. Our results show that TNF- α induces the activation of the transcriptionally active p65-p50 heterodimeric NF- κ B only in the presence of TSH, and that treatment of the cells with TSAb obtained from patients with Graves's disease enhances the activation of p65-p50 NF- κ B by TNF- α . We also show that TNF- α induces the expression of IL-6 only in the presence of TSH.

EXPERIMENTAL

Cell culture

FRTL-5 cells (A.T.C.C. CRL8305), passaged fewer than 10 times after purchase from A.T.C.C. (Manassas, VA, U.S.A.), were grown as described previously [25]. After 80–90% confluence, the cells were divided into two groups: one group was cultured in 6H medium; the other was cultured in medium without TSH (5H medium). After culture for 7 days, recombinant human TNF- α (2.5×10^3 i.u./ μ g; Asahi Chemical Industry Co., Tokyo, Japan) was added to the medium at a final concentration of 5–500 i.u./ml. The cells were then incubated for various durations (10 min to 24 h). In some experiments, the cells maintained in 5H medium were exposed to various doses of TSH (100 – 1000×10^{-6} i.u./ml) for 3–24 h or to forskolin (10μ M) for 6 h, followed by TNF- α (500 i.u./ml) for 10 min. The cells were then harvested for electrophoretic mobility-shift assay (EMSA) or Northern blot analysis as described below.

Preparation of immunoglobulin from TSAb-positive serum

Two sera obtained from patients with Graves's disease were provided by Dr T. Endo (Yamanashi Medical University, Kofu, Japan). The sera exhibited extremely high TSAb activities (Patient 1, 6000%; Patient 2, 3800%; measured by bioassay with cryopreserved pig thyroid cells at Ohtsuka Assay Laboratories, Tokyo, Japan). Immunoglobulins in 1 ml of each serum were precipitated with poly(ethylene glycol) (6000 Da) by the method of Kasagi et al. [32], then reconstituted with 4 ml of 5H medium. Confluent FRTL-5 cells maintained in 5H medium for 7 days were cultured for 18 h in 2 ml of this medium, followed by treatment with TNF- α for 10 min.

EMSA

The preparation of nuclear extracts and the EMSA procedures were as described previously [25]. In some experiments the nuclear extracts were treated with 30 units of bacterial alkaline phosphatase (Toyobo, Osaka, Japan) for 30 min at 37 °C. The κ Bwt oligonucleotide probe was prepared by annealing sense and anti-sense oligonucleotides (5'-TCGAGCGAGGGGGACTTTCCGAGAG-3' and 5'-TCGACTC-

TCGGAAAGTCCCCTCTGC-3' respectively) containing a canonical NF- κ B-binding site (underlined) in mouse Ig κ enhancer [22] and by extending the nucleotides with Klenow enzyme in the presence of [^{32} P]dCTP. The IL-6- κ B oligonucleotide probe was prepared by annealing sense and antisense oligonucleotides (5'-ATGTGGGATTTTCCCATGAGT-3' and 5'-GAGACTCATGGGAAAATCCCA-3' respectively) containing an NF- κ B-binding site (underlined) identified in rat IL-6 promoter [33]. The nuclear extracts (10 μ g of protein) were incubated at 25 °C for 15 min in EMSA buffer (20 μ l) containing 0.06 pmol of the labelled probe and 3 pmol of competitor oligonucleotide for displacement analysis. The reaction mixture without dye was loaded directly on a 4% (w/v) polyacrylamide gel and subjected to electrophoresis at 160 V at 25 °C. The gel was dried on a filter paper and exposed to X-ray film at -80 °C. In supershift analyses, 1 μ l of antibody or preimmune rabbit serum was added to the EMSA buffer before the addition of the labelled probe; the mixture was then incubated for 1 h at 25 °C. Antibodies directed against p50, p52, p65, c-Rel, RelB, Fos and Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The displacement analysis was performed with unlabelled κ Bwt oligonucleotide as well as κ Bmu oligonucleotide, in which the NF- κ B-binding site had been mutated [34]. The κ Bmu oligonucleotide was prepared by annealing with 5'-TCGAGCAGAGCTCACTTTCCGAGAG-3' and 5'-TCGACTCTCGGAAAGTGAGCTCTGC-3' (mutated sequences are underlined).

Determination of NF- κ B-dependent reporter gene activation

The construction of a NF- κ B-dependent reporter gene plasmid was described previously [25]. The plasmid named pGL3-3 κ Bpro contains three NF- κ B sites in tandem. In addition, the construction of rat p65-expressing plasmid (pp65) and pDNp65, which expresses a dominant-negative form of p65 (C-terminal deletion mutant), was described previously [35]. FRTL-5 cells were plated in six-well dishes (10^5 cells per well) and grown in 6H or 5H medium for 4 days. pGL3-pro (Promega, Madison, WI, U.S.A.) or pGL3-3 κ Bpro (each at 2 μ g per well), alone or in combination with pp65 and pDNp65 (each at 2 μ g per well), was transfected into the cells by using a LIPOFECTAMINE® reagent (Gibco-BRL, Grand Island, NY, U.S.A.) in accordance with the manufacturer's protocol. In all experiments a β -galactosidase-expressing plasmid (0.2 μ g per flask; pSV- β -gal; Promega) was also transfected. After incubation for 18 h with the liposome-DNA solution, it was replaced with fresh medium with or without TSH. After an additional 24 h of incubation, TNF- α (500 i.u./ml) was added to the medium and the cells were incubated for a further 8 h. The cells were then harvested and the luciferase and β -galactosidase activities in the cell lysates were determined with a luminometer (LB9501; Berthold, Pforzheim, Germany). The luciferase activity without TNF- α and TSH was taken arbitrarily as 100. Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed with a one-way analysis of variance followed by Student's *t* test. *P* < 0.05 was considered significant.

Northern blot analysis

Total RNA was extracted from FRTL-5 cells by the method of Chomczynski and Sacchi [36]. Northern blotting and hybridization were performed as described previously [37]. In brief, total RNA (15 μ g) was fractionated in 0.8% agarose gels. The RNA was transferred to a nylon membrane (Gene Screen Plus; New England Nuclear). Heat-denatured cDNA probes were labelled with [^{32} P]dCTP by using a random-primed DNA

labelling kit (Boehringer Mannheim, Mannheim, Germany). The preparation of rat IL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA species was described previously [25]. Rat p105 cDNA was provided by Dr T. Okamoto (Nagoya City University School of Medicine, Nagoya, Japan). After hybridization for 20 h at 42 °C, the membrane was washed and exposed to XAR film at -80 °C with an intensifying screen.

RESULTS

Modification of TNF- α -dependent activation of NF- κ Bs by TSH

FRTL-5 cells cultured in the absence (5H medium) or presence (6H medium) of TSH (10^{-3} i.u./ml) were treated with TNF- α (500 i.u./ml). The nuclear extracts were subjected to EMSA with κ Bwt oligonucleotide as a probe, which contained a canonical NF- κ B site (GGGACTTTC). As shown in Figure 1(A), TNF- α activated a single protein-DNA complex within 10 min in 5H medium (lane 2). The activation was sustained for 24 h (Figure 1A, lanes 3–5). In contrast, as shown in Figure 1(B), TNF- α induced the activation of two distinct complexes within 10 min in 6H medium (lane 2). The activation of both complexes persisted for 24 h (Figure 1B, lanes 3–5).

As shown in Figure 1(C), the complex observed in 5H medium (lane 1, complex A) was distinct from the faster-migrating complex in 6H medium (lane 2, complex B), because the mobility of the latter was slightly slower than that of the former. The slower-migrating complex in 6H medium was named complex C. These three complexes were also detected by using another EMSA probe, IL-6- κ B oligonucleotide (the sequence of the NF- κ B site was GGGATTTTC; Figure 1D). Complexes B and C

Figure 2 Time course and dose effects of TSH on activation of NF- κ B proteins by TNF- α

FRTL-5 cells maintained in the 5H medium for 7 days were exposed to TSH (10^{-3} i.u./ml) for various durations (A) or to various concentrations of TSH for 24 h (B). The cells were then treated with TNF- α (500 i.u./ml) for 10 min. The nuclear extracts were subjected to EMSA with oligonucleotide κ Bwt as a probe. Complexes A, B and C are indicated by arrowheads. Abbreviation: U, i.u.

were dose-dependently activated by TNF- α in 6H medium (Figure 1E).

To confirm this TSH effect, the duration and dose of TSH required for the activation of complexes B and C were evaluated, as shown in Figure 2. The concentration of TNF- α was fixed at

Figure 1 Effects of TSH on the activation of NF- κ B proteins by TNF- α in FRTL-5 cells

The cells were cultured in the absence (5H medium) or presence (6H medium) of TSH (10^{-3} i.u./ml) for 7 days. After incubation with TNF- α [500 i.u./ml in (A)–(D); 5–500 i.u./ml in (E)] for various durations in (A) and (B) or for 10 min in (C)–(E), the cells were harvested and the nuclear extracts were subjected to EMSA with oligonucleotide κ Bwt (A, B, C and E) or IL-6- κ B oligonucleotide (D) as probes. Three different complexes (complexes A, B and C) are indicated by arrowheads. Abbreviation: U, i.u.

Figure 3 Identification of NF- κ B subunits activated by TNF- α

FRTL-5 cells were cultured for 7 days in 5H medium (A) or 6H medium (B), then treated with TNF- α (500 i.u./ml) for 10 min. The nuclear extracts were subjected to EMSA with oligonucleotide κ Bwt as a probe. NF- κ B subunits were characterized by supershift analysis employing antibodies directed against p50, p52, p65, c-Rel and RelB, and also preimmune rabbit serum (pre). Complexes A, B and C are indicated by arrowheads. Several kinds of supershifted complex are indicated by single, double and triple asterisks.

500 i.u./ml. When the cells in 5H medium were treated with 10^{-3} i.u./ml TSH, a 6 h exposure of TSH resulted in the induction of these complexes (Figure 2A). In contrast, when the duration of TSH exposure was fixed at 24 h, 250×10^{-6} i.u./ml TSH was sufficient for the activation (Figure 2B). These results demonstrate that three distinct complexes binding to the NF- κ B site were activated by TNF- α in the presence or absence of TSH.

To characterize these complexes, the nuclear extracts prepared from FRTL-5 cells after a 10 min incubation with TNF- α were subjected to displacement and supershift analyses. All three complexes were completely displaced with a 50-fold molar excess of the unlabelled κ Bwt oligonucleotide, but not with the κ Bmu oligonucleotide, which harboured a mutated NF- κ B site (results not shown). As shown in Figure 3(A), supershift analyses revealed that complex A was supershifted by anti-p50 antibody, resulting in a dense band slightly shifted towards the anode from its original position (single asterisk) and a faint retarded band (double asterisk, lane 2). Note that no change in migration was

Figure 4 Activation of a p65-p50 heterodimer by TNF- α in the presence of forskolin and TSA

(A) FRTL-5 cells maintained in 5H medium for 7 days were treated with 10μ M forskolin for 6 h. (B) Immunoglobulin fractions purified from the sera of two patients with Graves's disease (Pt 1 and Pt 2) with high TSAb activities and from a normal healthy man were reconstituted with 5H medium. FRTL-5 cells maintained in 5H medium for 7 days were exposed to these media for 18 h. The cells were then treated with TNF- α (500 i.u./ml) for 10 min. The nuclear extracts were subjected to EMSA with oligonucleotide κ Bwt as a probe. Complexes A, B and C are indicated by arrowheads.

Figure 5 Effects of TSH on TNF- α -dependent expression of luciferase reporter gene in FRTL-5 cells

(A) pGL3-3 κ Bpro is a luciferase reporter plasmid driven by three canonical NF- κ B-binding sites (3 κ B). pGL3pro or pGL3-3 κ Bpro was transfected into the cells maintained in the presence or absence of TSH (10^{-3} i.u./ml) for 4 days. After incubation for 42 h, the cells were treated with TNF- α (500 i.u./ml) for 8 h. The luciferase activities, measured with a luminometer, were expressed as percentages of the luciferase activity without TNF- α and TSH. Results are means $\pm$ S.E.M. ($n = 3$). * $P < 0.01$ compared with the activity of pGL3-3 κ Bpro by TSH alone. Similar results were obtained from two separate experiments. (B) pp65 and pDnp65 are plasmids expressing wild-type p65 and a dominant-negative form of p65 respectively. pGL3-3 κ Bpro, alone or in combination with pp65 and pDnp65, was transfected into the cells cultured in the presence of TSH. After incubation for 42 h, the cells were treated with TNF- α (500 i.u./ml) for 8 h. The luciferase activities, measured with a luminometer, were expressed as percentages of the luciferase activity without TNF- α . Results are means $\pm$ S.E.M. ($n = 3$). * $P < 0.01$ compared with the activity of TNF- α alone. # $P < 0.01$ compared with the activity of pp65 alone. Similar results were obtained from a separate experiment.

observed with preimmune rabbit serum (Figure 3A, lane 1) or other antibodies (lanes 3–6). This indicates that complex A contained the p50 subunit of NF- κ B but not the other subunits.

In contrast, as shown in Figure 3(B), anti-p50 antibody shifted the mobility of both complexes B and C and produced a supershifted complex (double asterisk, lane 2), whereas anti-p65 antibody only supershifted complex C (triple asterisk, lane 4). These results indicate that complex C consisted of p65-p50 heterodimeric NF- κ B and that complex B contained the p50 subunit but not the other subunits. Similar results were obtained from the nuclear extracts prepared after 3 or 24 h of incubation with TNF- α (results not shown).

Taken together, these results demonstrate that TNF- α induced a rapid and sustained activation of p65-p50 heterodimeric NF- κ B as well as a complex containing p50 in FRTL-5 cells in the presence of TSH, whereas it activated another complex containing p50 in the absence of TSH.

TSAb and forskolin can mimic TSH action

To confirm the observed effects of TSH on the activation of different NF- κ Bs, we examined whether forskolin and TSAb could mimic the TSH action. As shown in Figure 4(A), treatment of FRTL-5 cells in 5H medium with 10μ M forskolin for 6 h induced the TNF- α -dependent activation of the p65-p50 heterodimer (complex C) and complex B. Similar results were obtained from the treatment with TSAb prepared from patients with Graves's disease. As shown in Figure 4(B), when the cells were cultured with immunoglobulins prepared from TSAb-negative serum of a normal healthy man, TNF- α induced only complex A. In contrast, when the cells were treated with TSAb-positive immunoglobulins, TNF- α induced the activation of both the p65-p50 heterodimer (complex C) and complex B.

Figure 6 Effect of TSH on the induction of IL-6 and p105 mRNA species by TNF- α

(A) FRTL-5 cells cultured in 5H or 6H medium for 7 days were treated with TNF- α (500 units/ml) for 3 h. (B) Cells cultured in 5H medium for 7 days or in 6H medium for 6–48 h were treated with TNF- α . Northern blot analysis was performed with IL-6, p105 and GAPDH cDNA species as probes. The sizes of the respective mRNA species (kb) are shown at the right. The experiments were performed on duplicate cell cultures. Similar results were obtained from two separate experiments.

It has been established that TSA β binds to the TSH receptor and stimulates adenylate cyclase activity, and that forskolin can activate the enzyme directly. Thus the stimulation of cAMP-protein kinase A pathway seems to be crucial for the activation of the p65–p50 heterodimeric NF- κ B by TNF- α in FRTL-5 cells.

Reporter gene activation by differentially induced NF- κ Bs

We next examined whether the differential induction of NF- κ Bs in the presence or absence of TSH alters the expression of the luciferase reporter gene driven by multimerized canonical NF- κ B sites (pGL3-3 κ Bpro). As shown in Figure 5(A), when the pGL3pro plasmid lacking NF- κ B sites was transfected into FRTL-5 cells, TNF- α and TSH, alone or in combination, had no effect on luciferase activity. In contrast, when the pGL3-3 κ Bpro plasmid was transfected, TNF- α markedly increased luciferase activity in the presence of TSH but not in its absence, suggesting that the induction of the p65–p50 heterodimer led to the activation of reporter gene expression. To confirm the involvement of p65 in the activation, we co-transfected a plasmid expressing the C-terminal deletion mutant of p65 (pDNp65) into FRTL-5 cells cultured in 6H medium (Figure 5B). This mutant lacked a transactivation domain but preserved a dimerization domain. It could therefore inhibit the function of wild-type p65. Co-transfection with pDNp65 resulted in a marked suppression of the luciferase activity that was increased by TNF- α . In addition, it attenuated the activity induced by transfection with pp65 expressing wild-type p65, indicating that DNp65 inhibited transactivation by wild-type p65.

Activation of endogenous NF- κ B-responsive genes by TNF- α in the presence of TSH

The expression of endogenous NF- κ B-responsive genes, IL-6 and p105, a precursor of p50, was also studied in the absence (5H medium) or presence (6H medium) of TSH. As shown in Figure 6(A), TNF- α did not increase the IL-6 mRNA level in 5H medium. Treatment with TSH alone slightly increased the level, whereas TNF- α markedly increased the mRNA levels in 6H medium. Two IL-6 mRNA species with sizes of 1.2 and 2.4 kb were comparable with a previous report by Northemann et al. [33].

In contrast with IL-6, TNF- α increased the p105 mRNA level even in the absence of TSH (5H medium). TSH alone did not affect the mRNA level. However, TNF- α , in the presence of TSH (6H medium), increased the mRNA level to a much greater extent than that in 5H medium. GAPDH mRNA levels were not altered by any treatment.

The duration of TSH treatment required for IL-6 expression is shown in Figure 6(B). After a 6 h incubation with TSH, an increase in IL-6 mRNA by TNF- α was detected. This observation was correlated with the induction of the p65–p50 heterodimer after a 6 h exposure to TSH, as shown in Figure 2(A).

DISCUSSION

The present study demonstrated that both the p65–p50 heterodimer and a complex containing the p50 subunit are activated by TNF- α in rat thyroid FRTL-5 cells cultured in the presence of TSH (6H medium), whereas only a complex containing the p50 subunit is activated in the absence of TSH (5H medium). Interestingly, the migration of this complex in 5H medium is different from that of the complex containing p50 in 6H medium. It might be reasonable to postulate that both complexes are composed of p50 homodimers, because they bind specifically to two different NF- κ B sites and are completely supershifted by anti-p50 antibody on EMSA but not by other antibodies directed against other NF- κ B subunits. If this is so, the difference in migration of both complexes might be due to the post-translational modifications of the p50 subunit. Because the p50 subunit was shown to be a phosphoprotein [38], the extent of phosphorylation status might affect the migration of the complexes. To test this possibility, we examined the effect of alkaline phosphatase on the binding and migration of the complexes. Our results showed that treatment of the nuclear extracts with alkaline phosphatase did not alter the migration and binding of these complexes (results not shown).

Another mechanism could be heterodimer formation of the p50 subunit with transcription factors other than NF- κ B. It has been reported that p50 can be complexed with Fos/Jun family protein in FRTL-5 cells [39]. We therefore tested this possibility with anti-Fos and anti-Jun antibodies, which can react with the known Fos and Jun family members respectively. However, these antibodies did not alter the mobilities of complexes containing p50 (results not shown), indicating that neither complex contains Fos or Jun proteins.

Although the basis of the different migrations of the complexes containing p50 is unclear, it is of particular interest that three distinct NF- κ Bs are induced by TNF- α in the presence or absence of TSH. We therefore examined whether TSH modifies the TNF- α -dependent expression of NF- κ B target genes. Our results clearly demonstrated that the maximal activation of the reporter gene and the endogenous NF- κ B-responsive genes, those encoding IL-6 and p105, was associated with the induction of the p65–p50 heterodimer. In addition, the expression of a

dominant-negative form of p65 decreased the TNF- α -dependent expression of the reporter gene. These results indicate that the heterodimer is important in TNF- α -dependent gene expression. This finding is compatible with previous reports that dimeric forms of NF- κ B containing the p65 subunit function as potent activators of gene expression [23,40].

In contrast, activation of the p50 homodimer alone resulted in a slight but significant increase in expression of the gene encoding p105. In the presence of TSH, TNF- α further increased its expression. Similar observations were reported by Cogswell et al. [41]. They showed that the p65–p50 heterodimer is a major activator for the human p105 gene promoter, whereas the p50 homodimer can also transactivate the promoter.

It was reported that a single set of NF- κ B was rapidly and transiently (within 2 h) activated by TNF- α in human papillary thyroid carcinoma cells, although the composition of NF- κ B subunits was not characterized [42]. In contrast, the present study revealed the rapid (within 10 min) and persistent (lasting for 24 h) activation of NF- κ B proteins in FRTL-5 cells. The rapid induction of NF- κ B might be due to the nuclear translocation of NF- κ B pre-existing in the cytoplasm. The persistent activation of NF- κ B might be caused by an increased synthesis of NF- κ B subunits [43,44] and/or a sustained degradation of I κ B [45].

The present study also demonstrated that the p65–p50 heterodimer was activated by TNF- α in the cells cultured with TSA or forskolin. These as well as TSH are known to stimulate the activity of adenylate cyclase and the production of intracellular cAMP and thereby to activate cAMP-dependent protein kinase (PKA) in thyroid follicular cells. It has been shown that the DNA-binding activity and transactivation property of p65 are positively regulated by PKA [46,47]. Because our preliminary study by Western blot analysis suggested that treatment with TSA did not alter the nuclear contents of p65 protein in FRTL-5 cells (results not shown), the activation of the p65–p50 heterodimer in the presence of TSH, TSA or forskolin could be ascribed to post-translational modification of p65 by PKA.

Rat IL-6 promoter contains functional *cis*-elements for cAMP-responsive element binding protein ('CREB') and NF- κ B [48]. In a human fibroblast cell line, an increase in intracellular cAMP production and PKA activity has been shown to activate the expression of the gene for IL-6 [49], whereas in HeLa cells NF- κ B activation is responsible for the increase in expression of the gene encoding IL-6 [50]. It is therefore likely that the increase in IL-6 mRNA levels by TSH in FRTL-5 cells might be due to the increase in intracellular cAMP production, whereas the further increase in the mRNA caused by TNF- α in the presence of TSH could be attributable to the induction of the p65–p50 heterodimer. It has also been reported that IL-1 β and TSH synergistically increase IL-6 production in FRTL-5 cells [51]. Because IL-1 β also induces the activation of NF- κ B [24], the synergistic effect of TSH and TNF- α could be explained by the induction of the p65–p50 heterodimer in the presence of TSH.

In conclusion, the present study has demonstrated that TSH and TSA modulate the TNF- α -dependent production of IL-6 in thyroid follicular cells. Because IL-6 is a multifunctional cytokine with key roles in the promotion of B-cell proliferation and differentiation and in the activation of T-cells [21], it is possible that TSH and TSA might modify the intrathyroidal immune responses in patients with ATD.

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