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Bacterial Lipopolysaccharide Stimulates the Thyrotropin-Dependent Thyroglobulin Gene Expression at the Transcriptional Level by Involving the Transcription Factors Thyroid Transcription Factor-1 and Paired Box Domain Transcription Factor 8

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The bacterial lipopolysaccharide (LPS) is a biological activator that induces expression of multiple genes in several cell types. LPS has been proposed as an etiopathogenic agent in autoimmune diseases. However, whether LPS affects the expression of autoantigens has not been explored. Thyroglobulin (TG) is a key protein in thyroid hormonogenesis and one of the major thyroid autoantigens. This study aimed to analyze the action of LPS on TG gene expression in Fisher rat thyroid cell line FRTL-5 thyroid cells. We demonstrate that LPS increases the TSH-induced TG protein and mRNA level. Evidence that the effect of LPS is exerted at the transcriptional level was obtained by transfecting the minimal TG promoter. The C element of the TG promoter, which contains sequences for paired box domain transcription factor 8 (Pax8) and thyroid transcription factor (TTF)-1 binding, is essential for full TG promoter expression under TSH stimulation. The transcriptional activity of a construct containing five tandem repeats of the C site is increased by LPS, indicating a possible involvement of the C site in the LPS-induced TG gene transcription. We demonstrate that the TG promoter mutated at the Pax8 or TTF-1 binding element in the C site does not respond to LPS. In band shift assays, binding of Pax8 and TTF-1 to the C site is increased by LPS. The Pax8 and TTF-1 mRNA and protein levels are augmented by LPS. The half-lives of TG, Pax8, and TTF-1 are increased in endotoxin-treated cells. Our results reveal the ability of LPS to stimulate the expression of TG, a finding of potential pathophysiological implication. (*Endocrinology* 147: 3260–3275, 2006)

THE ENDOTOXIN LIPOPOLYSACCHARIDE (LPS) is a major constituent of the outer membrane of Gram-negative bacteria. LPS is not intrinsically toxic but is one of the most potent biological response modifiers; picomolar concentrations are sufficient to stimulate cells from several systems. The endotoxin mainly induces immune cells to express a multiplicity of genes. The immunostimulation exerted by LPS principally comprises a marked activation of B cells with polyclonal antibody production as well as an induction of the expression of various kinds of mediators involved in cellular immunity (1–5). In conditions in which large amounts of LPS enter the bloodstream because of bacteriolysis, a systemic inflammatory overreaction occurs leading to septic shock (1, 3–5). Several studies have demonstrated that LPS plays a role as an environmental factor in some diseases in which autoantibodies or autoantigen-specific T cells are involved (2, 6). The property of LPS to facilitate autoimmunity has been applied to develop several experimental models of autoimmunity (2, 6, 7).

Thyroglobulin (TG) is a key protein for normal thyroid function. It is the matrix protein where thyroid hormone synthesis takes place, as well as one of the main thyroid autoantigens. Thyroid hormonogenesis involves the iodide uptake by the sodium iodide symporter and the iodination of TG catalyzed by thyroid peroxidase in the presence of hydrogen peroxide with later coupling of TG iodotyrosine residues (8). Thyroid-specific gene expression is predominantly under regulation of TSH and principally involves three transcription factors that have a central role, thyroid transcription factor (TTF)-1 (also named Titf1, NKX 2.1, or T/EBP), TTF-2 (also named FOXE1), and paired box domain transcription factor 8 (Pax8) (9, 10). These transcription factors are present in several tissues (11–14), although they are expressed together only in the developing and adult thyroid in a unique combination responsible for the early commitment, differentiation, and maintenance of the differentiated thyroid state (9, 10). The minimal rat TG promoter able to respond to TSH comprises a 207-bp fragment extending from −168 to +39 relative to the transcription initiation site of the gene. The elements necessary for cell type-specific transcription are contained in a smaller fragment extending from

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Abbreviations: CAT, Chloramphenicol acetyltransferase; CHX, cycloheximide; CMV, cytomegalovirus; DTT, dithiothreitol; FRTL, Fisher rat thyroid cell line; gal, galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; Luc, luciferase; Pax8, paired box domain transcription factor 8; PMSF, phenylmethylsulfonylfluoride; TG, thyroglobulin; TTF, thyroid transcription factor.

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−168 to −42. In this region, there are three binding sites for TTF-1, one site for TTF-2, and one for Pax8 that overlaps with the most 3′ sequence recognized by TTF-1 in the element known as the C site (15–17).

Infectious agents have been implicated in the induction of thyroid autoimmune disease (18, 19). Experimental autoimmune thyroiditis has been extensively induced by TG plus adjuvants including LPS in mice. However, it was observed that the disease developed by a different mechanism when LPS was used as the adjuvant (7, 20). This finding was partially explained by the direct induction of chemokine synthesis by LPS in thyroid cells (7). In a growing number of reports, an enhanced expression of proteins that act as self-antigens has been associated with the etiology and pathology of several autoimmune diseases (21–23). The possibility that LPS exerts a direct action on the expression of autoantigenic proteins has, however, not been explored.

This work aimed to study the influence of LPS on TG gene expression in Fisher rat thyroid cell line (FRTL)-5 thyroid cells and to examine the underlying mechanisms. Because LPS affects diverse cell types, FRTL-5 follicular thyroid cells are particularly useful to examine the direct effect of LPS on thyrocytes. Here, we demonstrate a novel action of LPS to increase TSH-induced TG gene expression at the transcriptional level in FRTL-5 thyroid cells. We provide evidence that the C site in the TG promoter is involved in the stimulatory action of LPS, possibly through an enhanced TTF-1- and Pax8-mediated transactivation. LPS induces an increment in the binding of the transcription factors TTF-1 and Pax8 to the C site of the TG promoter, as well as an increase in TTF-1 and Pax8 mRNA and protein expression. The half-lives of TG, Pax8, and TTF-1 are prolonged in LPS-treated cells. Taken together, these results indicate that LPS is able to increase the TG expression in the presence of TSH, a finding that could have implications for thyroid pathophysiology.

Materials and Methods

Cell culture

FRTL-5 rat thyroid cells (ATCC CRL 8305, American Type Culture Collection, Manassas, VA) were kindly provided by Dr. L. Kohn (Edison Biotech Institute, Ohio University, Athens, OH) and had the properties previously reported (24). Cells were grown in a 5% CO_2-95% air atmosphere in Coon's modified Ham F-12 medium supplemented with 5% calf serum (DMEM/Ham F-12, 1:1) (PAA Laboratories GmbH, Linz, Austria), 1 mIU/ml bovine TSH [a generous gift of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDKK) National Pituitary Hormone Program and Dr. A. F. Parlow, National Institutes of Health, Torrance, CA], 10 μg/ml bovine insulin, 5 μg/ml bovine transferrin, 2 μmol/ml glutamine, antibiotics, and antimycotics (Sigma, St. Louis, MO). Cells were passaged every 7–8 d and fed fresh medium every 2–3 d. When they reached 70% of confluence, cells were shifted to basal medium without TSH containing 0.2% calf serum and maintained for 5–7 d until the time of the experiments. Starved (basal) cells were treated with the indicated concentrations of LPS from *Escherichia coli* serotype 055:B5 (Sigma) in the presence of 0.5 mIU/ml TSH for different periods of time. All cultured cells were used before passage 20.

Immunocytochemistry

Cells were cultured in glass coverslips and air dried after the indicated treatments. Subsequently, coverslips were incubated overnight with an antihuman TG antibody (Sigma) in a solution containing Tris-buffered saline [50 mM Tris-HCl, 0.88% NaCl (pH 7.6)] and 0.5% BSA in PBS (1:1), 2 h with a biotinylated anti-IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ), followed by 2 h with an extrAvidin-Cy3 conjugate (Sigma). The slides were then counterstained with 1 μg/ml Hoechst solution (25). As a negative control, anti-TG antibody was omitted in a group of coverslips. The positivity of the reaction was observed as a red fluorescence by fluorescence microscopy (Eclipse E600, Nikon, Shinagawa-ku, Japan). The fluorescent reaction was also analyzed under a confocal laser scanning microscope (LSM 510, Carl Zeiss, Oberkochen, Germany), fitted with an inverted Axiovert 100M microscope (Carl Zeiss) and a 40× C-Apochromat objective (Carl Zeiss). Excitation on the laser scanning confocal microscope was at 543 nm with a 1-mW helium/neon laser. Optical sections were collected using a 560-nm-long pass filter emission to collect the fluorescence of Cy3. The fluorescence staining intensity was analyzed by internal LSM 510 and by MetaMorph softwares (Universal Imaging Corp., Downingtown, PA).

Whole and nuclear protein extract preparation

For total protein extract preparation, cells were harvested in PBS, centrifuged, resuspended in whole-cell lysate buffer [50 mM HEPES (pH 7), 2 mM $MgCl_2$, 250 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet 40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin] and incubated on ice for 15 min. The lysate was centrifuged, and the supernatant was collected (26). For nuclear protein extract preparation, cells were collected in buffer A [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF], incubated on ice for 10 min, and centrifuged. The nuclear pellet was resuspended in buffer C [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 min. Cellular debris was removed by centrifugation, and the supernatant was collected (27). Protein content was quantified by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA) using BSA as standard (28).

Western blot analysis

Proteins (25 μg) were resolved by 6 or 10% SDS-PAGE for TG or TTF-1, Pax8, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection, respectively. Proteins were electrotransferred to nitrocellulose membranes (Sigma). Ponceau S staining of the blots was used to control for equal protein loading. Membranes were blocked and incubated with antihuman TG (Dako, A/S, Golstrup, Denmark), TTF-1 (H-190), or Pax8 (F-19) antibodies cross-reacting with rat TG, TTF-1, or Pax8, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 0.1% Tween 20-Tris-buffered saline-5% skimmed milk at room temperature. Membranes were washed and then incubated with an anti-IgG-horseradish peroxidase conjugated antibody (Santa Cruz Biotechnology, Inc.). Immunoreactive bands were visualized with a luminol Western blot detection reagent (NEN Life Sciences Products, Boston, MA) as described by the manufacturer. The intensities of the bands were determined by scanning densitometry (Scion Image software, Scion Corporation, National Institutes of Health). Densitometric values were normalized in relation to the Ponceau S staining in each lane.

Protein half-life analysis

Cells were preincubated with TSH or TSH plus LPS for 24 h. Then, cells were treated with 10 μg/ml cycloheximide (CHX) for the indicated periods of time and harvested for whole-protein extract preparation and Western blot analysis as described above. Densitometric measurements at each time length were plotted and half-lives estimated from the best fitted equation in each case.

RNA isolation, Northern blot analysis, and RT-PCR

Total RNA was purified from FRTL-5 cells after treatment by the acid guanidinium thiocyanate/phenol/chloroform extraction procedure of Chomczynski and Sacchi (29). For Northern blot analysis, total RNA (20 μg) was subjected to electrophoresis on 1% agarose gels containing 0.66 M formaldehyde (Sigma). RNA was transferred to nylon membranes (Sigma) by capillary transblotting overnight. Membranes were baked for 2 h at 80 C and stained with methylene blue to reveal the integrity of

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RNA and sample loading. Hybridization was performed overnight at 42 C in a solution containing 50% formamide and α^{32}P-ATP probes labeled by the random primer method (30). Rat TG (31), TTF-1 (32), and Pax8 (33) cDNAs were used as probes. RT-PCR was done to estimate the expression of β-actin and GAPDH mRNAs. cDNA was synthesized from total RNA (1 μg), and PCR was performed in a 20-μl reaction volume containing 2 μl cDNA, 0.5 μM primers, 0.25 mM dNTPs, 1.5 mM $MgCl_2$, and 0.8 U Taq polymerase. For β-actin amplification, the mixed samples were heated to 95 C for 3 min, and then 21 cycles of amplification were performed at 95 C for 30 sec, 59 C for 30 sec, and 72 C for 30 sec followed by a 7-min extension at 72 C. For GAPDH amplification, samples heated to 94 C for 5 min were subjected to 18 cycles performed at 94 C for 1 min, 63 C for 1 min, and 72 C for 1 min followed by a 10-min extension at 72 C. The primer sequences for β-actin (560 bp) were: 5′ CGACGAGGCCCAGAGCAAGAGAGG (sense) and 5′CGTCAGGCAGCTCATAGCTCTTCTCCAGGG (antisense) and for GAPDH (513 bp) were: 5′ GAGTATGTCGTGGAGTCTACTG (sense) and 5′ GCTTCACCACCTTCTTGATGTC (antisense). PCR products were separated on 2% agarose gels and visualized with ethidium bromide. The intensities of the bands from Northern blot or RT-PCR assays were determined by scanning densitometry (Scion Image software, Scion Corp., National Institutes of Health).

Promoter constructs

The pTG luciferase (Luc) repoter plasmid contained the minimal wild-type promoter of the rat TG gene (−168 to +36 bp), linked to the Luc gene, and pTG Luc-PMT, the minimal TG promoter mutated in the Pax8 binding element, both in the pGL2-Basic plasmid (34). The 5C-CAT construct contained five tandem repeats of the TTF-1/Pax8 binding site (C site) from the rat TG promoter linked to the chloramphenicol acetyltransferase (CAT) gene (35). The pTACAT-3, referred to here as pTG CAT, corresponded to the minimal wild-type promoter of the rat TG gene (−168 to +39 bp) linked to the CAT gene, and the pTACAT-14, referred to here as pTG CAT-TMT, contained the minimal TG promoter mutated in the TTF-1 binding site (15). As negative controls, the promotorless pGL3-Basic (Promega, Madison, WI) and a TATA-box/CAT-containing plasmid were used. The cytomegalovirus (CMV) promoter-Luc and CMV-β galactosidase (gal) (CMV linked to β gal gene) plasmids (Promega) were used to monitor transfection efficiency (36, 37).

Transfection assays

FRTL-5 cells were plated at a density of 6×10^5 per 60-mm-diameter tissue culture dish 48 h before transfection. The calcium phosphate DNA coprecipitation method was used for transfections as previously described (36, 38). Cells were incubated with the precipitate containing 4-μg constructs of interest and 0.8 μg CMV-Luc or CMV-β-gal. Transfected cells were cultured in basal medium for 5 d and then treated with LPS. After the indicated time, cells were harvested in Tris-EDTA-NaCl solution [40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl], centrifuged, and resuspended in 100 mM KH_2PO_4 (pH 7.8) containing 1 mM DTT. Lysates were centrifuged, and the supernatants were assayed for Luc, CAT, or β-gal activities as described (37, 39, 40).

EMSA

EMSAs were performed as previously described (16). Synthesized double-stranded oligonucleotides C (5′-CACTGCCCAGTCAAGTGTTCTTGA-3′), derived from the specific TTF-1/Pax8 binding C site of the TG promoter, and two C mutants, Cp (5′-TCAGTCACGCGTGACTGGGCAGTG-3′), which does not bind TTF-1, and Ct (5′-ACGATGAGTGGCTCATAAATCG-3′), which does not bind Pax8 (41), were labeled with α^{32}P-ATP using the Klenow fragment of DNA polymerase and purified using Sephadex G25 columns (Sigma). Nuclear extracts (5 μg) were incubated in a 20-μl reaction volume on ice in binding mix buffer containing 40 mM HEPES (pH 7.9), 20 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 1 μg poly (deoxyinosine-deoxycytosine), and 5% Ficoll. Labeled oligonucleotide probe (50,000 cpm; approximately 50 pg DNA) was added, and incubation continued at room temperature. The resulting DNA-protein complexes were separated from free DNA on a 5% native polyacrylamide gel in 0.5× Tris borate-EDTA buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), then vacuum dried and exposed to x-ray film. Autoradiograms whose signal intensity was not in an oversaturated range were analyzed by scanning densitometry. For competition assays, a 100-fold excess of cold oligonucleotides C (oligo C), Cp (oligo Cp), or Ct (oligo Ct) was added to the reaction mixture before adding the labeled oligonucleotide. In supershift experiments using anti-TTF-1 or anti-Pax8 antibodies (2 μg), nuclear extracts were incubated with the antibodies 1 h on ice before adding the labeled probe and then processed as above.

Statistical analysis

Analysis of multiple intergroup differences was conducted by one-way ANOVA. As posttest, the Student-Newman-Keuls multiple comparisons test was used. Comparisons between two groups were made using the Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

LPS increases the TSH-induced TG protein and mRNA expression

To investigate LPS action on TG expression, basal FRTL-5 cells were treated with TSH in combination with different concentrations of LPS. TG levels were analyzed by immunofluorescence with confocal microscopy and by Western blot assays. In laser confocal microscopy pictures, we could detect TG in the cytoplasm of thyroid cells. Treatment with LPS did not modify the distribution of TG or the morphology of thyrocytes (Fig. 1A, a_1–d_1). The accumulation of TG in the cytoplasm of thyroid follicular cells was increased after TSH stimulation (48 h) (Fig. 1A, b_1). A greater accumulation of TG was observed in the presence of TSH plus LPS (Fig. 1A, c_1–d_1). Semiquantitative analyses using internal laser scanning microscope (Fig. 1A, a_2–d_2) and Methamorph softwares (Fig. 1B) confirmed that LPS induced an increment of TG expression. The maximal stimulatory effect (1.8-fold increase) was obtained with 0.1 μg/ml LPS (Fig. 1B). Treatment with higher LPS concentrations induced a lower increase (1 μg/ml LPS; 1.4-fold increase) or did not change (10 μg/ml LPS) TG levels compared with that induced by TSH (data not shown). Incubation with LPS alone (0.1–10 μg/ml) did not modify TG immunoreactivity in comparison with that of basal cells (data not shown). The LPS-induced increase of TG level was confirmed by Western blot (Fig. 2). TSH augmented TG expression at all analyzed time points with a maximal effect at 48 h. The presence of LPS stimulated TSH-induced TG level (24 and 48 h) with a maximal increase at 48 h. After 72 h of LPS incubation, there was no change in TG expression compared with the TSH-induced level (Fig. 2). Northern blot assays revealed that the treatment with 0.1 μg/ml LPS rapidly increased TSH-stimulated TG mRNA expression (3 and 6 h)m with a maximal effect at 3 h of treatment (Fig. 3). Then, LPS-induced TG mRNA progressively diminished and compared with the TSH-induced level a significant reduction was detected at 24 and 48 h of incubation, whereas no change was observed at 72 h of treatment (Fig. 3). In cells treated with TSH alone, TG mRNA augmented at all analyzed time points, with a maximal increment at 48 h (Fig. 3). Treatment with LPS alone did not modify TG protein (Fig. 2) or mRNA expression (data not shown) compared with basal values, indicating that the effect of LPS was dependent on the presence of TSH.

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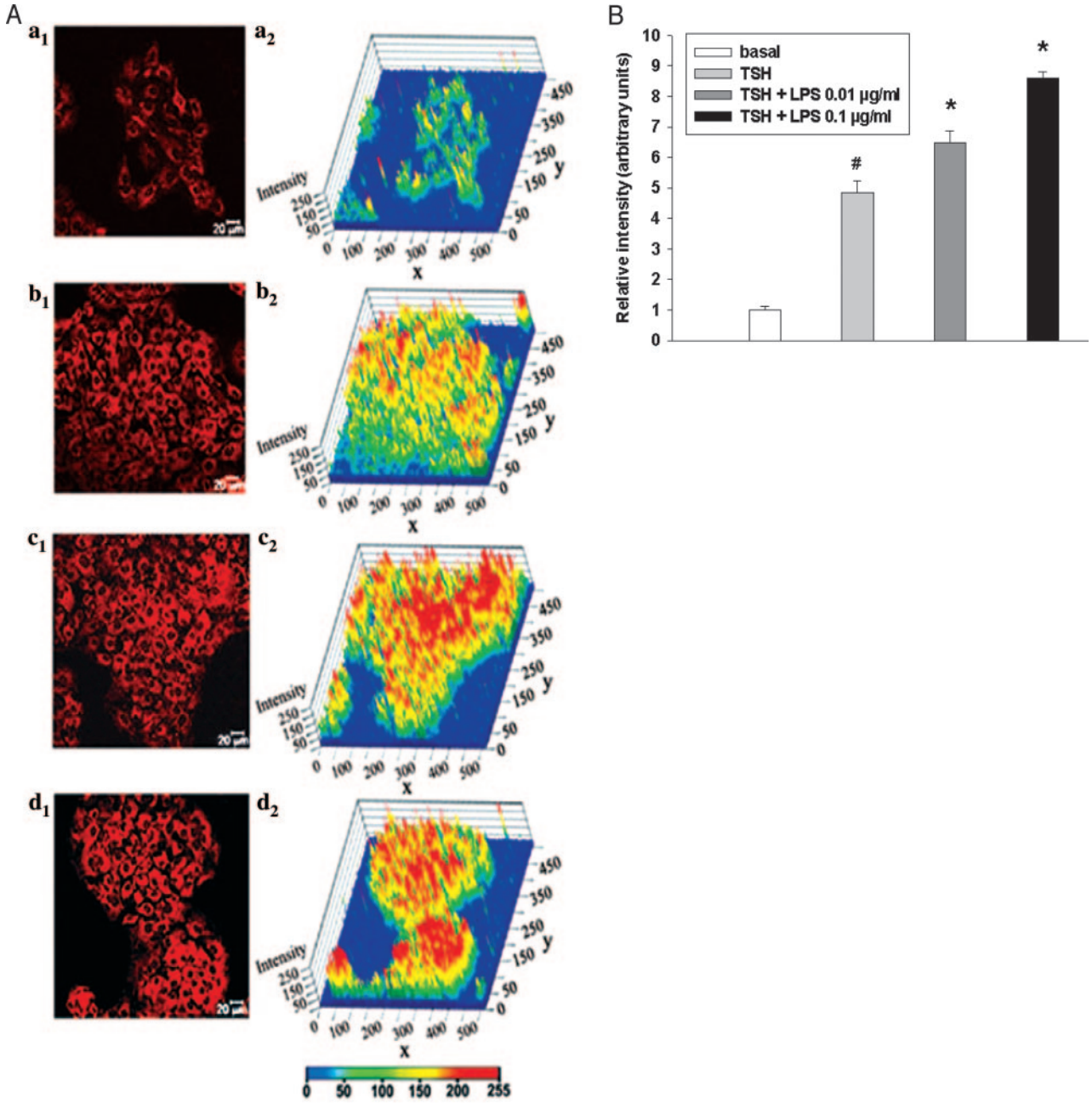

FIG. 1. LPS increases the TSH-stimulated TG accumulation in the thyroid cell. A, TG expression by immunofluorescence method in basal (a_1), TSH alone-treated (b_1), TSH + 0.01 μg/ml LPS-treated (c_1), and TSH + 0.1 μg/ml LPS-treated (d_1) cells incubated for 48 h, using confocal laser scanning microscopy (*bar*, 20 μm). Cy3 brightness intensity scale 0–250 was quantified by LSM 510 laser scanning microscope image system software (Carl Zeiss) (a_2–d_2). B, Relative intensity of Cy3 brightness quantified by MetaMorph softwares. Each value represents the mean ± SEM of at least three independent experiments.*, $P < 0.001$ *vs.* TSH alone; #, $P < 0.001$ *vs.* basal (taken as 1.00); Student-Newman-Keuls multiple comparisons test.

LPS stimulates TG promoter activity

To study the molecular mechanism by which LPS stimulates TG protein and mRNA expression, we analyzed the functional activity of the TG promoter. Transient transfection assays with a construct containing the hormone-responsive region from −168 to +36 bp of the minimal rat TG promoter linked to the Luc gene (pTG Luc) were performed. This promoter region has three TTF-1, one TTF-2, and one Pax8 binding elements (Fig. 4A) and is sufficient to promote transcription in response to TSH (15–17). We observed that TSH increased TG promoter activity with a maximal effect at 48 h (Fig. 4B). LPS (0.01 and 0.1 μg/ml) stimulated the TSH-mediated activation of the TG promoter in a dose-dependent manner after 18–24 h of treatment (Fig. 4B). The functional activation of the TG promoter by LPS was in accordance with the LPS-induced increase of TG protein and mRNA levels (Figs. 1–3). When LPS was added for 48 h, a significant repression of the TSH-stimulated promoter activity was ob-

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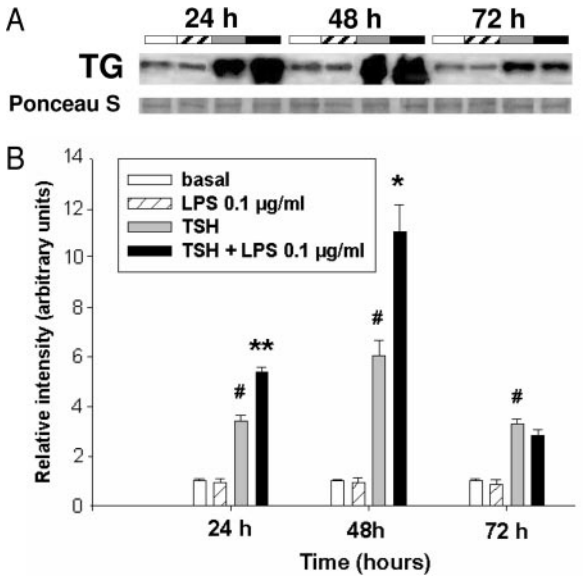

FIG. 2. LPS-induced increase in the TSH-stimulated TG accumulation varies with the time of incubation. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for different times (24–72 h). Then, cells then were harvested for whole-protein extract preparation. A, Representative Western blot of whole-cell extract (25 μg) probed with antihuman TG antibody. B, Densitometric analysis of the Western blots. Each value represents the mean ± SEM of at least three independent experiments. *, $P < 0.01$; **, $P < 0.001$ *vs.* TSH alone; #, $P < 0.01$ *vs.* basal (taken as 1.00); Student-Newman-Keuls multiple comparisons test.

served (Fig. 4B), a finding consistent with the reduction of TG protein and mRNA levels observed after longer times of endotoxin treatment (Figs. 2 and 3). No change in the TG promoter activity compared with that in basal cells was found in cells treated only with LPS (Fig. 4B).

The C site is involved in the LPS-induced stimulation of the TG promoter

Given the transcriptional regulation of TG gene expression by LPS, the involvement of the C site, one of the main elements for the binding of thyroid-specific transcription factors in the TG promoter, was examined. It is known that the C region is crucial for the full expression of the minimal TG promoter after TSH stimulation (15, 34). The activity of a construct containing five tandem repeats of the C site linked to a TATA box (5C-CAT) was analyzed in transfection assays (Fig. 5A). A concentration-dependent increase in the TSH-induced activity of 5C-CAT was observed at 24 and 48 h of LPS treatment (Fig. 5B). In the presence of LPS alone, the 5C-CAT activity was not modified in comparison with that obtained in basal conditions (Fig. 5B). These results indicate that the C element seems to be essentially involved in the LPS-induced stimulation of the TG promoter.

The Pax8 and TTF-1 binding elements are necessary for the LPS and TSH responsiveness of the TG promoter activity

Because the C site appears to participate in the LPS action on the TG promoter and because Pax8 and TTF-1 are known to be key regulators of TG gene expression through the C binding (41, 42), we investigated the functional relevance of the Pax8 and TTF-1 binding sites in the effect of LPS. The transcriptional activity of a construct with the minimal TG promoter mutated in the Pax8 binding element of the C site (pTG Luc-PMT) (34) was analyzed. Because TTF-1 and Pax8 binding sequences overlap at the C site in the TG promoter (43) and to avoid a potential influence of the mutation on TTF-1 binding, the construct had the core sequence for TTF-1 binding and four surrounding residues preserved (Fig 6A, *upper*). Under these conditions, binding of TTF-1, but not of Pax8, is possible (34). Confirmation of the specific binding of this construct by TTF-1 was done by supershift assays; only TTF-1 from FRTL-5 nuclear proteins binds a 24-bp oligonucleotide derived from the C site of TG promoter containing the mutation at the Pax8 binding site (oligo C-PMT, −79 to −55) (Fig. 6A, *lower*). In contrast to the observation with the wild-type TG minimal promoter, the activity of pTG Luc-PMT was not altered by LPS. As expected, activity of this construct was not stimulated by TSH (Fig. 6B). Although the activity of the mutated promoter in the absence of TSH and LPS was markedly lower than that of the wild-type, it was higher compared with the promotorless pGL3-Basic, indicating some TSH and Pax8-independent transcriptional activity that was not modified by LPS (Fig. 6B). To study the functional role of the TTF-1 binding site in mediating LPS action, we tested the activity of a construct containing the minimal TG promoter mutated in the core sequence for TTF-1 binding at the C element (pTG CAT-TMT) (Fig 6A, *upper*). Band shift assays with an oligonucleotide derived from the C site containing the mutation in the TTF-1 binding element (oligo C-TMT) confirmed that this construct allowed only the binding of Pax8 (Fig 6A, *lower*). Similar to the results observed with the response element mutated at the Pax8 binding site, pTG CAT-TMT did not respond to LPS or TSH stimulation (Fig. 6C). In the presence of LPS alone, no change in the activity of the two constructs, pTG Luc-PMT or pTG CAT-TMT, was observed when compared with the activity under basal conditions (Fig. 6, B and C). These results indicate that Pax8 and TTF-1 seem to be crucial mediators of the LPS-induced increase in TG promoter activity.

LPS increases the binding activity of Pax8 and TTF-1 to the C site in the TG promoter

EMSAs were performed to look for either qualitative or quantitative LPS-induced changes on the protein binding activity to the C site in the TG promoter. Nuclear extracts from cells cultured in the presence of LPS were tested for the ability to bind a 24-bp oligonucleotide (oligo C) derived from the C region (−82 to −58) of the TG promoter (see *Materials and Methods*) (16). We observed a pattern of protein-DNA complexes mainly composed of two bands that was similar with nuclear extracts collected under all different treatments conditions (Fig. 7A). Competition assays with cold oligo C showed that the two mobility shift bands represented specific binding to the C site sequence after TSH or TSH plus LPS treatment (Fig. 7C, lanes 4 and 8). However, only the upper band was stimulated by TSH in a time-dependent manner (Fig. 7A, lanes 3, 6, and 9), whereas the lower band presented a relatively stable intensity in all conditions, including the basal condition (Fig. 7A). It was apparent that LPS induced

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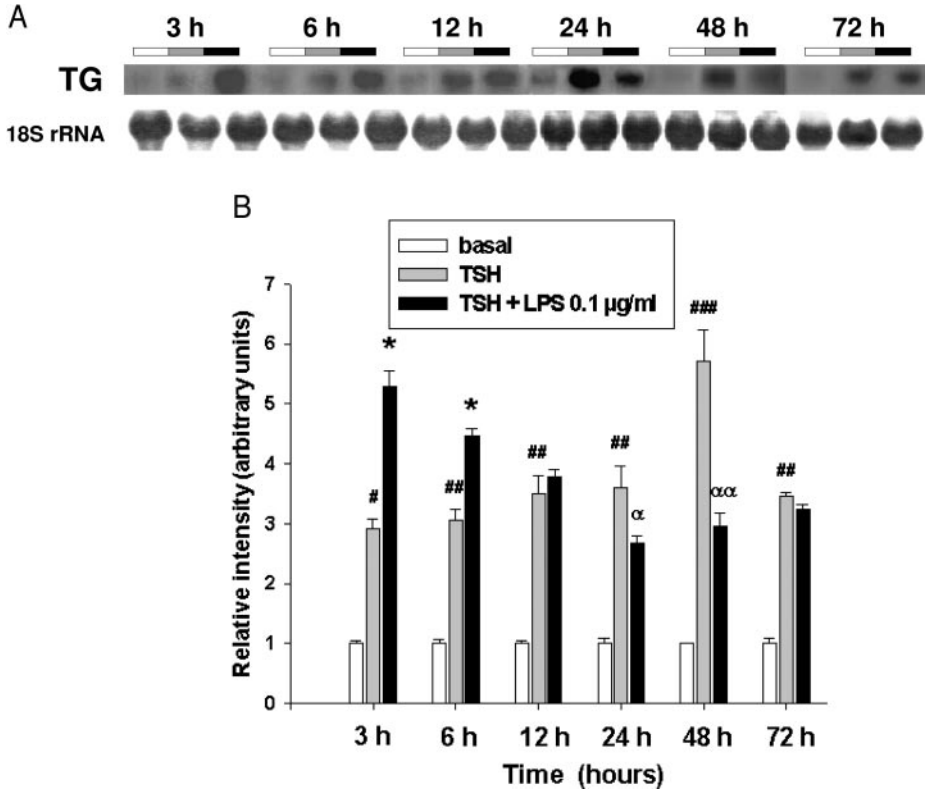

FIG. 3. LPS increases the TSH-induced mRNA TG level. Basal cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for different times (3–48 h) and then harvested for total RNA extraction. A, Representative Northern blot (20 μg) hybridized with a rat TG probe. B, Densitometric analysis of the Northern blots. Values were normalized with 18S rRNA. Each value represents the mean ± SEM of at least three independent experiments. *, $P < 0.01$ *vs.* TSH alone (increase); α, $P < 0.05$; $\alpha\alpha$, $P < 0.001$ *vs.* TSH alone (decrease); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

an increase in the intensity of the TSH-stimulated specific upper band at all analyzed time points (Fig. 7A, lanes 4, 7, and 10 *vs.* lanes 3, 6, and 9, respectively) and that it was statistically significant (Fig. 7B) with the highest stimulation compared with TSH alone at 3 h of treatment (Fig. 7, A and B). The intensity of the bands was not modified by LPS alone when compared with the one obtained under basal conditions (data not shown). Considering the TSH-dependent ability of TTF-1 and Pax8 to bind to the C site in the TG promoter demonstrated by some authors (33, 41), we suspected that the specific upper band obtained could represent complexes formed of TTF-1 and Pax8 with the C site. This assumption is in agreement with previous observations indicating that under TSH stimulation, both transcription factors form complexes binding to the C site with similar electrophoretic mobility that are detected as a single band by binding assays (33). Supershift assays with specific antibodies confirmed that the specific band certainly corresponded to a mixture of complexes of TTF-1 and Pax8 with the C site (Fig. 7C, lanes 2, 3, 6, and 7). The addition of anti-TTF-1 antibody resulted in a new retarded complex (Fig. 7C, lanes 2 and 6). Using an anti-Pax8 antibody in the binding reaction resulted in a reduction in the formation of the complex, but there was no retarded band (Fig. 7C, lanes 3 and 7) because the antibody used in these experiments recognizes the Pax8 N-terminal region implicated in DNA binding (43). Supershift assays corroborated the stimulatory effect of LPS on the binding activity to the C site for each of the transcription factors Pax8 and TTF-1 because the intensities of the remnant complexes containing Pax8 and TTF-1 in the presence of anti-TTF-1 or anti-Pax8 antibody, respectively, were increased by LPS compared with those obtained with TSH treatment alone (Fig. 7C, lanes 2 and 6; lanes 3 and 7). The intensity of the remnant Pax8-C site complex was lower when compared with the residual TTF-1-C site complex in the presence of anti-TTF-1 and Pax8 antibodies, respectively, suggesting a less abundant formation of Pax8-C site complexes in our system. Although both transcription factors are able to bind to the C site, the apparently low ability of Pax8 to bind to the C site is in agreement with previous observations indicating that Pax8 binds less efficiently than TTF-1 in most experimental conditions (33, 44). To further confirm that LPS stimulates the binding of both factors to the C site, we performed EMSAs using mutated oligonucleotides able to bind selectively TTF-1 (Ct) or Pax8 (Cp) (*Materials and Methods*) (41). The specificity of the oligonucleotides was confirmed in our experimental system by supershift assays; oligo Ct only binds TTF-1 and oligo Cp Pax8 in the presence of anti-TTF-1 (Fig. 7D, lanes 2, 4, and 10) or anti-Pax8 (Fig. 7D, lanes 5, 7, and 9) antibodies. In the presence of Ct and Cp, LPS increased the TSH-induced binding of TTF-1 (Fig. 7D, lane 3 *vs.* 1) and Pax8 (Fig. 7D, lane 8 *vs.* 6) to the C element, respectively. The absence of proteins other than TTF-1 and Pax8 binding to the C site was evident by the lack of remnant complexes when Ct plus anti-TTF-1 (Fig. 7D, lanes 2 and 4) or Cp plus anti-Pax8 (Fig. 7D, lanes 7 and 9) antibodies were tested. To further corroborate the binding specificity and the effect of LPS, we performed EMSAs using Cp and Ct as cold competitors. Shifted bands permit the estimation of the amount of TTF-1/oligo C complex and Pax8/oligo C complex in the presence of an excess of cold Cp and Ct, respectively. We found that under these conditions, LPS stimulated the TSH-induced binding of TTF-1 (Fig. 7E, lanes 2 and 5) and Pax8 (Fig. 7E, lanes 3 and 6) to the C site. Based on these results,

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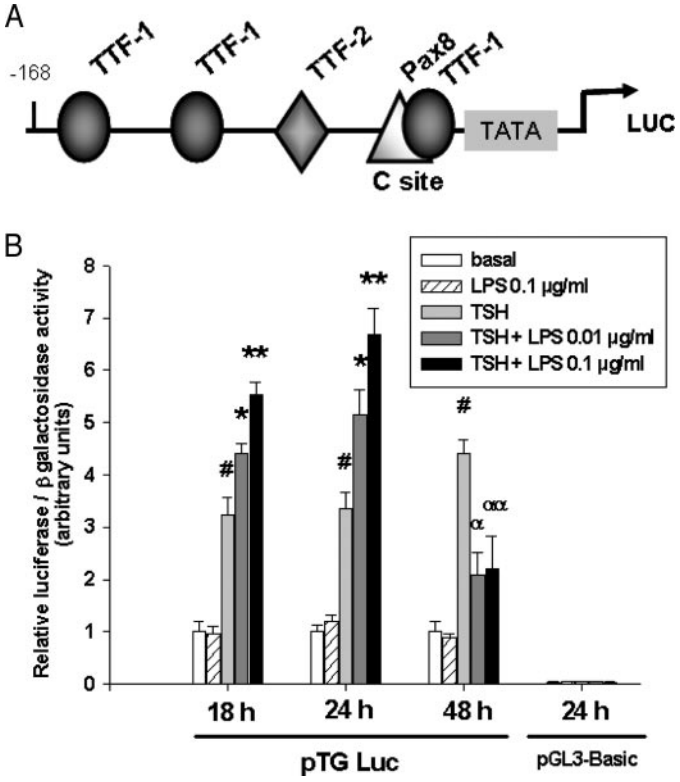

FIG. 4. LPS increases the TG promoter activity. A, Schematic drawing of pTG Luc containing the rat minimal TG promoter linked to Luc gene as reporter. B, FRTL-5 cells were transfected with 4 μg pTG Luc and 0.8 μg CMV-βgal. After transfection, cells were maintained for 5 d in basal medium and then treated with TSH alone or TSH plus LPS (0.01–0.1 μg/ml) for different times (18–48 h). Relative Luc/βgal activity is expressed as x-fold induction over the value of basal cells. The data represent the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* TSH alone (increase); α, $P < 0.05$; $\alpha\alpha$, $P < 0.01$ *vs.* TSH alone (decrease); #, $P < 0.05$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

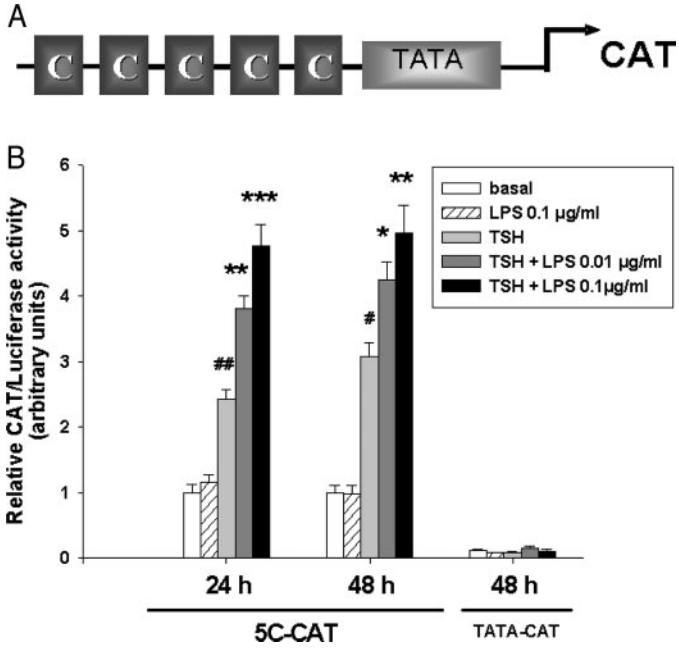

FIG. 5. The C element is involved in the LPS-induced increase of the TG promoter. A, Schematic drawing of 5C-CAT construct containing five tandem repetitions of the C site linked to TATA-box and CAT gene as reporter. B, FRTL-5 cells were transfected with 4 μg 5C-CAT and 0.8 μg CMV-Luc. After transfection, cells were maintained for 5 d in basal medium and then treated with TSH or TSH plus LPS (0.01–0.1 μg/ml) for 24 and 48 h. Relative CAT/Luc activity is expressed as x-fold induction over the value of basal cells. The data represent the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* TSH alone; #, $P < 0.05$; ##, $P < 0.01$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

we conclude that LPS treatment results in an increment of the TSH-stimulated binding of TTF-1 and Pax8 to the C site of the TG promoter. Because the pattern of the complexes detected with LPS was similar to that of TSH alone, no qualitative modifications in protein binding to the C site appear to be induced by the endotoxin. The ability of LPS to augment the binding of thyroid-specific factors to the C site in the TG promoter could be involved in an increased transactivation of the TG gene contributing to the higher LPS-induced TG expression demonstrated here.

LPS increases the TSH-induced Pax8 and TTF-1 mRNA and protein expression

The expression of Pax8 and TTF-1 mRNA and protein in response to LPS was also analyzed. TSH induced an up-regulation of Pax8 mRNA (Fig. 8A) and protein expression (Fig. 8B) from 3–72 and 12–72 h, respectively. The addition of LPS induced a rapid increment of the TSH-stimulated Pax8 mRNA level at 3 h that persisted at 6 h and did not differ from the TSH-induced level at 12 h (Fig. 8A). Then, LPS reduced Pax8 mRNA expression after 24 and 48 h of incubation, whereas no change was observed at 72 h of treatment compared with the TSH-stimulated expression (Fig. 8A). The endotoxin induced an increase of Pax8 protein levels at 12 h that was maintained at 24 and 48 h of treatment (Fig. 8B). LPS did not affect TSH-induced Pax8 level after 72 h (Fig. 8B). With regard to TTF-1 regulation, TSH induced a stimulation of TTF-1 mRNA at 3 h that persisted at 72 h (Fig. 9A), whereas an increment in the TTF-1 protein level was observed at 12 h and maintained until 72 h (Fig. 9B). Under LPS treatment, TSH-stimulated TTF-1 mRNA was rapidly increased at 3 and 6 h, then progressively decreased displaying a significant reduction at 24 and 48 h and no change after 72 h of incubation compared with those of TSH alone (Fig. 9A). In contrast, the TTF-1 protein amount was markedly increased at 12 h and then slightly decreased, although it remained higher at 24 and 48 h of incubation compared with TSH-alone treatment (Fig. 9B). No change in TSH-induced TTF-1 level was observed after incubation with the endotoxin for 72 h (Fig. 9B). Treatment with LPS alone did not modify the Pax8 or TTF-1 mRNA and protein expression compared with basal values (data not shown). These results indicate that LPS is able to stimulate TSH-induced Pax8 and TTF-1 expression in thyroid follicular cells. Because the endotoxin modifies the expression of TG, Pax8, and TTF-1 mRNA and protein in a rather similar way, we tested the specificity of the effect of LPS by analyzing the expression of the unrelated housekeeping β-actin and GAPDH genes. We observed that LPS

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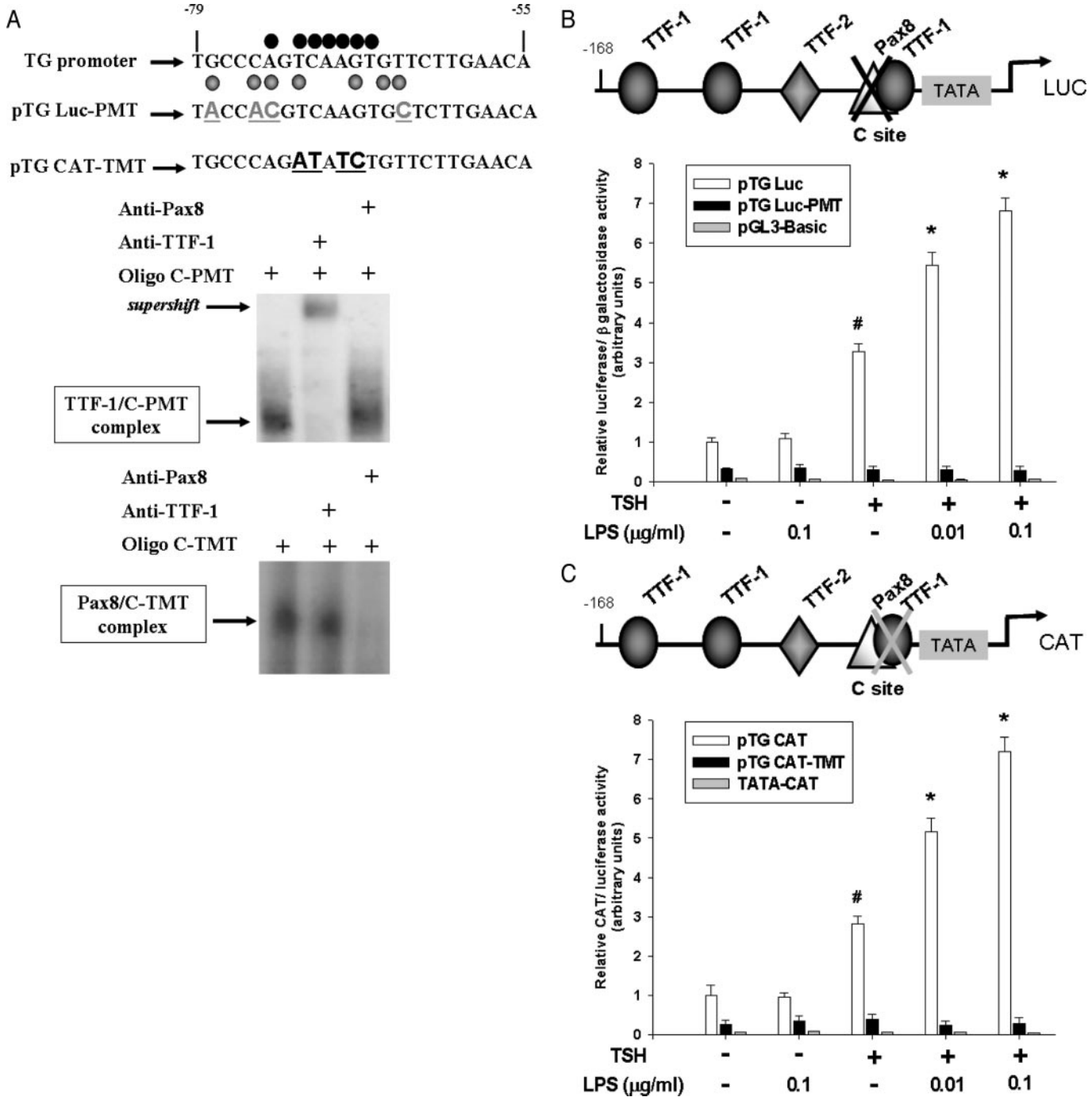

FIG. 6. The minimal TG promoter mutated in the Pax8 or TTF-1 binding site loses the LPS and TSH responsiveness. A, Region of minimal TG promoter containing the mutations (*underlined*) in the Pax8 binding site in *gray* (pTG Luc-PMT) and in the TTF-1 binding site in *bold black* (pTG CAT-TMT); the same region wild type is shown for comparison. *Black* and *gray dots* indicate the bases matching those of the TTF-1 and Pax8 consensus sequences, respectively (*upper*). The oligonucleotides derived from the mutated region (oligo C-PMT and oligo C-TMT) were radiolabeled and incubated with nuclear extracts from FRTL-5 cells treated with TSH alone for 24 h. By supershifts with anti-TTF-1 and anti-Pax8 antibodies, the binding of only TTF-1 to oligo C-PMT or Pax8 to oligo C-TMT was detected (*lower*). B, Schematic drawing of pTG Luc-PMT containing the minimal rat TG promoter mutated in the Pax8 binding site linked to Luc gene as reporter (*upper*). FRTL-5 cells were transfected with 4 μg pTG Luc or its mutant pTG Luc-PMT and 0.8 μg CMV-βgal. After transfection, the cells were maintained for 5 d in basal medium and then treated with TSH alone or TSH plus LPS (0.01–0.1 μg/ml) for 24 h. Relative Luc/βgal activity is expressed as x-fold induction over the value of basal cells (*lower*). C, Schematic drawing of pTG CAT-TMT containing the minimal rat TG promoter mutated in the TTF-1 binding site linked to CAT gene as reporter (*upper*). FRTL-5 cells were transfected with 4 μg pTG CAT or its mutant pTG CAT-TMT and 0.8 μg CMV-Luc. After transfection, cells were maintained for 5 d in basal medium and then treated with TSH alone or TSH plus LPS (0.01–0.1 μg/ml) for 24 h. Relative CAT/Luc activity is expressed as x-fold induction over the value of basal cells (*lower*). The data represent the mean ± SEM of at least three independent experiments. Cells transfected with pTG Luc or pTG CAT; *, $P < 0.001$ *vs.* TSH alone; #, $P < 0.01$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

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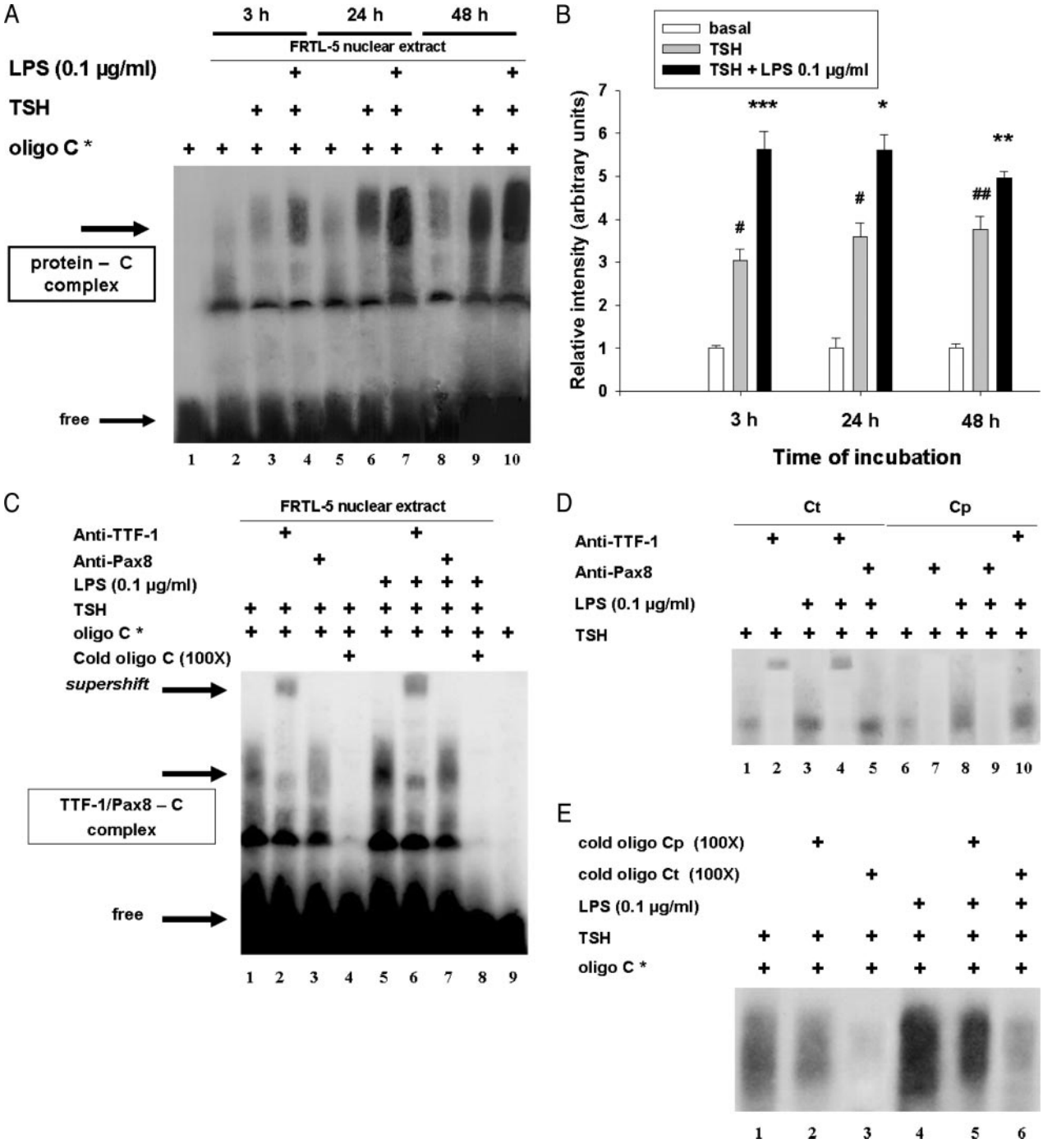

FIG. 7. LPS increases the TSH-stimulated binding of thyroid-specific transcription factors to the C site of the TG promoter. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for the indicated times. Cells were then harvested for nuclear extract preparation. EMSAs were performed with a ^{32}P-labeled oligonucleotide corresponding to the TTF-1/Pax8 binding C site (oligo C*) derived from the TG promoter or its mutants. A, Representative band shift assay. The probe was incubated without (lane 1) or with nuclear extracts from FRTL-5 cells (5 μg) (lanes 2–10). B, Densitometric analysis of the EMSAs. Autoradiograms whose signal intensity was into a range not oversaturated were analyzed by scanning densitometry. The data represent the mean ± SEM of at least three independent experiments.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* TSH alone; #, $P < 0.05$; ##, $P < 0.01$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test. C, Representative EMSA; the probe C was incubated without extracts (lane 9) or with nuclear extract from FRTL-5 cells treated for 24 h (5 μg) with TSH (lanes 1–4) or TSH plus LPS (0.1 μg/ml) (lanes 5–8) in the presence or absence of anti-hTTF-1 (lanes 2 and 6) or anti-hPax8 (lanes 3 and 7). For competition, a 100-fold excess of cold oligonucleotide C was used (lanes 4 and 8). D, Representative EMSA using the mutant probes of oligonucleotide C, Ct, which does not bind Pax8 (lanes 1–5) or Cp, which does not bind TTF-1 (lanes 6–10) incubated with nuclear extracts from FRTL-5 cells treated for 24 h (5 μg) with TSH (lanes 1–2 and 6–7) or TSH plus LPS (0.1 μg/ml) (lanes 3–5 and 8–10) in the presence of anti-hTTF-1 (lanes 2, 4, and 10) or anti-hPax8 (lanes 5, 7, and 9). E, Representative EMSA; the probe C was incubated with nuclear extract from FRTL-5 cells treated for 24 h (5 μg) with TSH (lanes 1–3) or TSH plus LPS (0.1 μg/ml) (lanes 4–6). In the presence of a 100-fold excess of cold oligonucleotides Cp and Ct, TTF-1 complex (lanes 2 and 5) and Pax8 complex (lanes 3 and 6) are visualized, respectively.

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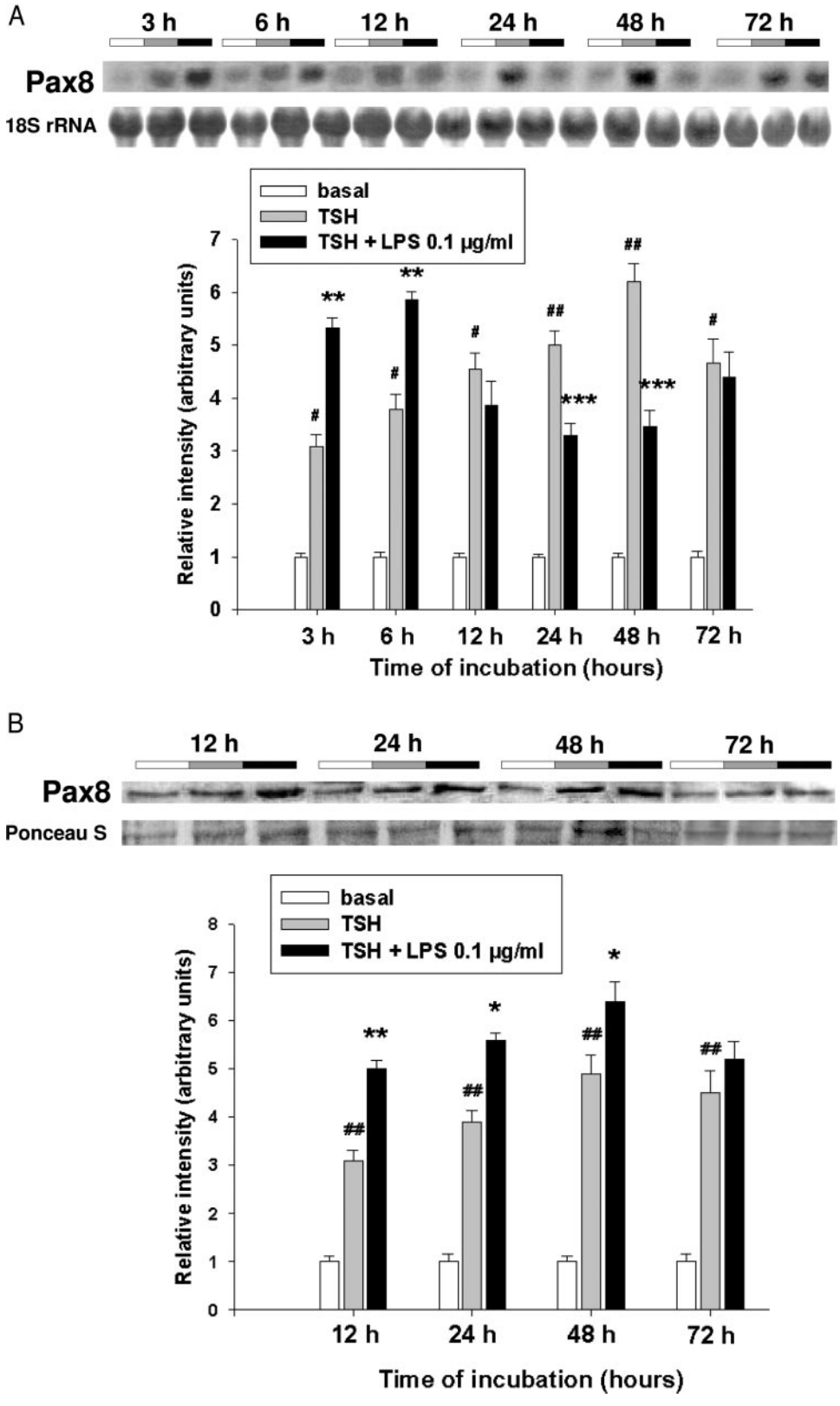

FIG. 8. LPS increases the TSH-stimulated Pax8 mRNA and protein expression. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for the indicated times and then harvested for RNA extraction (A) or whole-protein preparation (B). A, Representative Northern blot (20 μg) hybridized with a rPax8 probe (*upper*). Densitometric analysis of the Northern blots. Each value represents the mean ± SEM of at least three independent experiments. Values were normalized respect to 18S rRNA (*lower*). B, Representative Western blot of whole-cell protein (25 μg) probed with an anti-hPax8 antibody (*upper*). Densitometric analysis of the Western blots. Each value represents the mean ± SEM of at least six independent experiments. Ponceau S staining was used to correct loading differences (*lower*). *, $P < 0.01$; **, $P < 0.001$ *vs.* TSH alone; #, $P < 0.01$; ##, $P < 0.001$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

did not modify β-actin or GAPDH mRNA (Fig. 10, A and C) or protein (Fig. 10, B and D) levels at any time point analyzed. These results evidence that LPS does not exert a general unspecific effect on gene expression in thyrocytes but affects the expression of the thyroid-specific genes TG, Pax8, and TTF-1.

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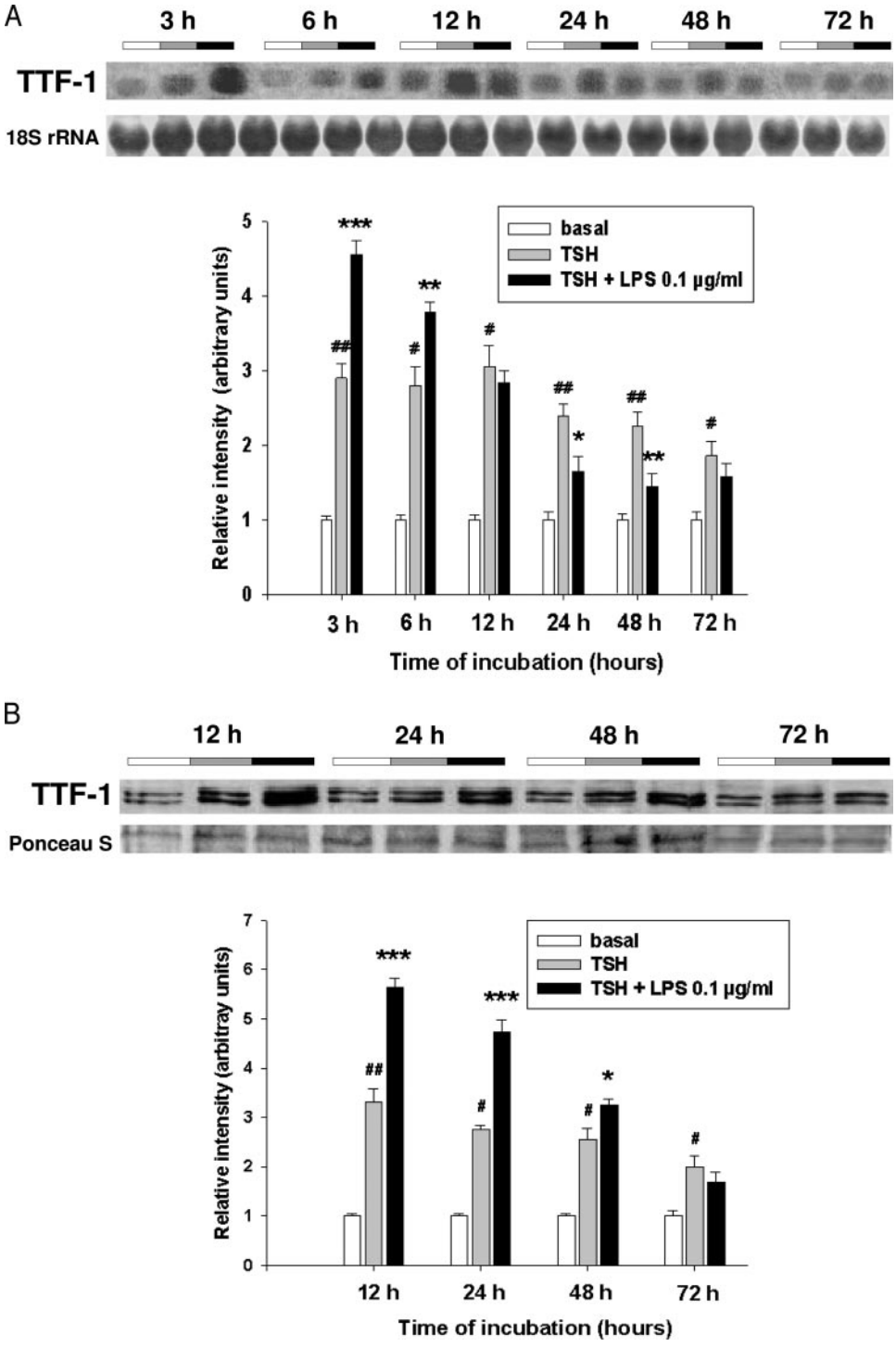

FIG. 9. LPS induces an increase in the TSH-stimulated TTF-1 mRNA and protein expression. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for the indicated times and then harvested for RNA extraction (A) or whole-protein preparation (B). A, Representative Northern blot (20 μg) hybridized with a rTTF-1 probe (*upper*). Densitometric analysis of the Northern blots. Values were normalized respect to 18S rRNA. Each value represents the mean ± SEM of at least three independent experiments (*lower*). B, Representative Western blot of whole-cell protein (25 μg) probed with an anti-hTTF-1 antibody (*upper*). Densitometric analysis of the Western blots. Each value represents the mean ± SEM of at least three independent experiments. Ponceau S staining was used to correct loading differences (*lower*). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* TSH alone; #, $P < 0.05$; ##, $P < 0.01$ *vs.* basal (taken as 1.00). Student-Newman-Keuls multiple comparisons test.

TG, Pax8, and TTF-1 half-lives are increased after LPS treatment

The observation that levels of TG, Pax8, and TTF-1 were increased while their mRNAs were decreased at the same time points (24 and 48 h) suggested that LPS could exert posttranscriptional or posttranslational actions on the expression of these genes. In an initial attempt to investigate this point, we evaluated the effect of LPS on the half-lives of the three proteins. Basal cells were pretreated with TSH or TSH plus LPS for 24 h and exposed to CHX to block protein synthesis. As shown in Fig. 11, the half-lives of TG, Pax8, and TTF-1 were increased in a rather similar proportion after LPS incubation compared with those of TSH alone. Under these experimental conditions, the three proteins appear to have a quite similar half-life after TSH-alone treatment. Values of half-lives were estimated to be approximately 5.0 h for TG (Fig. 11A), 5.7 h for Pax8 (Fig. 11B), and 6.9 for TTF-1 (Fig. 11C) in TSH-treated cells. These protein half-lives were in-

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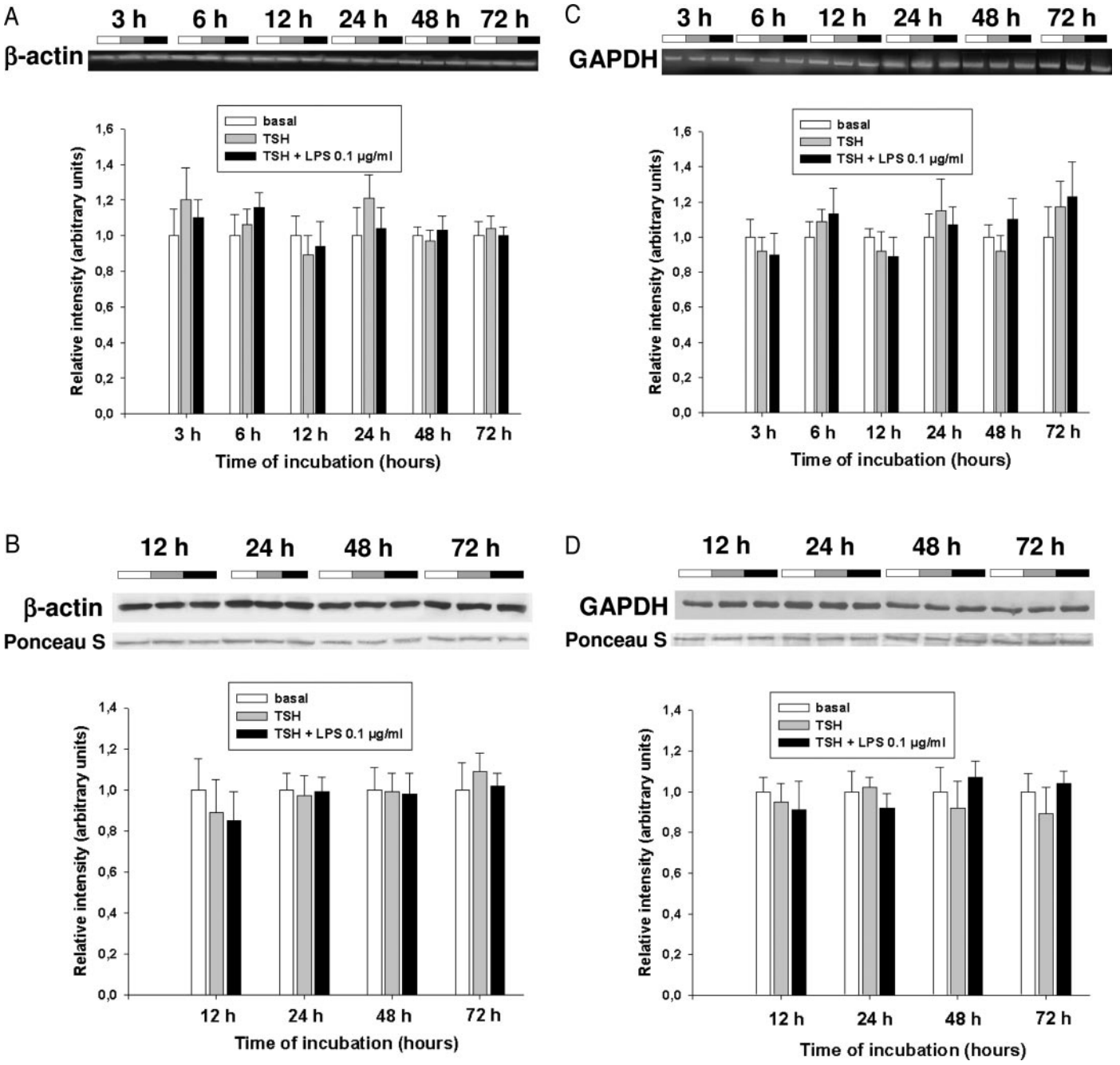

FIG. 10. LPS does not modify β-actin or GAPDH expression. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for the indicated times and then harvested for RNA extraction (A and C) or whole-protein preparation (B and D). A, Representative RT-PCR for β-actin (*upper*). Densitometric analysis of RT-PCR. Each value represents the mean ± SEM of at least three independent experiments (*lower*). B, Representative Western blot of whole-cell protein (25 μg) probed with an anti-β-actin antibody (*upper*). Densitometric analysis of the Western blots. Each value represents the mean ± SEM of at least three independent experiments. Ponceau S staining was used to correct loading differences (*lower*). C, Representative RT-PCR for GAPDH (*upper*). Densitometric analysis of RT-PCR. Each value represents the mean ± SEM of at least three independent experiments (*lower*). D, Representative Western blot of whole-cell protein (25 μg) probed with an anti-GAPDH antibody (*upper*). Densitometric analysis of the Western blots. Each value represents the mean ± SEM of at least three independent experiments. Ponceau S staining was used to correct loading differences (*lower*).

creased by LPS to reach values of approximately 12.0 h for TG (Fig. 11A), 11.5 h for Pax8 (Fig. 11B), and 16.2 h for TTF-1 (Fig. 11C). These observations are in favor of a posttranslational effect of LPS on TG, Pax8, and TTF-1 that would involve an increase of the stability of these proteins that could contribute to the increment of their levels.

Discussion

The present study provides evidence that the bacterial endotoxin LPS increases TG mRNA and protein levels by transcriptional activation of the TG gene. The action of LPS occurs only in TSH-stimulated thyroid cells, suggesting that

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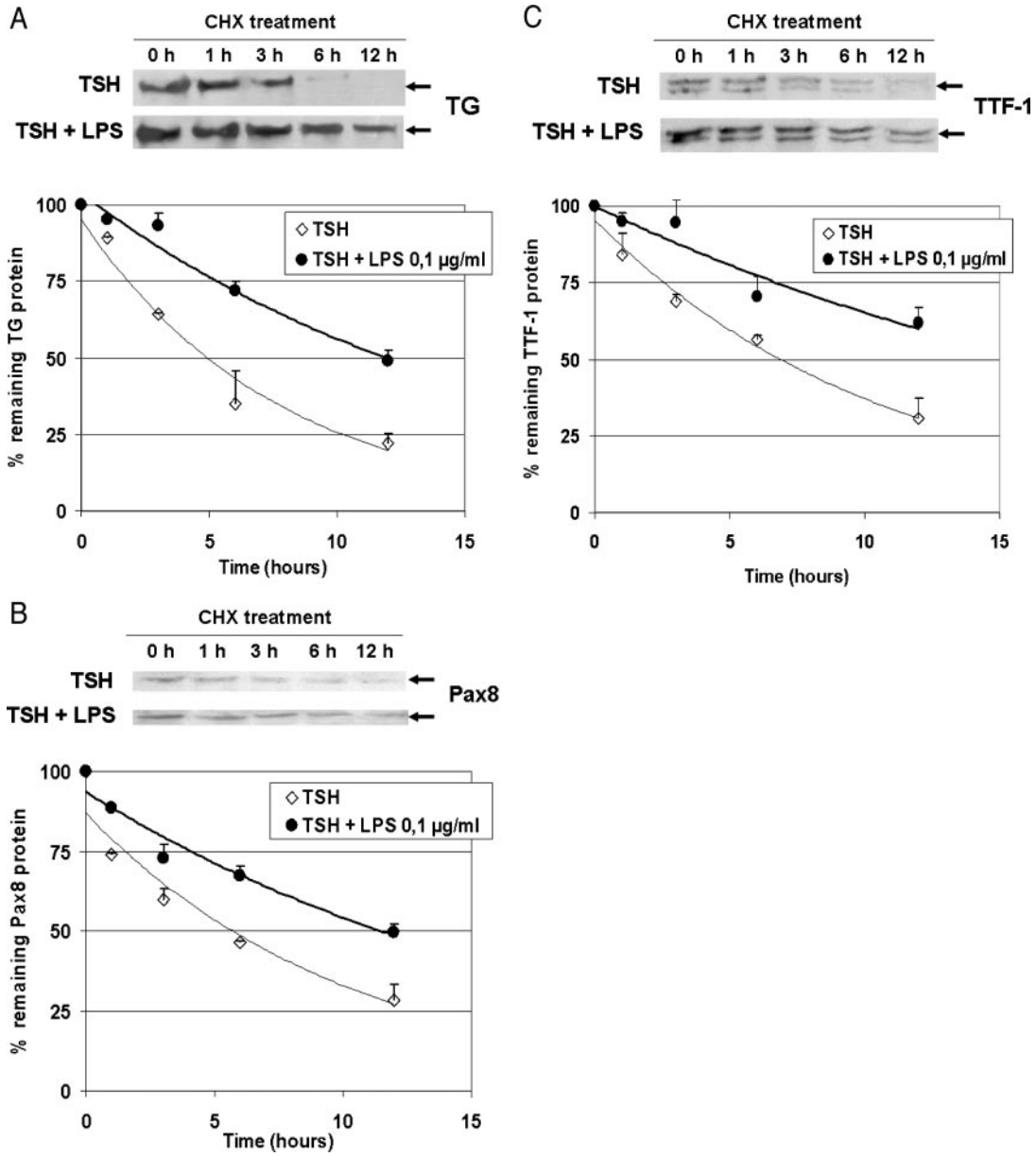

FIG. 11. LPS increases TG, Pax8, and TTF-1 half-lives. Basal FRTL-5 cells were treated with TSH alone or TSH plus LPS (0.1 μg/ml) for 24 h before incubation with CHX for the indicated times. Protein extracts were assayed for TG (A), Pax8 (B), and TTF-1 (C) levels by Western blot. Values from densitometric analysis were used for estimation of half-lives. Results are expressed as percent remaining protein; value at 0 h (CHX addition) is taken as 100%. Each value represents the mean ± SEM of at least three independent experiments. Ponceau S staining was used to correct loading differences.

a cross-talk between the signaling pathways induced by LPS and TSH may occur. The influence of bacterial endotoxins on the expression of thyroid-specific genes has not been reported previously. It has, however, been demonstrated that LPS is able to up-regulate diverse functions in other endocrine cells. For example, LPS increases GH mRNA and protein expression in rat adenohypophysial cells, as well as GH secretion *in vivo* (45). An increment in leptin expression and release from adipose tissue of various species is induced by LPS (46–48). Recently, evidence that LPS stimulates cortisol secretion in human adrenocortical cells has been reported (49). In the present study, the effects of LPS were observed at rather low, but not at high, concentrations of the endotoxin. We therefore expect that the moderate levels of circulating LPS present during certain infectious processes (50) could be able to induce TG expression in thyroid cells *in vivo*. In contrast, high levels of LPS, such as those found in endotoxic shock in humans, may not modify TG expression, although they have been reported to alter thyroid function by several other mechanisms (51, 52).

It is well established that LPS induces the expression of numerous genes in immune cells (1, 3–5). A role of the endotoxin through an immunostimulatory effect has been proposed in autoimmune diseases (2, 6, 7, 20). Several recent reports have associated the increased expression of proteins acting as autoantigens with the etiology and pathology of

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diverse autoimmune disorders (21–23). Despite the ability of LPS to act as a potent biological activator, the possibility that LPS could modify autoantigen expression in cells that are target for autoimmunity has not been explored. TG is a key protein involved in the biosynthesis of thyroid hormones, as well as one of the major thyroid autoantigens. Experimental autoimmune thyroiditis has been developed by administration of TG plus adjuvants in mice. However, a difference in the pathogenic processes was observed when LPS was used as the adjuvant (7, 20). This fact was in part explained by the finding that LPS interacts with the thyrocyte inducing the expression of several chemokines that, in turn, could facilitate lymphocytic infiltration in the thyroid tissue (7). In this paper, we demonstrate that LPS is able to increase the TSH-induced TG expression in thyroid cells. The involvement of TG as an important element to trigger the autoimmune thyroid response has been emphasized before (53). Thus, it is rational to speculate that the LPS-induced TG accumulation might contribute to antigenic processing of TG by thyroid cells in the presence of an activated immune system. These findings would favor the involvement of infectious agents in the induction of thyroid autoimmune disease (18, 19). Despite the intricate mechanisms operating in autoimmunity, the present results thus could contribute to disclose some etiopathogenic aspects of thyroid autoimmune disorders.

Here, we demonstrate the functional relevance of the C site in the TG promoter in the LPS-induced increase of TG gene transcription. The LPS-stimulating effect seems to be mediated by a higher TTF-1 and Pax8 binding activity to the C site. It is known that TTF-1 and Pax8 are the main transcription factors involved in TSH-dependent TG gene expression (9, 10). Accordingly, the present observations confirm the requirement of intact Pax8 and TTF-1 binding sites at the C site in the TG promoter for TSH- and LPS-induced TG gene expression. Our results show that the stimulatory action of LPS appears to take place through a similar mechanism to that involved in TG gene activation by TSH.

The increment in the C site binding activity of TTF-1 and Pax8 by LPS that we observed is consistent with the increased transcriptional activity of 5C-CAT and reinforces a possible stimulation of the C site-mediated transactivation by these transcription factors in the TG promoter. The increased binding of the transcription factors to the C site may be explained by the stimulatory action displayed by LPS on TTF-1 and Pax8 expression. However, other mechanisms facilitating the TTF-1 and Pax8 binding activity to the C site could be involved, although this hypothesis awaits confirmation. For example, it has been shown that redox state or phosphorylation can modify Pax8 and TTF-1 DNA binding activity through posttranslational modifications (17, 54, 55). The results presented here reveal that TTF-1 and Pax8 are the main factors responsible for the LPS-induced TG gene activation. Although the involvement of some other mediators should not be discarded, the lack of LPS action on the scarce residual activity of the TG minimal promoter mutated at the Pax8 or TTF-1 site argues against a significant role of other factors. Taking into account that TTF-1 and Pax8 are also expressed in nonthyroidal tissues where they regulate various target genes (9, 56–58), one wonders whether LPS could affect other TTF-1 and Pax8-transactivated genes in other cell types.

An apparent discrepancy between TG mRNA and protein levels after LPS treatment was observed in this study because the TSH-stimulated TG mRNA levels were reduced, whereas the TG protein levels were increased by the endotoxin at the same time points (24 and 48 h). This fact suggests that the action of LPS on TSH-induced TG expression is complex, possibly involving posttranscriptional modifications that could include changes in TG mRNA stability as well as changes of TG translation efficiency, degradation, and trafficking. Similar considerations could be made for the apparent lack of parallel changes in Pax8 and TTF-1 mRNA and protein levels obtained after LPS treatment. Thus, the endotoxin decreased TSH-stimulated Pax8 and TTF-1 mRNA levels at 24 and 48 h, whereas it increased simultaneously the amount of Pax8 and TTF-1 protein levels. These apparent contradictions may be partially explained by the increase in the protein half-lives of TG, Pax8, and TTF-1 demonstrated here. These observations suggest that LPS may alter posttranslational regulation and/or intracellular processing of these proteins, leading to an increase of protein stability. Another apparent contradiction was observed between the TSH-dependent promoter activity that was increased at 24 h and the TSH-dependent mRNA accumulation that was decreased at the same time point by LPS, which may be explained by the existence of posttranscriptional effects of LPS. Taken together, these observations support the existence of multiple levels for the action of LPS on TG, Pax8, and TTF-1 gene expression.

It is known that FRTL-5 cells chronically exposed to TSH enter a refractory state with no response to further stimulation, an effect explained by a down-regulation of the cAMP-dependent protein kinase A (59). This is probably one of the reasons for the decreased stimulatory action of TSH on TG protein expression after 3 d of TSH treatment observed in our study. However, at this time, we observed a higher reduction in the LPS-induced stimulation of TG, a finding that is consistent with the faster and marked diminution of the stimulatory action by LPS on TG mRNA expression and promoter transcription. This fact might, in part, be explained by a more rapid entry of cells into a refractory state due to a higher stimulation of LPS-induced signaling pathways. Several reports suggest that TG is able to autoregulate thyroid hormone biosynthesis by modulating sodium iodide symporter, thyroid peroxidase, and TG gene expression (8, 60, 61). Therefore, one might speculate that the LPS-induced elevation of TG protein levels observed here might also participate in its autorepression. Of note, the expression of the 5C construct was stimulated by LPS at the same time point we found a repression of the wild-type promoter (48 h), which suggests that the region responsible for repression is localized in the minimal promoter and might be situated upstream of the C site. This observation would be consistent with a possible involvement of TG in its autorepression because a previous report indicated that the TG-induced suppression of a TG-CAT construct was lost when most of the 5′-upstream TTF-1 and TTF-2 sites of the TG minimal promoter were deleted, but the C site was conserved (60). The apparent contradiction between the decrease of both TG promoter activity at 48 h and mRNA levels at 24 and 48 h induced by LPS, despite the LPS-induced increase of TTF-1 and Pax8 binding to the C site

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at 48 h, would favor negative regulation of the TG gene by regions upstream of the C site in the TG promoter.

In conclusion, our results reveal for the first time the ability of the endotoxin LPS to up-regulate TSH-stimulated TG gene expression at the transcriptional level in thyroid follicular cells. The presented evidence indicates that the stimulatory action of LPS could occur, at least in part, through a C site-mediated increase of TG promoter transactivation involving the transcription factors TTF-1 and Pax8. A further action of LPS on TG at the posttranslational level was evidenced. Because TG is an essential protein for thyroid hormone biosynthesis and one of the main thyroid autoantigens, these findings suggest a possible role of the endotoxin as a potential modifier of thyroid function, a fact of possible pathophysiological relevance. We also demonstrate a novel action of LPS to increase TTF-1 and Pax8 expression, an effect that may be relevant for TTF-1- and Pax8-regulated genes in other cell types.

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